UNIVERSIDADE ESTADUAL DE CAMPINAS

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EFEITO *IN VITRO* DA ADIÇÃO DO LEVONOGESTREL SOBRE A CONCENTRAÇÃO INTRACELULAR DE CÁLCIO EM ESPERMATOZÓIDES DE HOMENS FÉRTEIS

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural, na área de Histologia.

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Dedico este trabalho

Aos meus pais,

Como reconhecimento por todas às vezes

Que renunciaram aos seus sonhos

Para que pudessem realizar os meus.

Ofereço

Aos meus pais e ao meu irmão,
Por fazerem de nossa família
uma família perfeita para mim.
A vocês devo tudo o que sou.

"No meio da confusão, encontre a simplicidade.

A partir da discórdia, encontre a harmonia.

No meio da dificuldade reside a oportunidade."...

Albert Einstein

..."Pois a gente leva da vida a vida que a gente leva"

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RESUMO

O espermatozóide é uma célula altamente especializada, uma vez que apresenta diferenciações morfofisiológicas que garantem o sucesso da fecundação. Com o objetivo de adquirir a capacidade de fecundação precisam passar por três processos seqüenciais, maturação epididimária, capacitação e reação acrossômica (RA). A capacitação é um processo que acontece no trato reprodutor feminino e define-se como uma mudança bioquímica na membrana plasmática do espermatozóide, sendo a presença de progesterona e o aumento na concentração intracelular do cálcio (Ca^{+2}) eventos característicos e obrigatórios para a posterior RA. O levonogestrel (LNG) é um progestágeno sintético, semelhante a progesterona, o qual tem sido usado como contraceptivo feminino, seja na contracepção de emergência (CE), nas pílulas contraceptivas combinadas ou com o dispositivo intra-uterino (DIU). Entretanto, na literatura não há dados que relacionem a eficácia contraceptiva do lenovogestrel com possíveis efeitos deste hormônio sobre os espermatozóides humanos. Assim, com o objetivo de adicionar dados a respeito do mecanismo de ação contraceptiva do LNG, este estudo teve como objetivo avaliar o efeito de 5 concentrações de LNG, comparáveis as concentrações encontradas no plasma sanguíneo de mulheres, após a ingestão de LNG sob a forma de (CE) com a concentração intracelular do cálcio em espermatozóides capacitados de homens férteis. Um total de 24 amostras de sêmen, provenientes de 4 homens férteis foram avaliadas. Os espermatozóides foram selecionados através de um gradiente de Percoll, e foram submetidas a um período de capacitação. Às amostras foi adicionado o corante Fura2/AM e a

seguir estas foram expostas às concentrações de 8, 10, 14, 1.000 e 10.000 ng/mL de LNG e a dois controles, um positivo e outro negativo, sendo respectivamente a progesterona (32 μM = 11.000 ng/mL) e a meio de cultivo *human tubal fluid* (HTF). A seguir as amostras foram avaliadas através da espectrofotometria de fluorescência, com exitação de 340 nm e emissão de 510 nm. A taxa de fluorescência foi convertida em medidas de concentração intracelular de cálcio ($[Ca^{2+}]i$), utilizando a equação de Grynkiewicz et al. (1985), a qual demonstrou que as concentrações de 8, 10, 14 e 1.000 ng/mL de LNG foram capazes de induzir um aumento na $[Ca^{2+}]i$ significativamente maior quando comparados ao obtido com HTF, e significativamente menor que ao obtido com progesterona (o indutor natural), excetuando-se a concentração de 10.000 ng/ml (32 μM) de LNG, a qual foi estatisticamente similar que a progesterona 11.000 ng/ml (32 μM). Assim, é possível sugerir que o LNG *in vitro*, em baixas concentrações, aumente a $[Ca^{2+}]i$ nos espermatozóides. No entanto, este mecanismo, de maneira isolada, não deva ser o responsável pela indução da RA. Entretanto a $[Ca^{2+}]i$ obtida através de altas concentrações de LNG (10.000 ng/ml) pode ser sim, um possível mecanismo de ação do LNG como droga contraceptiva.

ABSTRACT

The spermatozoa is a highly specialized cell because it contains morfophysiological modifications that guarantees fecundation success. Spermatozoa must undergo three sequential changes before they acquire their fertilizing capacity: maturation in epididymis, capacitation and acrosome reaction (RA). Capacitation is a process that takes place in the female reproductive tract and is defined as a biochemical modification on the spermatozoa plasmatic membrane. The presence of progesterone (P) and the intracellular calcium concentration increase inside the spermatozoa are typical and obligatory events in the capacitation stage for further RA. Levonogestrel (LNG) is a synthetic progestin, similar to P, which has being used as a female contraceptive in several manners, among them they are: emergency contraception (EC), contraceptive pills combine and with the intrauterine system (IUS). Nevertheless, there are no available data or the scientific literature that correlate LNG contraceptive efficacy with possible effects of the hormones over human spermatozoa. Therefore, to add data over the contraceptive action mechanism of LNG, the aim of this study was to evaluate the effect of 5 concentrations of LNG somes comparable to the levels found in serum following ingestion of LNG as EC on the calcium intracellular concentration ($[Ca^{2+}]i$) rates of capacitated spermatozoa of fertile men. A total of 24 semen samples from 4 fertile men were evaluated. The spermatozoa were selected by Percoll gradient, following incubation with human tubal fluid medium supplemented with bovine serum albumin (HTF/BSA) under capacitating conditions. The sperm suspensions were incubated with Fura2/AM Subsequently the cells were exposed to the

following concentration of LNG: 8, 10, 14, 1,000 and 10,000 ng/mL, and two controls (positive and negative). The positive control was P (32 μM=11,000 ng/ml) and the negative one was medium (HTF). Cells were transferred to a quartz cuvette to make the reading in the spectrofluorometer at 340 nm excitation with emission at 510nm. Fluorescence measurements were converted to $[Ca^{2+}]i$ by the equation of Grynkiewicz *et al*. (1985). The Grynkiewicz equation shown that LNG concentration of 8, 10, 14, and 1,000 ng/mL were able to induce increase in the $[Ca^{2+}]i$ with significantly higher values, when compared with the rate obtained with HTF medium (as a negative control), and significantly lower than the rate obtained with natural P (as a positive control). However, the exception was the LNG concentration of 10,000 ng/mL (32 μM) in which values of $[Ca^{2+}]i$ were similar to those observed with P at a concentration of 11,000 ng/mL (32 μM). In conclusion, it is possible to suggest that although LNG *in vitro,* in low concentration, increase the $[Ca^{2+}]i$ in the spermatozoids. Despite the fact that this mechanism is not the only responsible for the RA induction the calcium obtained through high concentration of LNG (10,000 ng/ml) might have a contraceptive action in the spermatozoa.

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Capítulo I – *Introdução Geral*

INTRODUÇÃO

No processo de fertilização, estão envolvidos muitos fatores masculinos e femininos, que abrangem desde a formação do oócito e do espermatozóide, até mecanismos para interação e fusão entre ambas as células. Durante as etapas que antecedem a união das membranas citoplasmáticas dos gametas, o gameta masculino necessita adquirir a capacidade de fertilização, pois os espermatozóides que deixam os túbulos seminíferos são pouco móveis e incapazes de fecundar, dessa forma a mobilidade é potencializada após a passagem pelo epidídimo, num processo denominado maturação epididimária. Além disso, no trato reprodutor feminino ocorrem outros dois fenômenos com o gameta masculino, a capacitação e a reação acrossomal (RA), todos estes fundamentais para garantir o sucesso da fecundação dos espermatozóides (Bedford, 1967; Orgebin-Crist, 1967).

A capacitação pode ser definida como uma mudança bioquímica na membrana plasmática do espermatozóide (Brucker e Lipford, 1995), sendo caracterizada pelo aumento na concentração intracelular do íon cálcio (Baldi *et al*., 1991; Yanagimachi, 1994; Garcia e Meizel, 1999; Flesch e Gadella, 2000). Este íon possui um papel central no processo de fusão de membranas e o aumento de sua concentração intracitoplasmática é um evento obrigatório anterior à reação acrossomal (RA) (Bailey e Storey, 1994; Yanagimachi, 1994; Visconti *et al*., 1995; Brewis *et al*., 1996; Kaul *et al*., 1997; Wassarman, 1999).

Essas mudanças bioquímicas e estruturais que capacitam o espermatozóide ocorrem no útero e na tuba uterina, dentro dos quais existe um

fluído contendo proteínas tais como a taurina, a hipotaurina (Fellman *et al*., 1987; Huxtable, 1992), HCO_3^- (Okamura *et al*., 1987) e progesterona, as quais possuem comprovada atividade moduladora dos gametas. A tuba uterina também secreta, de maneira estrógeno-dependente, proteínas específicas denominadas oviductinas cuja função é de facilitar a união entre oócito e espermatozóide (Verhage *et al*., 1988; Mallette *et al*., 1995; Boatman, 1997).

Ainda na tuba uterina ocorre o encontro da membrana citoplasmática do espermatozóide com as células granulosas da *corona radiata*, resultando em formação de vesículas acrossomais e permitindo a liberação das enzimas *hialuronidase* e *acrosina* do acrossomo, facilitando a passagem do espermatozóide por esta camada. A RA (liberação de todo o conteúdo acrossômico) deverá acontecer somente quando a membrana citoplasmática do espermatozóide entrar em contato com as glicoproteínas da zona pelúcida (ZP) e culminará com a passagem do espermatozóide através desta camada. No entanto, para que a RA aconteça é necessário que o espermatozóide entre previamente em contato com as células granulosas (Yanagimachi, 1994).

