JOSIANE APARECIDA ANDRADE DO NASCIMENTO

MODELO *IN VIVO* PARA AVALIAR A REAÇÃO ACROSSOMAL DE ESPERMATOZÓIDES HUMANOS E A EXPRESSÃO DE GLICODELINA-A NO ENDOMÉTRIO APÓS A ADMINISTRAÇÃO ORAL DE LEVONORGESTREL PARA ANTICONCEPÇÃO DE EMERGÊNCIA

Tese de Doutorado

ORIENTADOR: Prof. Dr. LUIS GUILLERMO BAHAMONDES

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ORIENTADOR: Prof. Dr. LUIS GUILLERMO BAHAMONDES

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Curso de Pós-Graduação em Tocoginecologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas

Data: 26/02/2007

Dedico este trabalho...

...Aos meus familiares que são tudo para mim.

...À minha mãe biológica Maria Augusta de Andrade (in memoriam)
que tinha como sonho primeiro possibilitar os meus estudos...

Agradecimentos

Ao Dr. Luis Bahamondes, meu orientador, a oportunidade de realização deste trabalho e também a orientação, o apoio, o exemplo de profissionalismo, de competência, disciplina, generosidade e ética.

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"Cem vezes por dia eu me lembro de que minha vida interior e minha vida exterior dependem do trabalho que outros homens estão fazendo agora. Por causa disso, preciso me esforçar para retribuir pelo menos parte dessa generosidade – e não posso deixar nenhum minuto vazio".

(Albert Einstein)

Sumário

Símbolos, Siglas e Abreviaturas

AE	Anticoncepção de emergência
AR	*Acrossomal reaction*
CAISM	Centro de Atenção Integral à Saúde da Mulher
EC	*Emergency contraception*
Kda	QuiloDalton
LH	Hormônio luteinizante
LNG	Levonorgestrel
OMS	Organização Mundial da Saúde
RA	Reação acrossomal
SEM	*Standard error of the mean*
SIU-LNG	Sistema intrauterino liberador de LNG
Unicamp	Universidade Estadual de Campinas
WHO	*World Health Organization*

Resumo

Os objetivos foram avaliar a reação acrossomal (RA) de espermatozóides recuperados depois de lavado uterino, medir o levonorgestrel (LNG) no soro e no lavado uterino e avaliar a expressão da glicodelina-A endometrial após a administração do LNG para anticoncepção de emergência (AE). **MATERIAIS E MÉTODOS:** Quarenta e oito experimentos foram realizados em 14 mulheres menstruando regularmente. Quatro grupos foram formados diferindo entre eles os intervalos coito–tratamento e tratamento–recuperação dos espermatozóides e obtenção da biópsia. **RESULTADOS:** A concentração dos espermatozóides recuperados a partir da cavidade uterina 24 ou 48 horas após o tratamento foi de 14,5 $\pm$ 3,9 x 10^6 e 17,3 $\pm$ 6,8 x 10^6 células/ml, respectivamente. Não houve diferenças entre a taxa de RA e a intensidade da expressão da glicodelina-A endometrial nos ciclos tratados com LNG ou placebo. Após 24 horas da administração do LNG o valor no fluido de lavagem da cavidade uterina representou 1,38% dos valores observados no soro. **CONCLUSÕES:** A RA ou a expressão da glicodelina-A endometrial não foram influenciadas pelo LNG após 24 ou 48 horas da sua administração para AE. O LNG não prejudicou o muco

cervical uma vez que espermatozóides viáveis foram encontrados no trato genital feminino 36-60 horas após o coito e 24-48 horas após a ingestão do LNG. O mecanismo de ação do LNG para AE permanece enigmático.

Palavras-chave: anticoncepçaão de emergência/ levonorgestrel/ espermatozóide humano/ reação acrossomal/ glicodelina-A.