As células granulosas, ao redor do oócito, são impregnadas pela progesterona, esteróide capaz de desencadear o estímulo fisiológico para RA nos espermatozoides. Assim, aqueles espermatozóides que sofrem esta reação precocemente, pelo contato com as células granolusas na *corona radiata* facilitam a passagem de outros espermatozóides através desta barreira, permitindo que um espermatozóide interaja seus receptores com a zona pelúcida, faça a RA e finalmente fecunde o oócito (Cheng *et al*., 1998; Flesch e Gadella, 2000).

A concentração fisiológica de progesterona estimada ao redor do oócito logo após a ovulação é de 0,3 μg/ml, e esta concentração está em uma faixa-dose apropriada para desencadear a RA nos espermatozóides (Blackmore *et al*., 1990; Flesch e Gadella, 2000). Na ausência de um estímulo fisiológico (Progesterona), apenas uma baixa porcentagem de espermatozóides libera o conteúdo acrossomal (Leyton e Saling, 1989; Wassarman, 1992; Yanagimachi, 1994; Baldi *et al*., 2000).

A progesterona tem sido apontada como indutora da RA por induzir o aumento na concentração do cálcio intracelular (Osman *et al*., 1989; Blackmore *et al*., 1990; Baldi *et al*., 1991; Foresta *et al*., 1993; Melendrez *et al*., 1994). Aumentos significativos na concentração do íon cálcio no citoplasma do espermatozóide foram observados, *in vitro,* com apenas 10 ng/ml de progesterona, enquanto que o efeito máximo foi observado com 1.000 ng/ml (Blackmore *et al*., 1990). Embora a concentração de progesterona suficiente para desencadear a entrada de Ca^{2+} na célula com posterior RA *in vivo* seja de 32μM (11000ng/ml)(TESARIK, 1992).

O Levonogestrel (LNG) é um progestógeno sintético (Blackmore *et al*., 1990) usado atualmente como um contraceptivo feminino, seja na contracepção de emergência (CE) (pílula do dia seguinte), nos contraceptivos orais combinados, nos anéis vaginais ou em conjunto com um dispositivo intra-uterino (DIU-LNG). A CE é um método hormonal contraceptivo utilizado após o coito, quando não

tenham sido utilizados outros métodos contraceptivos adicionais (El-Mahgoub, 1980).

O DIU-LNG foi desenvolvido visando reduzir alguns problemas encontrados com o uso de DIU de cobre, principalmente o aumento da quantidade de sangramento menstrual (Luukkainen *et al*., 1986). Tudo indica que este objetivo foi alcançado, uma vez que diferentes autores mostraram que durante o uso do DIU-LNG a quantidade de fluxo menstrual foi significativamente reduzida (Nilsson, 1978; El-Mahgoub, 1980; Nilsson *et al*., 1983; Heikkilä *et al*., 1982). O DIU-LNG apresenta taxa de gravidez muito baixa, uma vez que nenhuma gestação foi observada em até 7 anos de uso (Faundes *et al*., 1993; Diaz *et al*., 2000), entretanto, não há dados na literatura que relacionem a eficácia contraceptiva do DIU-LNG, com algum possível efeito sobre os espermatozóides humanos após estes entrarem em contato com o LNG, liberado constantemente no endometrio das usuárias.

Baseando-se no pressuposto de que a progesterona poderia aumentar a $[Ca^{2+}]i$ resultando em RA, Nikkanen e colaboradores (2000) desenvolveram um estudo que se constituiu na aplicação de pequenas doses de LNG diretamente na cauda do epidídimo de cobaias, com a finalidade de buscar um modelo contraceptivo pós-testicular. Este estudo se baseou na suposição de que os espermatozóides, sofreriam RA quando em contato com o LNG e deste modo ficariam impossibilitados de fertilizar os ovócitos. Nesse estudo, amostras de espermatozóides foram retiradas da vagina das fêmeas após a cópula, e foi observado que todos os espermatozóides estavam imóveis. Entretanto, não foi

realizada uma análise mais detalhada para se verificar a presença da capacitação ou RA nesses espermatozóides, de modo a constatar-se uma possível associação entre o efeito do LNG e a imobilidade dos espermatozóides.

O LNG além de ser utilizado como contraceptivo feminino, também tem sido testado em combinação com a testosterona para o controle da fertilidade masculina (Foegh *et al*., 1980), já que para a contracepção masculina o bloqueio da espermatogênese não é totalmente necessário, bastando apenas a eliminação da capacidade de fertilização do espermatozóide. Isso pode ser conseguido através da indução precoce dos processos cruciais para a fertilização desenvolvidos pelo espermatozóide, como por exemplo, a capacitação e a RA (Nikkanen *et al*., 2000).

Um estudo preliminar realizado no Laboratório de Reprodução Humana do Centro de Atenção Integral à Saúde da Mulher (CAISM) da UNICAMP mostrou que o LGN é um bom indutor da RA em espermatozóide humanos. Foram testadas, *in vitro*, diferentes concentrações desse progestógeno: 200 ng/ml; 400 ng/ml; 800 ng/ml e 1.000 ng/ml em incubações durante 15 e 30 minutos com 2-8x10^6 espermatozóides. O fluido folicular (FF), que possui uma alta concentração de progesterona natural, e o meio de cultura puro, foram utilizados como controle positivo e negativo da RA, respectivamente. Considerando-se o índice de RA induzido pelo FF como 100%, diferentes concentrações de LNG incubadas com espermatozóides, não aumentaram significativamente o índice de RA num intervalo de 15 minutos de incubação. Entretanto, com uma incubação de 30 minutos as taxas de RA foram de 54,1% (com 200 ng/ml de LNG) ; 55,9% (com

400 ng/ml de LNG); 57,6% (com 800 ng/ml de LNG) e 70,5% (com 1000 ng/ml de LNG), respectivamente, em relação ao controle positivo (FF). Concluiu-se que era necessário um tempo de pelo menos 30 minutos para que o LNG atuasse sobre esses espermatozóides e induzisse a RA (Bahamondes *et al*., 2003). Esse resultado está de acordo com a eficiência do DIU-LNG e CE com LNG como contraceptivo, uma vez que os espermatozóides permanecem no canal endocervical por algumas horas ou dias após o coito, antes de ascender pelo trato reprodutor feminino (Diaz *et al*., 2000).

Baseado no fato do LNG ser um progestógeno sintético muito semelhante a progesterona e esse esteróide natural ter sido apontado como o estímulo fisiológico indutor da RA por induzir o aumento na concentração do cálcio intracelular (Osman et al., 1989; Blackmore et al., 1990; Baldi et al., 1991; Foresta et al., 1993; Melendrez et al., 1994), o objetivo deste projeto de pesquisa foi investigar um dos possíveis mecanismos bioquímicos pelo qual o LNG age como uma droga contraceptiva. Para isto foi realizado um estudo *in vitro,* utilizando o corante Fura2/AM e espectrofluorometro.

O corante Fura2/AM penetra no interior dos espermatozóides, onde sofre a ação de enzimas esterases, as quais hidrolisam seu grupamento AM., impedindo assim que o corante deixe a célula. Em seguida o corante se liga ao cálcio e dessa forma o complexo Fura2/AM-Ca^{2+} passa a emitir fluorescência. Através da espectrofotometria de fluorescência, a concentração do complexo Fura2/AM-Ca^{2+} intracelular é quantifica, dessa forma a técnica foi utilizada para analisar a $[Ca^{2+}]i$

em espermatozóides humanos, de homens férteis, incubados com diferentes concentrações de LNG.

OBJETIVOS

Os objetivos deste projeto de pesquisa consistiram em:

1- Avaliar *in vitro* a $[Ca^{2+}]i$ em espermatozóides já capacitados de homens férteis; após uma hora de incubação com diferentes concentrações do progestógeno LNG;

2- Determinar *in vitro* uma curva dose-resposta, relacionando a concentração de LNG e a $[Ca^{2+}]i$ espermatozóides *in vitro* já capacitados de homens férteis;

Capítulo II - ***Manuscrito científico decorrente da dissertação e submetido à publicação***

The *in vitro* effect of levonorgestrel on the Ca^{2+} intracellular concentration of human spermatozoa

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Abstract

Objectives: To understand the mechanisms involved in the contraceptive effect of levonorgestrel (LNG) on spermatozoa by evaluating Ca^{2+} intracellular concentration ($[Ca^{2+}]i$) in the human spermatozoa of fertile men exposed to different concentrations of LNG.

Materials and Methods: Twenty-four samples of human semen from normospermic volunteers were loaded with 2 µmol/L Fura-2/AM following 2 h of capacitation. Cells were exposed to 8, 10, 14, 1,000, and 10,000 ng/mL of LNG, to P (32 µM = 11,000 ng/mL), control medium, and human tubal fluid (HTF) for 1 h at 37ºC and 5% CO_2. Evaluation was carried out by spectrofluorometer at 340 nm excitation with emission at 510 nm. Fluorescence measurements were converted to $[Ca^{2+}]i$ using the equation proposed by Grynkiewicz et al. (1985).

Results: Samples of human capacitated spermatozoa exposed to 8, 10, 14, and 1,000 ng/mL LNG had significantly lower $[Ca^{2+}]i$ rates compared to progesterone and higher rates when compared to HTF medium. However, when the $[Ca^{2+}]i$ of natural progesterone was compared to that of 10,000 ng/mL of LNG, results were similar.

Conclusions: The 10,000 ng/mL dose of LNG induced a similar rate of $[Ca^{2+}]i$ as natural progesterone and may be one of the mechanisms of action of contraception of LNG.

Key words: Ca^{2+}/ human spermatozoa/ levonorgestrel.