Summary

The objectives were to assess acrosomal reaction (AR) of spermatozoa following flushing the uterus, to measure levonorgestrel (LNG) in serum and in the flushing fluid of the uterus and the endometrial glycodelin-A expression after administration of LNG as emergency contraception (EC). **MATERIALS AND METHODS:** Forty-eight experiments were conducted on 14 regularly menstruating women. Four groups were formed based on different intercourse - treatment interval and treatment – recovery of spermatozoa and the biopsies. **RESULTS:** Spermatozoa recovered from uterus 24 or 48 hours after treatment were 14.5 $\pm$ 3.9 x 10^6 and 17.3 $\pm$ 6.8 x 10^6 cells/ml, respectively. There were no differences between the AR rate and the endometrial glycodelin-A staining intensity on LNG or placebo treated cycles. LNG at uterine flushing medium represents 1.38% of the values observed in serum at 24 hours after LNG intake. **CONCLUSIONS:** AR status or glycodelin-A were not influenced after 24 or 48 hours of administration of 1.5 mg of LNG as EC indicating no significant effect. LNG did not impair the cervical mucus either because viable spermatozoa were found in the genital

tract 36-60 hours after coitus and 24-48 hours after LNG intake. The mechanism of action of LNG as EC remains enigmatic.

Key-words: emergency contraception/ levonorgestrel/ human spermatozoa/ acrosome reaction/ glycodelin-A.

1. Introdução

O Levonorgestrel (LNG) é um progestágeno sintético (Blackmore *et al*., 1990) usado como contraceptivo feminino. Durante as décadas de 70 e 80, muitos estudos foram conduzidos usando-se várias doses do componente para a investigação da eficácia e dos efeitos colaterais do LNG quando ele era administrado pós-coito (Kesserü *et al*., 1973; Canzler *et al*., 1984).

O LNG é comercializado em vários países em pílulas para anticoncepção de emergência (AE) por mulheres que tiveram coito sem proteção anticoncepcional, ou que sofreram abuso sexual, ou por mulheres que possuem razões para acreditar em uma eventual falha do método anticoncepcional adotado. Um estudo desenvolvido pela organização mundial da saúde (OMS) avaliou a taxa de gravidez após o uso do regime de Yuzpe (com contraceptivos orais combinados) e o regime com LNG puro para AE, e mostrou que o segundo regime foi mais eficaz, evitando um maior número de gravidezes. Esse estudo, também mostrou que o regime de LNG foi mais eficaz quanto mais próximo do coito ele era administrado (Von Hertzen e Van Look, 1998).

O LNG é administrado oralmente na forma de dois comprimidos de 0,75 mg cada um, ingeridos em intervalos de 12 horas, e até as 72 horas seguintes ao coito não protegido (Tremblay *et al.*, 2001). Também o LNG pode ser administrado em uma única dose de 1,5 mg, conforme recentes estudos demonstraram que este regime pode ser seguramente simplificado sem nenhuma diferença na eficácia ou aumento nos efeitos colaterais (WHO, 1998; Von Hertzen, *et al.*, 2002; Cheng *et al.*, 2004; Devoto *et al.,* 2005; Gainer *et al.,* 2006). Um estudo que avaliou a farmacocinética do LNG para AE mostrou que os níveis plasmáticos do progestágeno permaneceram constantes após 12 e 24 horas da administração das pílulas (Tremblay *et al.*, 2001).

Embora o LNG seja amplamente utilizado como AE, o seu exato mecanismo de ação é ainda pouco compreendido e tem sido alvo de extensas discussões (WHO, 1998; Croxatto *et al.*, 2003; Muller *et al.*, 2003; Gemzell-Danielsson e Marions, 2004). Provavelmente, como todos os contraceptivos hormonais, as pílulas de LNG para AE agem através de mecanismos múltiplos que dependem do tempo de sua administração e da fase do ciclo menstrual em que a mulher se encontra (Grimes *et al.*, 2002).

Os mecanismos de ação especulados se referem a alterações no surgimento do pico do hormônio luteinizante (LH) e na ovulação, no desenvolvimento folicular e do corpo lúteo, no espessamento do muco cervical o qual poderia interferir na penetração espermática e transporte, e também na interferência com os eventos da fertilização (Swahn *et al.*, 1996; Durand *et al.*, 2001; Hapangama *et al.*, 2001; Croxatto *et al.*, 2001; Marions *et al.*, 2002).

Possíveis hipóteses que explicariam especificamente o efeito contraceptivo do LNG seriam o efeito local desse progestágeno (Nilsson *et al.*, 1978a) sobre o endométrio, o muco cervical, a penetração dos espermatozóides no muco cervical e a motilidade da tuba uterina (Nilsson e Luukkainen, 1977). Isso, porque a supressão da ovulação é um efeito observado em apenas 50% dos casos (Nilsson *et al.,* 1980c).