1. Introduction

Sexual reproduction requires the fusion of sperm cell and oocyte during fertilization to produce the diploid zygote. In mammals, including humans, spermatozoa must undergo a number of changes before they acquire their fertilizing capacity. The two most important steps are capacitation and acrosome reaction (AR) [1]. A delicate reorientation and modification of plasma membrane molecules, including changes in ion permeability that prepare the cell to undergo AR, take place in the female reproductive tract when sperm cells are activated by the so-called capacitation factors [2].

AR begins immediately following primary binding of a sperm cell to the zona pellucida (ZP). The apical plasma membrane of the sperm head starts to fuse with the underlying acrosome membrane at multiples sites, resulting in the dispersal of the acrosomal content. An early event that takes place in AR is an obligatory increase in free cytosolic calcium (Ca^{+2}). Ca^{+2} entrances into spermatozoa during capacitation has been demonstrated in several species using fluorescent intracellular Ca^{+2} indicator [3].

In mammalian spermatozoa, progesterone has been shown to induce Ca^{+2} entrance into spermatozoa and AR [4], and studies confirm the presence of a non-nuclear progesterone receptor on the plasma membrane of human spermatozoa [1]. The presence of two classes of progesterone receptors has been demonstrated in human spermatozoa. One class has an elevated affinity constant in the nanomolar range and is specific for progesterone, whereas the other class

has an affinity constant in the micromolar range and binds equally well to other hydroxylated progesterone derivatives [5].

Levonorgestrel (LNG) is a progestin that has been widely used as emergency contraception (EC) and its mechanisms of action have been discussed extensively in the literature, particularly those regarding the compound's capacity to interfere or not with pre-fertilization events [6]. One hypothesis is that LNG interferes with sperm migration and/or fertilizing capacity. Sperm migration in women consists firstly of migration of some spermatozoa to the fallopian tube, aided by propulsive contractions of the genital tract, and secondly, over a period of several days, spermatozoa that have been stored in the uterine cervix migrate in successive cohorts to the fallopian tube. Only spermatozoa that have been stored in the crypts of the uterine cervix are able to fertilize the ovum following capacitation [7].

Although the mechanism of action of LNG-containing EC on spermatozoa is still poorly understood, investigators have reported that the local application of LNG in the tail of the epididymis of rats impaired the *in vivo* fertilizing potential, suggesting that the drug has a direct effect on spermatozoa [8]. Our group has reported that *in vitro* exposure of human spermatozoa to low concentrations of LNG, similar to serum levels observed after oral LNG intake for EC [9], fails to induce AR and does not interfere with other sperm functions [10-12]; however, high concentrations similar to those observed in users of the levonorgestrel-releasing intrauterine system (LNG-IUS) induced AR [13].

The objective of this study was to contribute to the understanding of the mechanisms involved in the contraceptive effect of LNG on human spermatozoa by evaluating Ca^{2+} intracellular concentration ($[Ca^{2+}]i$) in the capacitated human spermatozoa of fertile men exposed to different concentrations of LNG.

2. Materials and methods

This study was conducted at the Department of Histology and Embryology, Institute of Biology and at the Human Reproduction Unit, Department of Obstetrics and Gynecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil and was approved by the Ethical Committee of the institution. All volunteers signed an informed consent form prior to initiation of the study.

Samples of human semen were donated by 4 healthy volunteers with normal sperm analysis according to World Health Organization (WHO) criteria [14] and according to the strict definition criteria of normal sperm morphology ($\geq$14%) [15, 16]. Semen was collected in sterile plastic jars following masturbation after 3 to 5 days of sexual abstinence. Each volunteer provided 6 semen samples, making a total of 24 samples. The samples were collected at weekly or biweekly intervals, and only 4 donors were enrolled in order to reduce the known person-to-person variation that occurs in sperm capacitating speed and AR rate [17].

2.1. Procedures

2.1.1. Sperm preparation

Following complete liquefaction, the samples were washed from seminal plasma, and the motile spermatozoa were selected using a discontinuous 90-50% Percoll density gradient (Sigma, St. Louis, MO, USA; P4937), over which one layer of semen was added [18]. The sample was centrifuged for 20 min at 275 g. The bottom layer, containing the motile sperm fraction, was then washed by the same centrifugation for 10 min with 2 mL of HAM-F10 medium. After centrifugation, the resulting sperm pellet was resuspended in human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA; 9962) supplemented with 35 mg/mL bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA; A8806) and the concentration of spermatozoa in the sample was adjusted to 5 x 10^6/mL. The cells were then incubated at 37ºC under 5% CO_2 in air for 2 h in order to permit appropriate capacitation [19].

Post-incubation sperm viability was assessed by mixing one drop of sperm suspension with one drop of eosin Y solution (0.5% in phosphate buffer saline – PBS, ICN Pharmaceuticals Biochemical Division, Aurora, OH, USA) on a slide, and examining 100 spermatozoa at 400x [14].

2.1.2. Incubation with Fura/2AM

Following the capacitation procedure, sperm suspensions were incubated in the dark with 2µmol/L Fura-2/AM (Sigma, St. Louis, MO, USA; F0888) for 45 min at 37^{o}C and 5% CO_2. The extracellular Fura-2/AM was removed by centrifugation with HTF at 300g for 5mim and the pellet was resuspended in human tubal fluid

medium supplemented with 35 mg/mL bovine serum albumin (HTF-BSA). Cells were incubated for a further 15 min at 37°C and 5% CO_2, centrifuged, and resuspended at a concentration of 2.5 x 10^6 cells/mL. The Fura-2/AM loading protocols did not affect sperm motility, as determined by Baldi et al., [19]. Samples with Fura-2/AM were maintained in a darkened environment throughout the experiment.

2.1.3. Preparation of samples for incubation with LNG

After Fura-2/AM preparation, each one of the 24 samples was divided into 9 aliquots to which the following were added: tube 1: nothing was added; tube 2: LNG(13 beta-ethyl-17-alpha-ethinyl-17-beta-hydroxygon-4-en-3-one) 8 ng/mL (0.026 μM); tube 3: LNG 10 ng/mL (0.032 μM); tube 4: LNG 14 ng/mL (0.045 μM); tube 5: LNG 1,000 ng/mL (3.2 μM); tube 6: LNG 10,000 ng/mL (32 μM); tube 7: progesterone (Sigma, St. Louis, MO, USA; E8783) 11,000 ng/mL (32 μM) [20]; tube 8: EGTA (Ethylene glycol bis-β-amino-ethyl ether, Sigma, St. Louis, MO, USA; E8145) 0.1 mM, and tube 9: triton (t-octyl phenoxy polyethoxyethanol, Sigma, St. Louis, MO, USA; X100) 0.01%. Aliquots were exposed to treatment for 1 h at 37ºC and 5% CO_2.

2.1.4. Processing of $[Ca^{2+}]i$ measurement

Cells were transferred to a quartz cuvette for spectrofluorometer evaluation. Fluorescence was measured using a spectrofluorometer, (Hitachi, Tokyo, Japan,

model F-2000) set at 340 nm excitation with emission at 510 nm. Fluorescence measurements were converted to $[Ca^{2+}]i$ using the equation proposed by Grynkiewicz et al. [21]: $[Ca^{2+}]\ i = Kd\ (F- Fmin) / (Fmax-F)$ in which Kd was assumed to be a dissociation constant of Fura2/AM for Ca^{2+} of 224 nmol/L, and maximal fluorescence (Fmax) is measured by lysing the cells with 0.01% triton X100 followed by minimal fluorescence (Fmin) with 10 mmol/L EGTA, pH 10.

2.1.5. Statistical analysis

Statistical analysis was performed using the Friedman test (ANOVA nonparametric tests), and the InStat Software Package. Significance level was established at $p < 0.05$. The values are presented as mean ± standard error of the mean (SEM).

3. Results

Twenty-four samples were evaluated. The samples with human capacitated spermatozoa that were exposed for 1 h to the 5 different concentrations of LNG showed that concentrations of 8, 10, 14, and 1,000 ng/mL presented a rate of induction of $[Ca^{2+}]i$ significantly higher than the rate observed with HTF medium (as a negative control) and significantly lower than the rate obtained with natural progesterone (as a positive control). However, the exception was the LNG concentration of 10,000 ng/mL (32 μM) in which values of $[Ca^{2+}]i$ were similar to

those observed with progesterone at a concentration of 11,000 ng/mL (32 μM), (Table I).

4. Discussion

The mechanism of action of levonorgestrel-only EC is still unclear although its use is expanding worldwide, in view of its high efficacy and few reported adverse events [22]. Nikkanen et al. [8] reported that LNG applied to the epididymis of rats impaired spermatogenesis and decreased the *in vivo* fertilizing potential. Based on these findings, the possibility of LNG having an effect on some functions of spermatozoa cannot be excluded. Therefore, one of the mechanisms of action of LNG as EC may relate to its effect on spermatozoa function and their ability to interact with the oocyte. Nevertheless, several studies have evaluated the *in vitro* effect of LNG [11, 12] at doses that mimic serum levels following ingestion of the steroid as EC [9], and failed to find significant differences compared to progesterone, the natural inductor.

The present study evaluated the *in vitro* influence of different concentrations of LNG [9, 11, 12, 18] on the $[Ca^{2+}]i$ of capacitated spermatozoa because the increases $[Ca^{2+}]i$ is one of the first events described in the signal transduction cascade leading to AR in response to follicular fluid (FF), progesterone or ZP [21]. When human spermatozoa are exposed to ZP, to human FF with a high amount of natural progesterone, a pronounced rise in intracellular Ca^{2+} concentration occurs that involves the activation of voltage-dependent Ca^{2+} channels as well as the release of Ca^{2+} from intracellular stores [22].