Entre os possíveis alvos da AE hormonal as alterações histológicas e bioquímicas no endométrio são fatores de grande interesse para estudo uma vez que podem interferir com a implantação do embrião. Entretanto, os resultados disponíveis falharam em mostrar algum impacto biológico consistente sobre os marcadores de receptividade uterina (Landgren *et al.,* 1989; Durand *et al.*, 2001; Ugocasi *et al.,* 2002).

Foi especulada a hipótese de que o LNG pudesse interferir com a histologia e morfologia do endométrio, diminuindo desta forma a receptividade para a implantação embrionária (Kesserü *et al.*, 1974; Yuzpe *et al.*, 1974; Ling *et al.*, 1979; Ling *et al.*, 1983; Kubbar *et al.*, 1986; Young *et al.*, 1994), mas os resultados de um estudo (Durand *et al.,* 2001) não apoiaram essa idéia de que o LNG usado em AE causaria um efeito contraceptivo anti-implantacional, pois não foram observadas alterações histológicas no endométrio após a administração da medicação por um curto tempo.

Outro possível efeito do LNG na AE seria o de alterar a expressão de algumas proteínas no endométrio. Uma dessas proteínas é a glicodelina-A, cuja

expressão é regulada pelos níveis de progesterona (Seppala *et al*., 2002). Glicodelina é uma glicoproteina de 28 KDa contendo 180 aminoácidos (Julkunen *et al*., 1988) e sintetizada a partir de um único gene localizado no cromossomo 9 (região cromossômica 9q34) (Van Cong *et al*., 1991). A Glicodelina-A é uma de três isoformas existentes (Dell *et al*., 1995) e é localizada no epitélio superficial e glandular do endométrio (Julkunen *et al*., 1986a). Consiste no principal produto das células epiteliais do endométrio na fase secretória e fica bastante diminuída na fase folicular do ciclo menstrual (Richlin *et al.,* 2002)**.** Durante a fase peri-ovulatória normal a glicodelina-A não está presente no endométrio, e tem sua expressão máxima apenas durante as duas últimas semanas da fase lútea (Julkunen, *et al.,* 1986; Brown *et al.,* 2000). Entre os dias 4-5 pós-ovulação a secreção de glicodelina está associada com um aumento na secreção de progesterona ovariana. O nível máximo de glicodelina é observado no dia 12 pós-ovulatório (Julkunen *et al*., 1986a).

A Glicodelina é secretada dentro da cavidade uterina e é considerada, entre outras proteínas, como uma facilitadora da implantação embrionária (Clark *et al*., 1996). As variações na expressão da glicodelina durante os ciclos ovulatórios são decorrentes da produção de progesterona (Julkunen *et al*., 1986b).

A ausência da glicodelina no período próximo da ovulação (janela fértil estrógeno-dominante) é importante para o sucesso da concepção (Clark *et al*., 1996). Fisiologicamente, a glicodelina está relacionada a alguns processos biológicos, pois inibe, de maneira dose-dependente, a ligação do espermatozóide humano na zona pelúcida do oócito (Oehninger *et al*., 1995).

E além da propriedade contraceptiva da glicodelina há também a hipótese de que a glicodelina possua atividade imunossupressora e bloqueie a atividade das células *natural killer* (NK) (Okamoto *et al*., 1991). No momento da implantação embrionária os níveis de glicodelina são elevados (Julkunen *et al*., 1985) e isso pode proteger o embrião da destruição por parte das células NK (Li *et al*., 1993).

A síntese da glicodelina-A pode ser induzida durante a janela fértil através da administração de progestágenos (Seppala, 2004). Neste período de janela fértil normal, a expressão da glicodelina-A no endométrio não está presente, e uma expressão inadequada da mesma foi observada em mulheres usuárias do SIU-LNG (sistema intra-uterino liberador de levonorgestrel) e de implantes subdérmicos liberadores de LNG em decorrência da entrega constante de LNG no organismo dessas mulheres (Mandelin *et al.,* 1997; 2001). Esta informação é de grande relevância porque uma vez que a glicodelina-A é uma potente inibidora da ligação entre espermatozóide e zona-pelúcida (Oehninger *et al.,*1995) e a sua expressão continuada em função do LNG pode propiciar um efeito contraceptivo.

Em acréscimo aos estudos dos possíveis mecanismos de ação do LNG em AE, o nosso grupo trabalhou com a hipótese de que o LNG pudesse interferir com a capacidade fertilizante dos espermatozóides (Bahamondes *et al.,* 2003; Brito *et al.,* 2005; Munuce *et al*., 2006) através de um efeito direto sobre as funções espermáticas. Esta especulação foi decorrente: i) do conhecimento de que a progesterona natural possui efeito direto sobre os espermatozóides; ii) de um estudo prévio (Bahamondes *et al.,* 2003) ter nos mostrado que altas concentrações de LNG foram capazes de desencadear reação acrossomal (RA)

em espermatozóides capacitados *in vitro* após 30 minutos de exposição ao progestágeno*;* e iii) não haver relatos na literatura sobre a quantificação da concentração de LNG especificamente no fluido da cavidade uterina após a administração de pílulas de LNG para AE.

A progesterona natural tem sido relatada como promotora de alterações nas funções espermáticas relacionadas com a fertilização, como os fenômenos de capacitação, ativação espermática, RA, ligação do espermatozóide na zona pelúcida e penetração no oócito (Osman *et al.*, 1989; Blackmore *et al.*, 1990; Sueldo *et al.*, 1993; Oehninger *et al.*, 1994; Bray *et al.*, 1999; Baldi *et al.*, 1998; 2000).

A progesterona é capaz de desencadear o estímulo fisiológico da RA em uma pequena população de espermatozóides que possuem o receptor de membrana específico (Meizel E Turner, 1996; Cheng *et al.*, 1998; Luconi *et al.*, 1998; Sirivadyapong *et al.*, 1999; Flesch e Gadella, 2000) e especificamente tem sido apontada como indutora da RA através do aumento na concentração do cálcio intracelular nos espermatozóides (Osman *et al.*, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Foresta *et al.*, 1993; Melendrez *et al.*, 1994).

Apenas entre 10% e 30% dos espermatozóides apresentaram receptores para a progesterona (Tesarik *et al.* 1992), e há hipóteses de que talvez seja essa a sub-população de espermatozóides capaz de sofrer a capacitação e posteriormente fertilizar.

Aqueles espermatozóides que completaram a RA precocemente tornam-se incapazes de penetrar a zona pelúcida do oócito pelo fato de eles já terem

perdido seus conteúdos acrossomais enzimáticos (Blackmore *et al*., 1990; Flesch e Gadella, 2000). Por isso vale observar que a possibilidade de se manipular o fenômeno da RA seria uma vantagem potencial, uma vez que tal reação é uma etapa necessária para o processo de fertilização (Roblero *et al.*, 1988).

Contudo, poucos estudos abordaram a questão do LNG agir sobre o espermatozóide como uma possibilidade do mecanismo de ação contraceptivo do LNG quando administrado como AE (Kesseru *et al*., 1974; Nikkanen *et al.,* 2000*;* Bahamondes *et al.,* 2003; Yeung *et al.,* 2002).

Um estudo (Nikkanen *et al.,* 2000) mostrou que a aplicação local do LNG na cauda do epidídimo de ratos prejudicou o potencial fertilizante dos espermatozóides *in vivo*, sugerindo que a droga afetou diretamente a mobilidade dos espermatozóides.

Em contraposição, nosso grupo (Brito *et al.,* 2005) apresentou dados de que a exposição de espermatozóides capacitados *in vitro* a baixas concentrações de LNG e similares aos níveis séricos da droga após a administração oral para AE (Johansson *et al.,* 2002) não apresentou indução da RA, enquanto que altas concentrações de LNG compatíveis com aquelas observadas em usuárias de SIU-LNG foram capazes de induzir a RA (Bahamondes *et al.,* 2003).

Imediatamente após a ejaculação, os espermatozóides dos mamíferos são incapazes de fertilizar o oócito e esta capacidade é adquirida como conseqüência de uma série de alterações fisiológicas e funcionais denominadas

de capacitação. A capacitação espermática ocorre durante a permanência e migração do espermatozóide no trato genital feminino (Yanagimachi, 1988).

A migração espermática consiste em duas fases: na primeira, alguns espermatozóides ajudados pelas contrações do trato genital, chegam até o útero, tubas de Falópio e cavidade peritoneal. Na segunda fase, de duração de alguns dias, aqueles espermatozóides que permaneceram estocados nas criptas cervicais migram em sucessivos grupos para a tuba de Falópio. Apenas os espermatozóides estocados nas criptas cervicais são capazes de fertilizar o oócito após a própria capacitação (Croxatto, 1996; 2003).