Our group has evaluated the *in vitro* effect of LNG at different concentrations on different spermatozoa functions. The effect of the concentration 200, 400, and 800 ng/mL of LNG on the AR of capacitated spermatozoa failed to observed significant effect of LNG on AR after 15 min of exposure to the steroid [10]. However, the higher LNG concentrations and 30 min of exposure showed higher AR rates in spermatozoa *in vitro* [10]. Another experiment was carried out [11] with capacitated and non-capacitated human spermatozoa over different durations of exposure to concentrations of 1, 10, and 100 ng/mL of LNG diluted in HTF medium, and the results showed no effect on AR rates when compared to spermatozoa incubated with HTF medium alone.

These results were similar to the findings of Yeung et al. [18], who also failed to observe any effect of LNG on AR at similar doses to those used by our group [11]. However, they observed that human spermatozoa exposed to LNG underwent a dose-dependent decrease in curvilinear velocity, a significant decrease in straight-line velocity, average path velocity and linearity with the maximum concentration treatment of 100 ng/mL of LNG. In addition, the authors observed a marginal decrease in the ZP binding capacity that allowed them to conclude that LNG may affect fertilization through its direct action on sperm functions[12].

Our study showed that LNG concentrations of 8, 10, 14, and 1,000 ng/mL induced a higher rate of $[Ca^{2+}]i$ when compared to the HTF medium alone but a lower rate when compared to progesterone alone. However, when the $[Ca^{2+}]i$ induced by natural progesterone (32 μM =11,000 ng/mL, equivalent concentration

to present in the FF) was compared to that of 10,000 ng/mL of LNG, the results were similar [23]. Consequently, it may be hypothesized that at these doses, LNG *in vitro* increases [Ca^{2+}]i in human spermatozoa.

Furthermore, a recent study [12] showed that neither 1, 10, nor 100 ng/mL of LNG was able to influence the expression of specific α-D-mannose binding sites and failed to show any significant effect on spermatozoa–ZP binding or on the early development of the mouse embryo. It is therefore possible to speculate that LNG, at low concentrations, has a weak agonistic effect on progesterone receptors in human spermatozoa, reinforcing the theory that there are two classes of progesterone receptors in human spermatozoa, one class with an elevated affinity constant at the nanomolar range specific for progesterone, and another with an affinity constant at the micromolar range that binds equally well to other hydroxylated progesterone derivatives [23, 24].

Progesterone present in FF induces hyper-activation, increases spermatozoa-ZP binding, and spermatozoa AR, and, consequently, increase [Ca^{2+}]i [5]. The exact mechanism through which progesterone stimulates an increase in Ca^{2+} in spermatozoa remains to be established, but it is known that the dose-response in the relationship of progesterone action on spermatozoa intracellular Ca^{2+} concentration suggests the presence of specific receptors [5]. Indeed, [Ca^{2+}]i increased in capacitating spermatozoa in a time-dependent fashion and this effect is relevant during the *in vivo* capacitation of sperm in the human female genital tract [19].

It is therefore possible to understand why the lower concentrations of LNG tested in this study (8, 10, 14 and 1,000 ng/mL) showed a lower $[Ca^{2+}]i$ than the higher LNG concentration used (10,000 ng/mL). The 10,000 ng/mL LNG concentration may be acts in the micromolar range and results in a $[Ca^{2+}]i$ rate in the spermatozoa of approximately 90% of the total $[Ca^{2+}]i$ induced by progesterone. This finding suggests that the LNG effect may be dose-dependent effect on spermatozoa functions and fertilizing ability and this hypothesis is supported by the fact that relatively small modifications in the progesterone molecule result in a loss of potency of this response [25]. This may be one of the reasons why low concentrations of LNG, binding in a unperfected way to the progesterone receptor, fail to induce the same response as progesterone [10].

In the present study, higher concentrations of LNG induced approximately 90% of total $[Ca^{2+}]i$ when compared to natural progesterone [23]. Moreover, in a previous study [13], we observed a significantly higher expression of α-D-mannose (type III), binding sites when a dose of 10,000 ng/mL of LNG was compared to a dose of 1,000 ng/mL. A significant increase in the percentage of acrosome-reacted spermatozoa compared to controls was also observed following LNG exposure. These results confirm the hypothesis that doses of LNG >400 ng/mL stimulate AR [10], and are in agreement with the findings of Yeung et al. [18]. Consequently, LNG may also bind to the sperm progesterone receptor, stimulating $[Ca^{2+}]$ uptake and the occurrence of AR. Zona binding, $[Ca^{2+}]i$ and AR are some of the prerequisites for the interaction between spermatozoa and the oocyte

and also to fertilization [3, 24]. The fact that high doses of LNG increase the number of acrosome-reacted pattern cells [10, 13] increase $[Ca^{2+}]i$, reduce motility, and ZP binding [18] suggests that LNG may decrease sperm fertilizing capacity [26].

The present results suggest that $[Ca^{2+}]i$ may be a possible mechanism of action both of EC pills and the LNG-IUS since the concentration of 32 μM of LNG (10,000 ng/mL) used in this study to induce $[Ca^{2+}]i$ is equivalent to a progesterone concentration of 10 μg/mL which it is present in the FF. However, it has to be remembered that LNG is not a natural progesterone and other investigators have reported that even small modifications in the progesterone molecule result in a loss of the potentiality of promoting increases $[Ca^{2+}]i$ [23].

The relevance of LNG in the mechanism of action of LNG EC pills and the LNG-IUS depends on the concentration of the steroid in the uterine fluid and cervical mucus after ingestion of EC pills or following the insertion of an LNG-IUS. A large intersubject variation in plasma LNG concentrations following ingestion has been described [9], and it is known that body mass index and sex hormone-binding globulin (SHBG) can affect LNG bioavailability. Devoto et al. [27] observed that endometrial concentrations of LNG fell to almost zero at 168 h after ingestion of 1.5 mg and Miles et al. [28] showed that the endometrium responds to a critical plasma level of progesterone that is not reflected in the peripheral plasma level of the hormone. We can speculate that the same may occur in the case of LNG, and therefore the progesterone receptor and endometrial SHBG expressed in the

human endometrium and which bind LNG may play a role in the regulation of local endometrial LNG concentrations [29].

When the LNG concentration is <10,000 ng/mL (as tested), the relative relevance of the contraceptive effect of the LNG based on $[Ca^{2+}]i$ may be low. However, when the concentration of LNG used is similar to the high doses tested (10,000 ng/mL), increases $[Ca^{2+}]i$ and consequently AR may form an important component in the mechanism of action of both contraceptive methods. In conclusion, these results showed that LNG affects $[Ca^{2+}]I$ only at high concentrations, acting in the same way as progesterone, and the contribution of this effect to the mechanism of action of EC pills is still under discussion.

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References

[1] Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. Hum Reprod Update 1995;1:41-62.

[2] Flesch, FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 2000; 1469:197-235.

[3] Yanagimachi R. Mammalian fertilization. In: Knobil, E, Neill, JD, editors. The physiology of reproduction. New York: Raven Press; 1994. p. 189-318.

[4] Aitken R. Molecular mechanisms regulating human sperm function. Mol Hum Reprod 1997; 3:169-73.

[5] Calogero AE, Burrello N, Barone N, Palermo I, Grassa U, D'Agata R. Effects of progesterone on sperm function: mechanisms of action. Hum Reprod 2000;15 (Suppl1):28-45.

[6] Task Force on Postovulatory Methods of Fertility Regulation. Randomised controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. Lancet 1998;352:428-33.

[7] Croxatto HB. Gamete transport. In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, surgery, and technology. Philadelphia: Lippincott-Raven; 1996. p. 385-402.

[8] Nikkanen V, Soderstrom K, Tuusa S, Jaakkola UM. Effect of local epididymal levonorgestrel on the fertilizing ability of male rat, a model for post-testicular contraception. Contraception 2000;61:401-6.

[9] Johansson E, Brache V, Alvarez F, et al. Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. Hum Reprod 2002;17:1472-6.

[10] Bahamondes L, Nascimento JA, Munuce MJ, Fazano F. Faundes A. The *in vitro* effect of levonorgestrel on the acrosome reaction of human spermatozoa from fertile men. Contraception 2003;68:55-9.

[11] Brito KS, Bahamondes L, Nascimento JA, De Santis LH, Munuce MJ. The *in vitro* effect of emergency contraception doses of levonorgestrel on the acrosome reaction of human spermatozoa. Contraception 2005; 72:225-8.

[12] Munuce MJ, Nascimento JAA, Rosano G, Faúndes A, Saboya-Brito K, Bahamondes L. *In vitro* effect of levonorgestrel on sperm fertilizing capacity and mouse embryo development. Contraception 2005;72:71-6.

[13] Munuce MJ, Nascimento JAA, Rosano G, Faundes A, Bahamondes L. Doses of levonorgestrel comparable to that delivered by the levonorgestrel-releasing intrauterine system can modify the in vitro expression of zona binding sites of human spermatozoa. Contraception 2005; in press.

[14] World Health Organization. WHO laboratory manual for examination of human semen and semen-cervical mucus interaction. Cambridge (UK): Cambridge University Press, 1999.

[15] Krueger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology *in vitro* fertilization. Fertil Steril 1988;49:112-7.

[16] Krueger TF, Menkveld R, Stander FSH, et al. Sperm morphological features as a prognostic factor *in vitro* fertilization. Fertil Steril 1986; 46:1118-23.

[17] Perreault SD, Rogers BJ. Capacitation pattern of human spermatozoa. Fertil Steril 1982;38:258-60.

[18] Yeung WSB, Chiu PCN, Wang CH, Yao YQ, Ho PC. The effects of levonorgestrel on various sperm functions. Contraception 2002;66:453-7.

[19] Baldi E, Casano R, Falsetti C, Krauz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl 1991;12:323-30.

[20] Tesarik JMD, Mendoza C, Carreras A. Selective expression of a progesterone receptor on the human sperm surface. Fertil Steril 1992;58:784-92.

[21] Grynkiewicz G, Poenie M, Tsien RY. A new generation of ca 2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440-50.

[22] Croxatto HB, Ortiz ME, Muller AL. Mechanism of action of emergency contraception. Steroids 2003;68:1095-8.

[23] Blackmore PF, Beebe SJ, Danford DR, Alexander N. Progesterone and 17-alpha hydroxyprogesterone: novel stimulators of calcium influx in human sperm. J Biol Chem 1990; 265:1376-80.

[24] Harper CV, Kirkman-Brown JC, Barratt CL, Publicover SJ. Encoding of progesterone stimulus intensity by intracellular [Ca2+] ([Ca2+]i) in human spermatozoa. J Bioch 2003;372:407-17.

[25] Luconi M, Bonaccorsi L, Maggi M, Pecchioli P, Krausz C, Forti G, Baldi E. Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. J Cl Endocr Metabol 1998;83:877-85.

[26] Liu DY, Baker HW. Inducing the human acrosome reaction with a calcium ionophore A23187 decreases sperm zona pellucida binding with oocytes that failed to fertilize in vitro. J Reprod Fertil 1990;89:127–34.

[27] Devoto L, Fuentes A, Palomino A, Espinoza A, Kohen P, Ranta S, von Hertzen H. Pharmacokinetics and endometrial tissue levels of levonorgestrel after administration of a single 1.5-mg dose by the oral and vaginal route. Fertil Steril. 2005;84:46-51.

[28] Miles R, Paulson R, Lobo R, Press M, Dahmoush L, Sauer M. Pharmacokinetics and endometrial tissue levels of progesterone after administration by intramuscular and vaginal routes a comparative study. Fertil Steril 1994;62:485–90.

[29] Misao R, Itoh N, Mori H, Fujimoto J, Tamaya T. Sex hormone-binding globulin mRNA levels in human uterine endometrium. Eur J Endocrinol 1994;131:623–9.

Table I - Effect of different treatments on $[Ca^{2+}]i$ in human spermatozoa, measured after 1h of incubation

Exposure of capacitated spermatozoa to different treatments (N = 24 samples)	Mean ± SEM	P-value	
		HTF vs.	P vs.
Human Tubal Fluid medium	741.1 ± 19.2		
Progesterone (32 µM)	2099.7 ± 58.5	<0.001	
Levonorgestrel 8 ng/mL (0.026 µM)	981.0 ± 24.5	<0.05	<0.001
Levonorgestrel 10 ng/mL (0.032 µM)	1278.1 ± 4.7	<0.05	<0.001
Levonorgestrel 14 ng/mL (0.045 µM)	1196.7 ± 2.7	<0.05	<0.001
Levonorgestrel 1,000 ng/mL (3.2 µM)	1459.8 ± 1.3	<0.001	<0.05
Levonorgestrel 10,000 ng/mL (32 µM)	1911.5 ± 89.6	<0.001	NS

HTF: Human Tubal Fluid medium; P: Progesterone; NS: Not significant

Capítulo III - *Finalização*

CONSIDERAÇÕES E CONCLUSÕES FINAIS

Este trabalho permite as seguintes conclusões:

(1) As concentrações de 8, 10, 14 e 1.000 ng/ml de LNG foram capazes de induzir um aumento na concentração intracelular de cálcio significativamente superior ao HTF isolado, porém significativamente menor que com progesterona. Assim, é possível sugerir que embora o LNG *in vitro*, aumente a concentração intracelular de cálcio nos espermatozóides, este mecanismo, de maneira isolada, não deva ser o responsável pela indução da RA.

(2) A concentração máxima avaliada neste estudo (10.000 ng/ml de LNG) foi capaz de desencadear em torno de 90% do efeito máximo do aumento na concentração intracelular de cálcio nos espermatozóides, este mecanismo, induzido por uma concentração similar de progesterona, sugerindo que o efeito do LNG pode ser dose-dependente.

(3) As concentrações mais baixas de LNG testadas no presente trabalho (8 ng/ml, 10 ng/ml, 14 ng/ml e 1.000 ng/ml) induziram um aumento na concentração intracelular de cálcio em menor proporção quando comparadas com a maior concentração de LNG utilizada (10.000 ng/ml), a qual corresponde a ordem de micromolar, reforçando a teoria de Luconi *et al*., (1998), a qual mostrara a presença de duas classes distintas de receptores de progesterona na membrana plasmática dos espermatozóides; uma classe constituída por receptores específicos para progesterona e que atua na faixa de concentrações nanomolares e outra classe com receptores

com baixa constante de afinidade que se ligam aos progestágenos, e atuam na faixa de concentrações micromolares.

(4) Os resultados do presente trabalho permitiram compreender a idéia de uma ação adicional e complementar o LNG, sendo ele um progestágeno sintético e não progesterona natural, assim, a possível ligação de baixas concentrações de LNG no receptor específico e exclusivo da progesterona, é feita de maneira “imperfeita”, não sendo capaz de estimular um efeito igual ao da própria progesterona presente no FF.

REFERÊNCIAS BIBLIOGRÁFICAS

Aitken, R. Molecular mechanisms regulatoring human sperm function. **Molecular Human Reproduction, 1997; 3:169-173.**

Bahamondes, L., Nascimento, J. A. A.; Munuce, M. J.; Fazano, F.; Faundes, A. The *in vitro* effect of levonorgestrel on the acrosome reaction of human spermatozoa from fertile men. **Contraception, 2003; 68:55-59.**

Bailey, J. L.; Storey, B. T. Calcium influx into mouse spermatozoa activated by solubilized mouse zona pellucida, monitored with the calcium fluorescent indicator, fluo-3. Inhibition of the influx by three inhibitors of the zona pellucida induced acrosome reaction: tyrphostin A48, pertussis toxin, and 3-quinuclidinyl benzilate. **Molecular Reproduction Development, 1994; 39:297-308.**

Baldi, E.; Casano, R.; Falsetti, C.; Krauz, C.; Maggi, M.; Forti, G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. **Journal of Andrology, 1991; 12:323-30.**

Baldi, E.; Luconi, M.; Bonaccorsi, L.; Muratori, M.; Forti, G. Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. **Frontiers in Bioscience, 2000; 5:110-23.**

Bedford, J.M. Effect of duct ligation on the fertilizing ability of spermatozoa in the epididymis of the rabbit. **Journal of Experimental Zoology, 1967; 166:271-82.**

Blackmore, P. F.; Beebe, S. J.; Danford, D. R.; Alexander, N. Progesterone and 17-alpha hydroxyprogesterone: novel stimulators of calcium influx in human sperm. **The Journal of Biological Chemistry, 1990; 265:1376-80.**

Boatman, D. E. Responses of gametes to the oviductal environment. **Human Reproduction, 1997; 12 Suppl-11:133-49.**

Brito KS, Bahamondes L, Nascimento JA, De Santis LH, Munuce MJ. The *in vitro* effect of emergency contraception doses of levonorgestrel on the acrosome reaction of human spermatozoa. **Contraception 2005; 72:225-8.**

Brewis, I. A.; Clayton, R.; Barrat, C. L.; Hornby, D. P.; Moore, H. D. M. Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. **Molecular Human Reproduction, 1996; 2:583-89.**

Brucker, C.; Lipford, G. B. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. **Human Reproduction Update, 1995; 1:41-62.**

Calogero, A. E.; Burrello, N.; Barone, N.; Palermo, I.; Grassa, U.; D'agata, R. Effects of progesterone on sperm function: mechanisms of action. **Human Reproduction 2000;15 (Suppl1):28-45.**

Cheng, F. P.; Gadella, B. M.; Voorhout, W. F.; Fazeli, A. R.; Bevers, M. M.; Colenbrander, B. Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. **Biology of Reproduction, 1998; 59:733-42.**

Croxatto, H.B.; Gamete transport. **In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, Surgery, and Technology. Philadelphia: Lippincott-Raven; 1996. p. 385-402.**

Croxatto H. B, Ortiz M. E, Muller A. L. Mechanism of action of emergency contraception. **Steroids 2003;68:1095-8.**

Devoto L, Fuentes A, Palomino A, Espinoza A, Kohen P, Ranta S, von Hertzen H. Pharmacokinetics and endometrial tissue levels of levonorgestrel after administration of a single 1.5-mg dose by the oral and vaginal route. **Fertility and Sterility. 2005;84:46-51.**

Diaz, J.; Bahamondes, L.; Monteiro, I.; Petta, C.; Hidalgo, M. M.; Arce, X. E. Acceptability and performance of the levonorgestrel-releasing intrauterine system (Mirena®) in Campinas, Brazil. **Contraception, 2000; 62:59-61.**

El-Mahgoub, S. "The norgestrel-T DIU". **Contraception, 1980; 22:271-86**

Fellman, C W; Green T R; Eicher A L. The oxidation of hypotaurine to taurine: bis-aminoethyl-alfaldisulfone, a metabolic intermediate in mammalian tissue. **Advanced Experimental Medicine Biology, 1987; 210:39-48.**

Faundes, A.; Alvarez, F.; Diaz, J. A. Latin American experience with Levonorgestrel IUD. **Annals of Medicine, 1993; 25:149-53.**

Flesch, F. M.; Gadella, B.M. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. **Biochimica et Biophysica Acta, 2000; 1469:197-235.**

Foegh, M.; Nicol, K.; Petersen, I. B.; Schou, G. Clinical evaluation of long-term treatment with levonorgestrel and testosterone enanthate in normal men. **Contraception, 1980; 21:631-40.**

Foresta, C.; Rossato, M.; Di Virgilio, F. Ion fluxes through the progesterone-activated channel of the sperm plasma membrane. **Biochemical Journal, 1993; 294:279-83.**