A duração da capacidade fertilizante do espermatozóide humano após seu depósito na fêmea é desconhecida, e sob o aspecto fisiológico e funcional muito pouco foi estudado em relação a este período de vida no qual o espermatozóide humano permanece dentro do trato reprodutivo feminino (Gould *et al.,* 1984).

Estimativas prévias da vida fértil máxima (24 – 48 horas) e da motilidade (de 2 a 7 dias) do espermatozóide humano (Perloff e Steinberger, 1964; Gould *et al.,* 1984) foram baseadas na recuperação desses gametas móveis a partir do muco cervical, do útero, dos oviductos e do fluido peritoneal (Gould *et al.,*1984; Bielfeld *et al.,* 1992; Zinaman *et al.,* 1989; Williams *et al*., 1993).

Em humanos, em que a receptividade sexual feminina é contínua e os tempos de duração da ovulação podem ser variáveis, estimativas confiáveis da durabilidade da fertilidade dos espermatozóides não são disponíveis decorrentes

de problemas logísticos e éticos para o trabalho com seres humanos, e também das dificuldades técnicas associadas com a recuperação do conteúdo uterino humano (Williams *et al*. 1993).

Estudos prévios recuperaram espermatozóides humanos durante procedimentos de histerectomia (Moyer, 1970), de esterilização tubárea cirúrgica (Alvarez-Sanchez *et al*., 1978) ou através de uma lavagem da cavidade uterina após inseminação artificial (Mortimer, 1982; Williams, 1993), e a quantidade dos espermatozóides recuperados nestes casos foi bastante variável, além do fato de que poucos trabalhos fizeram a avaliação das funções espermáticas (por exemplo, da capacitação e o status da RA) nestes espermatozóides retirados de dentro do trato reprodutivo feminino (Gould, 1984; Zinaman, 1989) em conseqüência das dificuldades técnicas e das restrições éticas.

Os métodos naturais de contracepção para planejamento familiar se baseiam em certas suposições em torno da ovulação e também de uma estimativa da longevidade do oócito e do espermatozóide. Geralmente acredita-se que os espermatozóides humanos sejam capazes de fecundar o oócito por um período de 48 – 72 horas (Gould *et al.,* 1984).

Em mulheres foi demonstrado que os dias férteis do ciclo menstrual constituem o dia do pico de LH e também os 5 dias precedentes da ovulação (Wilcox *et al.,* 1995). Conseqüentemente, os espermatozóides depositados na vagina de mulheres que receberem o LNG para AE poderão ser expostos a

concentrações desconhecidas do progestágeno e assim interferir com a capacidade fertilizante deles.

O mecanismo de ação do LNG como AE é ainda pouco compreendido e a maioria dos estudos têm focado o efeito sobre os espermatozóides e a maiora dos estudos são *in vitro.* Por isso, entendemos que era necessário o estudo do efeito do LNG, como AE, sobre os espermatozóides *in vivo* através de um procedimento de lavagem da cavidade uterina após coito e após administração de LNG para AE. Também, avaliar a RA, a concentração de LNG no útero após sua administração oral e também analisar a expressão do padrão de glicodelina-A após AE com LNG.

2. Objetivos

2.1. Objetivo geral

Avaliar o efeito do LNG como AE sobre espermatozóides recuperados *in vivo* do útero humano através de um procedimento de lavagem da cavidade uterina após coito, sobre o muco cervical e sobre a glicodelina-A endometrial.

2.2. Objetivos específicos

- Avaliar a taxa de RA *in vivo* nos espermatozóides recuperados da cavidade uterina e do muco cervical após a administração de LNG para AE ou placebo a diferentes tempos entre o coito e a toma do LNG e entre a administração do LNG e o lavado.
- Medir a concentração de LNG no soro e no fluido do lavado uterino após 24 – 48 horas da administração oral de 1,5 mg de LNG para AE.
- Analisar a expressão do padrão de glicodelina-A no endométrio humano após diferentes tempos da administração de 1,5 mg de LNG para AE.