Garcia, M. A.; Meizel, S. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. **Biology of Reproduction, 1999; 60:102-09.**

Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of ca2+ indicators with greatly improved fluorescence properties. **The Journal of Biological Chemistry, 1985; 260:3440-50.**

Harper, C.V.; Kirkman-Brown, J.C.; Barratt, C.L.; Publicover, S.J. Encoding of progesterone stimulus intensity by intracellular [Ca2+] ([Ca2+]i) in human spermatozoa. **Journal of Biochemie, 2003 Jun 1;372(Pt 2):407-17.**

Heikkilä, M.; Nylander, P.; Luukkainen, T. Body iron stores and patterns of bleeding after insertion of levonorgestrel- or a copper-releasing intrauterine device. **Contraception, 1982; 26:465.**

Johansson E, Brache V, Alvarez F *et al.* Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. **Human Reproduction, 2002;17:1472-6.**

Kaul, G.; Singh, S.; Gandhi, K. K.; Anand, S. R. Calcium requirement and time course of capacitation of goat spermatozoa assessed by chlortetracycline assay. **Andrologia, 1997; 29:243-51.**

Kruger, I. F.; Menkvel, R., Stander, E. S. H., LOmbard, C. J., Van Der Merwe, J. P., Van zyl, J. A. e Smith, K. Sperm morphological features as a prognostic factors in *in vitro f*ertilization. **Fertility and Sterility, 1986, 46:1118-23.**

Krueger, T.F.; Acosta, A.A.; Simmons, K.F.; Swanson, R.J.; Matta, J.F.; Oehninger, S. Predictive value of abnormal sperm morphology *in vitro* fertilization. **Fertility and Sterility, 1988;49:112-7.**

Leyton, L.; Saling P. 95Kd sperm proteins bind ZP3 and serves as tyrosine substrates in response to zona binding. **Cell, 1989; 57:1123-30.**

Liu DY, Baker HW. Inducing the human acrosome reaction with a calcium ionophore A23187 decreases sperm zona pellucida binding with oocytes that failed to fertilize in vitro. **Journal of Reproduction and Fertility 1990;89**:**127–34.**

Luconi M, Bonaccorsi L, Maggi M, PecchioII P, Krausz C, Forti G, Baldi E. Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. **The Journal of Clinical Endocrinology and Metabolism, 1998;83:877-85.**

Luukkainen, T.; Allonen, H.; Haukkamaa, M.; Lähteenmäki, P.; Nilsson, C. G.; Toivonen, J. Five year's experience with levonorgestrel-releasing IUDs. **Contraception, 1986; 33:139-48.**

Malette, B.; Paquete, Y.; Merlen, Y.; Bleau, G. Oviductins possess chitinase- and mucin-like domains: a lead in the search for the biological function of these oviduct-specific ZP-associating glycoproteins. **Molecular Reproduction Development, 1995; 41:384-97.**

Melendrez, C. S.; Meize,L S.; Berger, T. Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction *in vitro*. **Molecular Reproduction Development, 1994; 39:433-38.**

Miles R, Paulson R, Lobo R, Press M, Dahmoush L, Sauer M. Pharmacokinetics and endometrial tissue levels of progesterone after administration by intramuscular and vaginal routes a comparative study. **Fertility and Sterility. 1994;62:485–90.**

Misao R, Itoh N, Mori H, Fujimoto J, Tamaya T. Sex hormone-binding globulin mRNA levels in human uterine endometrium. **European Journal of Endocrinology. 1994;131:623–9.**

Munuce MJ, Nascimento JAA, Rosano G, Faundes A, Bahamondes L. Doses of levonorgestrel comparable to that delivered by the levonorgestrel-releasing

intrauterine system can modify the in vitro expression of zona binding sites of human spermatozoa. **Contraception 2005; in press.**

Munuce MJ, Nascimento JA, Rosano G, Faundes A, Saboya-Brito K, Bahamondes L. In vitro effect of levonorgestrel on sperm fertilizing capacity and mouse embryo development. **Contraception, 2005;72:71-6.**

Nikkanen, V.; Söderström, K.; Tuusa. S.; Jaakkola, U. Effect of local epididymal Levonorgestrel on the fertilizing ability of male rat, a model for post-testicular contraception". **Contraception, 2000; 61:401-6.**

Nilsson, C. G.; Allonen, H.; Diaz, J.; Luukkainen, T. Two year's experience with two levonorgestrel-releasing intrauterine devices and one copper-releasing intrauterine device: A randomized comparative performance study. **Fertility and Sterility, 1983; 39:187-92.**

Orgebin-Crist, M.-C. Maturation of spermatozoa in the rabbit epididymis. Fertilising ability and embryonic mortality in does inseminated with epididymal spermatozoa. **Annales de Biologie Animale Biochimie Biophysique, 1967; 373-89.**

Okamura, N.; Tajima, Y.; Sugita, Y. Regulation of mammalian sperm activity by bicarbonate in genital fluids. In: **New Horizons in Sperm Cell Research**. Mohri H. (ed.). Japan Sci. Soc. Press1987. pp.197-203.

Osman, R. A.; Andria, M. L.; Jones, A. D.; Meizel, S. Steroid induced exocytosis: the human sperm acrosome reaction. **Biochemical and Biophysical Research Communications, 1989; 160:828-33.**

Perreault, S.D.; Rogers, B.J. Capacitation pattern of human spermatozoa. **Fertility and Sterility, 1982;38:258-60.**

Task Force on Postovulatory Methods of Fertility Regulation. Randomised controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. **Lancet, 1998;352:428-33.**

Tesariki, J. M. D.; Mendoza, C.; Carreras, A. Selective expression of a progesterone receptor on the human sperm surface. **Fertility and Sterility, 1992; 58:784-92.**

Verhage, H. G.; Fazleabas, AT.; Donnelly, K. The *in vitro* synthesis and release of proteins by the human oviduct. **Endocrinology, 1988; 122:1639-45.**

Visconti, P. E.; Bailey, J. L.; Moore, G. D.; Pan, D.; Olds-Clarke, P. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. **Development, 1995; 121:1129-37.**

Wassarman, P. M. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. **Cell, 1999; 96:175-83.**

Wassarman, P. M. Mouse gamete adhesion molecules. **Biology of Reproduction, 1992; 46:186191.**

World Health Organization. ***WHO laboratory manual for examination of human semen and semen-cervical mucus interaction*. Cambridge (UK): Cambridge University Press, 1999.**

Yanagimachi, R. **Mammalian fertilization**. In: Physiology of Reproduction**.** Eds: Knobil E; Neill J. Raven Press, New York (1994) p.189-317.

Yeung, W.S.B.; Chiu, P.C.N.; Wang, C.H.; Yao, Y.Q.; Ho, P.C. The effects of levonorgestrel on various sperm functions. **Contraception, 2002;66:453-7.**

Capítulo IV - *Anexos*

ANEXO I – Aprovação pelo Comitê de Ética.

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA
Caixa Postal 6111
13083-970 Campinas-SP
(0_19) 3788-8936
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cep@head.fcm.unicamp.br

PARECER CIRCUNSTANCIADO DE PROJETO DE PESQUISA ANALISADO PELO COMITÊ DE ÉTICA EM PESQUISA DA FCM/UNICAMP

IDENTIFICAÇÃO		
1. Título do Projeto: Efeito da adição de levonorgestrel (LNG) sobre o influxo de cálcio em espermatozóides de homens férteis.		
2. Pesquisador Responsável: Luciana Helena de Santis		
3. Instituição do Pesquisador: Departamento de Histologia e Embriologia do Instituto de Biologia - UNICAMP		
4. Local onde está será realizada a Pesquisa: Laboratório de Embriologia Experimental do Departamento de Histologia e Embriologia		
5. Nº de inscrição no CEP/FCM: **420**/2003.	**6.** Grupo:	**7.** Data de apresentação ao CEP: / /200 .

OBJETIVOS
8. - Avaliar "in vitro" o efeito dediferentes concentrações do progestágeno LNG sobre o influxo de cálcio em espermatozóides já capacitados de homens férteis - Determinar "in vitro" uma curva dose-resposta, relacionando a concentração de LNG e o influxo de cálcio em espermatozóides "in vitro" já capacitados de homens férteis. - Sugerir uma concentração de LNG para administração intra-útero ou sistemicamente que induza reação acrossômica em 100% dos espermatozóides - Frente aos resultados, discutir o mecanismo de ação do efeito contraceptivo do LNG.

SUMÁRIO DO PROJETO
9. Os autores darão continuidade a uma linha de pesquisa envolvendo utilização e avaliação da eficácia do LNG como método contraceptivo, já utilizado como DIU-LNG. Os autores demonstraram em estudo anterior em que o LNG induz a reação acrossômica em espermatozóides capacitados de homens férteis, mas o mecanismo de ação desse evento ainda não foi elucidado .Serão estudados espermatozóides de voluntários sadios, com paternidade comprovada , fornecidos em 6 amostras de sêmen, sendo uma por semana, por masturbação, em local apropriado do Centro de assistência à Saúde da Mulher- CAISM - UNICAMP. Os pacientes serão ressarcidos dos gastos com a locomoção.

COMENTÁRIOS DO RELATOR
10. O estudo está descrito adequadamente. O Termo de Consentimento está adequado e satisfatório, apenas será necessário acrescentar os números de telefone para contato com o autor e Comitê de Ética em Pesquisa - FCM - UNICAMP..

PARECER FINAL		
☒ Recomendo a aprovação	☐ Não recomendo a aprovação	☐ Em pendência
11.		

Campinas, 9 de outubro de 2003.	Nome e assinatura do(s) membro(s) relator(es) do CEP:

ANEXO II – Carta de consentimento informado.

UNICAMP

PESQUISADORA RESPONSÁVEL:
Luciana Helena de Santis
☎ (0__19) 3788-9358
(0__19) 97457179

COMITÊ DE ÉTICA EM PESQUISA
✉ Caixa Postal 6111
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Eu, _____ fui informado que a finalidade deste estudo é melhorar o conhecimento em relação ao efeito de um componente hormonal usado em muitos anticoncepcionais (Levonogestrel) sobre a capacidade de fertilização dos espermatozóides humanos.

Entendo que serei convidado a fornecer seis amostras de sêmen, sendo uma por semana, em dias e horas a serem determinados. Por cada amostra de sêmem serei ressarcido com um valor de R$ 50,00 visando compensar os meus gastos com transporte e alimentação a cada dia de coleta. Também me foi esclarecido que estas amostras deverão ser obtidas por masturbação, em ambiente próprio existente no Centro de Assistência a Saúde da Mulher (CAISM) da UNICAMP. Esta pesquisa não trará nenhum benefício para mim e apenas estarei contribuindo para o progresso da ciência. Também me foi esclarecido que o sêmen não será utilizado para outros fins que o desta pesquisa, sendo descartado ao fim de cada experimento.

Também me foi esclarecido que serei compensado com os gastos de transporte e que me será fornecida uma declaração de comparecimento para que eu possa apresentá-la no meu trabalho a cada dia de coleta do sêmen.

Fui esclarecido e informado a respeito deste estudo. Eu tenho e continuarei tendo oportunidade de esclarecer qualquer dúvida sobre a pesquisa e sobre minha participação nela, devendo receber respostas satisfatórias às perguntas que nesse sentido forem feitas por mim. Eu concordo em participar voluntariamente do estudo anteriormente citado. Também fui informado que posso interromper minha participação a qualquer momento da pesquisa ou que posso me recusar a participar, e que isto não trará nenhum prejuízo nos atendimentos futuros na UNICAMP. Todos os dados em relação a meu nome serão mantidos de forma sigilosa.

_____ _____

Assinatura do voluntário Assinatura do pesquisador

Data

_____ _____/_____/_____

Testemunha

ANEXO III: Demais Produção Científica durante o Mestrado.

ELSEVIER

Contraception

Contraception 72 (2005) 225–228

Original research article

The in vitro effect of emergency contraception doses of levonorgestrel on the acrosome reaction of human spermatozoa

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Abstract

Introduction: The aim of this study was to evaluate the effect of three concentrations of levonorgestrel (LNG) comparable to the levels found in serum following ingestion of LNG as emergency contraception (EC) on the acrosome reaction (AR) of capacitated and noncapacitated spermatozoa of fertile men.

Materials and Methods: A total of 24 semen samples from three fertile men were evaluated. The spermatozoa were selected by Percoll gradient. Twelve samples were subsequently incubated with human tubal fluid medium supplemented with bovine serum albumin (HTF/BSA) for 20 h under capacitating conditions. The capacitated spermatozoa and the spermatozoa from the remaining 12 samples were exposed to LNG at 1, 10 and 100 ng/mL, to follicular fluid (FF) (20 %v/v) and to HTF medium. The ratio of live to dead spermatozoa was assessed after 1, 2 and 3 h of incubation at 37°C and 5% CO_2. After 30 min of exposure to the different LNG concentrations, aliquots were divided into two parts. In the first part, spermatozoa were immediately stained with Hoescht 33258 and fluorescein isothiocyanate–pisum sativum agglutinin (FITC-PSA) in order to assess AR rate and to repeat evaluation of the live-to-dead ratio. After 3 h of incubation, the remaining part of the aliquots were submitted to the same procedures. Each concentration of LNG was then compared with FF and HTF medium as positive and negative controls, respectively.

Results: The results showed that in vitro exposure to the three different LNG concentrations did not induce AR.

Conclusion: This study failed to show any in vitro effect on AR of LNG concentrations similar to those found in serum following intake of LNG as EC. If this effect exists or if there is any other that influences sperm fertilizing capacity, in vitro experiments are probably not an appropriate way of testing it.

Keywords: Acrosome reaction; Human spermatozoa; Levonorgestrel

1. Introduction

The mechanisms of action of levonorgestrel (LNG) alone as emergency contraception (EC) are widely discussed in the literature, particularly those regarding the compound's capacity to interfere or not with prefertilization events [1]. The period during which EC may be able to exert an effect consists of the 5 days preceding ovulation and the day of ovulation itself. This is the period during which women are able to become pregnant [2]. One hypothesis is that LNG interferes with sperm migration and/or fertilizing capacity. Sperm migration in women consists firstly of migration of some spermatozoa, aided by propulsive contractions of the genital tract to the fallopian tube, and, secondly, over a period of several days, spermatozoa that have been stored in the uterine cervix migrate in successive cohorts to the fallopian tube. Only spermatozoa stored in the crypts of the uterine cervix are able to fertilize the ovum after capacitation [3].

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A previous study has shown that administration of 400 μg of LNG 3–10 h after coitus affects sperm migration between 3 and 9 h following treatment, reduces the number of spermatozoa recovered from the uterine cavity, increases the pH of the uterine fluid (immobilizing spermatozoa) and increases the viscosity of the cervical mucus (preventing further passage of sperm cells into the uterine cavity) [4].

Emergency contraception with LNG is more effective than the Yuzpe regimen in preventing accidental pregnancies. In addition, it is more effective if administered within 72 h after intercourse and if the intake occurs shortly after sexual intercourse [5–7]. Although the mechanism of action of EC with LNG is still poorly understood, there is some evidence that it interferes with certain ovulatory functions [8–11], and that it affects cervical mucus and sperm penetration [12].

Our group has reported that in vitro exposure of human spermatozoa to different concentrations of LNG induces acrosome reaction (AR) [13]; however, the concentrations used in that experiment were higher than the levels observed in serum after intake of LNG as EC [7]. Acrosome reaction is part of the fertilization process of the human ovum, involving the fusion of sperm membranes and the release of the hydrolytic acrosomal enzymes required for egg penetration [12].

Recently, Yeung et al. [14] failed to observe any influence of LNG in concentrations that mimic serum levels of LNG following ingestion as EC, either on AR or on zona pellucida (ZP) penetration. Our hypothesis was that these findings were different from those observed in our previous publication [13] because Yeung et al. [14] did not capacitate spermatozoa overnight, which is a prerequisite for both partial and complete AR [15], and also because they did not use positive controls (with progesterone or follicular fluid (FF) [16]) to test whether all experiments had been adequately conducted. Moreover, Calvo et al. [17] showed that a 30-min exposure to an AR inducer is sufficient to achieve AR induction, and Tesarik et al. [18] showed that most of the spermatozoa undergoing ionophore-induced AR retain the material that reacts with pisum sativum agglutinin (PSA) in the equatorial acrosomal region for at least 30 min postreaction, but that a considerable loss of this material occurs during further incubation. Therefore, we hypothesized that a 3-h exposure to controls and to LNG concentrations, as in the protocol of Yeung et al. [14], would be too long and that the AR rate found may not have been accurate because cells that had reacted earlier would not have been accounted for. Therefore, we conducted the present study with the objective of evaluating the AR rate in the capacitated and noncapacitated human spermatozoa of fertile men exposed to the same concentrations of LNG used previously by Yeung et al. [14] at different times of exposure.

2. Materials and methods

This study was conducted at the Human Reproduction Unit, Department of Obstetrics and Gynecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil, and was approved by the Ethical Committee of the institution. All volunteers signed an informed consent form prior to initiation of the study.

Twenty-four semen samples were obtained from three healthy volunteers with normal sperm analysis according to World Health Organization (WHO) criteria [19] and according to the strict definition criteria of normal sperm morphology (≥14%) [20,21]. Semen was collected by masturbation in sterile plastic containers after 3 to 5 days of sexual abstinence. Each volunteer provided eight semen samples. Only three donors were enrolled in order to reduce the known person-to-person variation that occurs in sperm capacitating speed and the rate of AR [22].

2.1. Procedures

2.1.1. Sperm preparation

Motile spermatozoa were selected using a two-step Percoll technique according to the protocol followed by Yeung et al. [14]. After sperm selection, the resulting sperm pellet was resuspended in 4 mL of human tubal fluid medium supplemented with bovine serum albumin (HTF-BSA) in order to promote optimum nutrient content for the incubation period as well as dilution of the spermatozoa [13]. Twelve samples were then incubated at 37°C under 5% CO_2 atmosphere for 20 h for capacitation. The remaining 12 samples were used in the experiments without undergoing capacitation.

2.1.2. Preparation of samples for incubation with LNG

After sperm preparation, each of the 24 samples was divided into five aliquots and incubated as follows: (1) 500 μL HTF medium with spermatozoa; (2) 499 μL HTF medium with spermatozoa and 1 μL (1 ng/mL) LNG work solution (500 ng/mL); (3) 490 μL HTF medium with spermatozoa and 10 μL (10 ng/mL) LNG work solution (500 ng/mL); (4) 400 μL HTF medium with spermatozoa and 100 μL (100 ng/mL) LNG work solution (500 ng/mL); and (5) 400 μL HTF medium with spermatozoa and 100 μL follicular fluid (FF) (20% v/v). Aliquots with capacitated and noncapacitated spermatozoa were exposed to treatment for 3 h at 37°C at 5% CO_2, and live-to-dead ratio was assessed every hour.

2.1.3. Assessment of spermatozoa AR

Samples were stained with Hoescht 33258 (bis-Benzidime; Sigma) at 37°C under 5% CO_2 in air for 10 min. Fluorescein isothiocyanate–pisum sativum agglutinin staining and AR evaluation were conducted as previously described [13,14]. Acrosomal reaction was assessed at 30 min and at 3 h of exposure to the different LNG concentrations.