3. Publicação

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An *in vivo* model to assess acrosome reaction of human spermatozoa and the expression of glycodelin-A in human endometrium after levonorgestrel-emergency contraception pill administration

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Running title: *In vivo* acrosome reaction after LNG-EC pill intake

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Abstract

BACKGROUND: The objectives were to assess acrosomal reaction (AR) of spermatozoa following flushing the uterus, to measure levonorgestrel (LNG) in serum and in the flushing fluid of the uterus and the endometrial glycodelin-A expression after administration of LNG as emergency contraception (EC).

MATERIALS AND METHODS: Forty-eight experiments were conducted on 14 regularly menstruating women. Four groups were formed based on different intercourse - treatment interval and treatment – recovery of spermatozoa and the biopsies.

RESULTS: Spermatozoa recovered from uterus 24 or 48 hours after treatment were $14.5 \pm 3.9 \times 10^6$ and $17.3 \pm 6.8 \times 10^6$ cells/ml, respectively. There were no differences between the AR rate and the endometrial glycodelin-A staining intensity on LNG or placebo treated cycles. LNG at uterine flushing medium represents 1.38% of the values observed in serum at 24 hours after LNG intake.

CONCLUSIONS: AR status or glycodelin-A were not influenced after 24 or 48 hours of administration of 1.5 mg of LNG as EC indicating no significant effect. LNG did not impair the cervical mucus either because viable spermatozoa were found in the genital tract 36-60 hours after coitus and 24-48 hours after LNG intake. The mechanism of action of LNG as EC remains enigmatic.

Key-words: emergency contraception/ levonorgestrel/ human spermatozoa/ acrosome reaction/ glycodelin-A

Introduction

Levonorgestrel (LNG) is widely used as an emergency contraceptive (EC). It is usually administered in two oral doses of 0.75 mg, given 12 hours apart, or in a single dose of 1.5 mg (WHO, 1998; von Hertzen, *et al*., 2002; Devoto *et al.,* 2005; Cheng *et al.*, 2004; Gainer *et al.,* 2006). The mechanism of action of LNG as an EC is still poorly understood (WHO, 1998; Croxatto *et al*., 2003; Müller *et al.*, 2003; Gemzell-Danielsson and Marions, 2004).The postulated mechanisms of action include an effect on the luteinizing hormone (LH) surge and ovulation, follicular or corpus luteum development, thickening of the cervical mucus affecting sperm penetration and transport, and interference with fertilization (Swahn *et al*., 1996; Durand *et al*., 2001; Hapangama *et al*., 2001; Croxatto *et al*., 2001; Marions *et al*., 2002).

Another proposed mechanism is that LNG interferes with endometrial function or with endometrial protein expression. One of such protein is glycodelin-A, a secretory progesterone (P)-regulated glycoprotein (Seppala *et al.*, 2002). Inappropriate expression of glycodelin-A by sustained delivery of LNG has been observed in women using LNG-releasing intrauterine system (LNG-IUS) and subdermal contraceptive implants (Mandelin *et al.,* 1997; 2001). Additionally, the administration of LNG for EC prior to the LH surge alters the luteal phase secretory pattern of glycodelin-A in serum and endometrium (Durand et al., 2005).

Because it has been reported that the local application of LNG into the tail of the epididymis of rats impairs the *in vivo* fertilizing potential, suggesting that the drug has a direct effect on spermatozoa (Nikkanen *et al.,* 2000), our group has been working on the hypothesis that LNG as EC could interferes with the fertilizing capacity of spermatozoa (Bahamondes *et al.,* 2003; Brito *et al.,* 2005; Munuce *et al*., 2005). However, there was

no association between in vitro exposures to LNG.

The duration of the fertilizing capacity of human spermatozoa in the woman is unknown (Gould *et al.,* 1984). Previous studies based on the recovery of spermatozoa from the cervical mucus, uterus, oviducts, and peritoneal fluid reported a maximal fertile life of 24 to 48 hours, with a motile life from 48 h to 7 days (Perloff and Steinberger, 1964; Gould *et al.,* 1984; Zinaman *et al.,* 1989; Bielfeld *et al*., 1992; Williams *et al*., 1993). In humans, reliable estimates of the duration of spermatozoa fertility are not available due to the ethical and logistic constraints, and to the technical difficulties associated with retrieving the contents of the uterus (Williams *et al*. 1993). Spermatozoa deposited in the vagina during intercourse may remain in the endocervix for many hours or even days before ascending to the Fallopian tubes, and only these cells have the ability to fertilize (Croxatto, 1996). Consequently, spermatozoa deposited in the vagina of women who receive LNG for EC could be exposed to unknown concentrations of LNG for hours or days, and this may influence their fertilizing capacity.