2.2. Statistical analysis

Means and standard error of the means (SEM) were calculated for each concentration for both capacitated and noncapacitated samples. ANOVA nonparametric tests were

Table 1
Effect of different treatments and different exposure times on sperm acrosome reaction (percentage) ($n=24$)

Treatments	Mean±SEM	p value	
		HTF vs.	FF vs.
Capacitated spermatozoa (20 h of incubation)+30 min of exposure to treatments (N=8 samples)			
HTF	2.63±0.28		
FF	6.13±0.69	<.001	
LNG			
1 ng/mL	3.19±0.41	NS	<.05
10 ng/mL	3.44±0.43	NS	<.05
100 ng/mL	3.75±0.39	NS	NS
Capacitated spermatozoa (20 h of incubation)+3 h of exposure to treatments (N=12 samples)			
HTF	3.95±0.57		
FF	4.82±0.39	NS	
LNG			
1 ng/mL	3.36±0.69	NS	NS
10 ng/mL	3.82±0.40	NS	NS
100 ng/mL	4.05±0.52	NS	NS
Noncapacitated spermatozoa+30 min of exposure to treatments (N=8 samples)			
HTF	3.19±0.33		
FF	7.00±0.42	<.001	
LNG			
1 ng/mL	5.25±0.40	NS	NS
10 ng/mL	3.88±0.35	NS	<.05
100 ng/mL	3.19±0.73	NS	<.001
Noncapacitated spermatozoa+3 h of exposure to treatments (N=12 samples)			
HTF	3.29±0.33		
FF	4.25±0.50	NS	
LNG			
1 ng/mL	4.54±0.42	NS	NS
10 ng/mL	4.21±0.58	NS	NS
100 ng/mL	3.88±0.46	NS	NS

performed to assess differences between treatments. The level of significance was established at $p<.05$.

3. Results

The results showed that the samples with capacitated and noncapacitated spermatozoa, which were exposed to controls (HTF medium and FF) and to the three different measurements of LNG for 30 min, presented significant induction of AR by FF when compared to the others. However, when the AR rate was assessed after 3 h of exposure to LNG, the values found for all measurements were similar, and there was no statistically significant difference. Nonetheless, exposure to different doses of LNG failed to induce significant AR comparable to the values induced by FF at either of the incubation periods (Table 1).

4. Discussion

The present study supports the findings of Calvo et al. [17] and Tesarik et al. [18] that a 30-min exposure to an AR inducer is sufficient to induce AR, and that most of the spermatozoa undergoing ionophore-induced AR retain the material that reacts with PSA in the equatorial acrosomal region for at least 30 min postreaction. However, a considerable loss of this material occurs during further incubation, which explains the low AR rates found when the same sample was assessed after 30 min and following longer periods of incubation.

Our study also confirmed the data reported by Yeung et al. [14] that concentrations of 1, 10 and 100 ng/mL failed to induce AR in vitro, although that study design was not totally appropriate since the time of exposure to the different LNG measurements was inadequate, and the samples were not exposed to FF as positive control.

The mechanism of action of EC with LNG alone is still unclear, although its use is expanding worldwide, especially because of its high efficacy and few reported adverse events [1]. Few studies have evaluated the influence of LNG on sperm function [4,13,14]; however, it is possible to speculate that one of the mechanisms of action may be its effect on spermatozoa. In women, it has been demonstrated that the fertile days of the menstrual cycle are the day of LH peak and the 5 days preceding ovulation [2]. Spermatozoa deposited in the vagina during intercourse remain in the endocervix for many hours or days before ascending to the fallopian tubes, and only these cells have the ability to fertilize [3]. Consequently, spermatozoa deposited in the vagina of women who receive LNG as EC could be exposed to unknown concentrations of LNG for hours or days, and this may influence their fertilizing capacity.

In accordance with the study carried out by Kesseru et al. [4], who observed a reduction in the number of spermatozoa recovered from the uterus after administration of LNG in vivo, Yeung et al. [14] observed that 1 to 100 ng/mL of LNG in vitro provoked a decrease in sperm motility and in the number of spermatozoa bound to the ZP, as well as lower fusion capacity as tested by the zona-free hamster oocyte test.

However, a recent study conducted by our group showed that neither 1, 10 nor 100 ng/mL of LNG was able to influence the expression of specific α-D-mannose binding sites. Moreover, these doses were unable to modify the number of spermatozoa tightly bound to the human ZP or influence the mouse embryo development [23].

Progesterone present in FF induces sperm AR, hyperactivation, and increases sperm binding to the ZP [3]. Although LNG has a weak agonistic effect on the progesterone receptors in sperm, we have previously reported that when capacitated spermatozoa were incubated for up to 30 min with 200 to 800 ng/mL of LNG, spermatozoa underwent induced AR [6]. Thus, it is possible to speculate that at this range of concentrations, the progesterone receptor is able to recognize the progestin molecule and exert its effect, causing spermatozoa to lose their acrosomes and, consequently, their fertilizing capacity [7]. However, the plasmatic concentrations of LNG following its administration in the form of EC pills [7] were 8.4 and 12.5 ng/mL after ingestion of two

doses of 0.75 mg, 12 h apart, or after the ingestion of a single dose of 1.5 mg, respectively. These levels are lower than those tested by us in a previous study [13]. At this low range of LNG concentrations, the progesterone receptor is probably unable to recognize the progestin molecule and, consequently, cannot exert its effect, AR does not occur and there is no influence in the fertilizing capacity of spermatozoa [24].

In conclusion, our study showed that exposure to different concentrations of LNG, similar to those observed in the serum of women following ingestion of LNG as EC, was unable to induce sperm AR in vitro. If this effect exists or if there is another effect that influences sperm fertilizing capacity, in vitro experiments are probably not the best approach for testing it.

Acknowledgments

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References

[1] Croxatto HB, Ortiz ME, Muller AL. Mechanism of action of emergency contraception. Steroids 2003;68:1095–8.
[2] Wilcox AJ, Weinberg CR, Baird DD. Timing of sexual intercourse in relation to ovulation: effects on the probability of conception, survival of the pregnancy, and sex of the baby. N Engl J Med 1995;333:1517–21.
[3] Croxatto HB. Gamete transport. In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, surgery, and technology. Philadelphia: Lippincott-Raven; 1996. p. 385–402.
[4] Kesseru E, Garmendia F, Westphal N, Parada J. The hormonal and peripheral effects of DL-norgestrel in post-coital contraception. Contraception 1974;10:411–24.
[5] Hertzen HV, Van Look PFA. Randomised controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. Task force on postovulatory methods of fertility regulation. Lancet 1998;353:428–33.
[6] Piaggio G, von Hertzen H, Grimes DA, van Look PFA. Timing of emergency contraception with levonorgestrel or the Yuzpe regimen. Lancet 1999;353:721.
[7] Johansson E, Brache V, Alvarez F, et al. Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. Hum Reprod 2002;17:1472–6.
[8] Hapangama D, Glasier AF, Baird DT. The effects of peri-ovulatory administration of levonorgestrel on the menstrual cycle. Contraception 2001;63:123–9.
[9] Durand M, Craviotto MC, Raymond EG, et al. On the mechanisms of action of short-term levonorgestrel administration in emergency contraception. Contraception 2001;64:227–34.
[10] Landgren BM, Johannisson E, Aedo AR, Kumar A, Shi YE. The effect of levonorgestrel administered in large doses at different stages of the cycle on ovarian function, and endometrial morphology. Contraception 1989;39:275–89.
[11] Croxatto HB, Fuentealba B, Brache V, et al. Effects of the Yuzpe regimen, given during the follicular phase, on ovarian function. Contraception 2002;65:121–8.
[12] Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. Physiology of reproduction. New York: Raven Press; 1994. p. 189–318.
[13] Bahamondes L, Nascimento JA, Munuce MJ, Fazano F, Faundes A. The in vitro effect of levonorgestrel on the acrosome reaction of human spermatozoa from fertile men. Contraception 2003;68:55–9.
[14] Yeung WSB, Chiu PCN, Wang CH, Yao YQ, Ho PC. The effects of levonorgestrel on various sperm functions. Contraception 2002;66:453–7.
[15] Bijay SJ, Cohen-Dayag A, Tur-Kaspa I, Eisenbach M. Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction. FEBS Lett 1998;427:309–13.
[16] Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. Hum Reprod Update 1995;1:51–62.
[17] Calvo L, Vantman D, Banks MS, et al. Follicular fluid-induced acrosome reaction distinguishes a subgroup of men with unexplained infertility not identified by semen analysis. Fertil Steril 1989;52:1048–54.
[18] Tesarik J, Mendoza C, Carreras A. Fast acrosome reaction measure: a highly sensitive method for evaluating stimulus-induced acrosome reaction. Fertil Steril 1993;59:424–30.
[19] World Health Organization. WHO laboratory manual for examination of human semen and semen–cervical mucus interaction. Cambridge (UK): Cambridge University Press; 1999.
[20] Krueger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in vitro fertilization. Fertil Steril 1988;49:112–7.
[21] Krueger TF, Menkveld R, Stander FSH, et al. Sperm morphological features as a prognostic factor in in vitro fertilization. Fertil Steril 1986;46:1118–23.
[22] Perreault SD, Rogers BJ. Capacitation pattern of human spermatozoa. Fertil Steril 1982;38:258–60.
[23] Munuce MJ, Nascimento JAA, Rosano G, Faúndes A, Saboya-Brito L, Bahamondes L. In vitro effect of levonorgestrel on sperm fertilizing capacity and mouse embryo development. Contraception 2005;72:71–6.
[24] Liu DY, Baker HW. Inducing the human acrosome reaction with a calcium ionophore A23187 decreases sperm zona pellucida binding with oocytes that failed to fertilize in vitro. J Reprod Fertil 1990;89:127–34.