Since the mechanism of action of EC is still poorly understood, the objectives of this study were to assess the acrosomal reaction (AR) status of spermatozoa recovered *in vivo* following flushing of the human uterine cavity after sexual intercourse, to measure the LNG concentrations in serum and uterine flushings in respect of the endometrial glycodelin-A expression after the administration of LNG as EC, or placebo.

Subjects and Methods

The study was conducted at the Human Reproduction Unit, Department of Obstetrics and Gynaecology, School of Medicine, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil. A total of 14 women aged 35.1 ± 3.2 years (mean ±

SEM) (range 30 to 41) and parity 2.6 ± 0.2 (mean ± SEM) were studied. The admission criteria included surgical sterilization, regular menstrual periods (25-35 day intervals), negative screening for Chlamydia and gonorrhea, no use of any hormone therapy, no breastfeeding or pregnancy in the 3 months preceding the study, and a partner with a normal semen analysis performed two weeks before the experiments, assessed according to the World Health Organization Manual (World Health Organization, 1999). All the couples gave their written informed consent and the study protocol was approved by the Institutional Review Board.

Study Design

This was a double blind, placebo-controlled study designed to evaluate the effects of LNG as EC upon the AR of human spermatozoa and the expression of glycodelin-A in human endometrium at different times after LNG intake. The participants were randomly assigned to four groups (see below) and the difference between them in respect of the intercourse to treatment interval and the time-elapsed to spermatozoa recovery from the uterine cavity was addressed. All the groups were studied during two consecutives cycles, a control cycle (placebo administration) and the treatment cycle with 1.5 mg of LNG. Forty-eight experiments were conducted, 12 in each group. For randomization, sealed envelopes were used and the pills were prepared in opaque vials by a person not involved in the study. The coded envelopes were kept outside of the institution.

In group I the women took LNG or placebo 12 hours after coitus and the uterine flushing was performed 24 hours after the pill intake. Group II was given LNG or placebo 12 hours after coitus and the uterine flushing was performed 48 hours after the

pill administration. Group III received the LNG or placebo treatment 36 hours after coitus and the uterine flushing was performed 24 hours after the pill administration. Group IV received the treatment (LNG or placebo) 24 hours after artificial vaginal insemination and the uterine flushing was performed 24 hours after the pill administration.

Experiments

In all women, follicular development was monitored daily by ultrasound using a 5.0 MHz vaginal probe (Justavision 400, Toshiba, Toshigi-Ken, Japan), according to the characteristics of the cervical mucus as defined in the WHO Manual (World Health Organization, 1999) and by serum progesterone. The couples were asked to abstain from sexual intercourse during the 5 days preceding the experiment, a behavior confirmed by absence of spermatozoa in daily assessment of the cervical mucus. The volunteers were instructed to have sexual intercourse at the night of the day when the greatest follicular diameter showed positive correlation with cervical mucus i.e., spinnbarkheit $\geq$ 10 cm and crystallization > 2+ (World Health Organization, 1999). In group IV, they were asked to have an artificial insemination in the next morning. In the following morning, a cervical mucus sample was taken in order to carry out a post coital test and the experiment was performed only if the post coital test was adequate (World Health Organization, 1999).

At 12 or 36 hours after sexual intercourse LNG or placebo was administered at the clinic. Uterine flushing was performed 24 or 48 hours after the pill intake. All the experiments were performed in the middle of the menstrual cycle before ovulation. To confirm this event a daily blood sample was collected and the serum was separated. Presence of the follicular phase of the cycle was confirmed by daily serum progesterone

147 levels below 3 ng/ml (Israel *et al.,* 1972). The samples of serum were stored at -20°C
148 until the measurement of LNG.

149 Uterine flushing was performed using the following technique. To minimize
150 discomfort, one tablet of 5 mg of midazolam (Dormonid®, Roche, São Paulo, Brazil)
151 was administered 20 min before the procedure. Next, the cervix was exposed using a
152 speculum, and the cervical mucus was gently removed using a syringe and placed in a
153 sterile tube with 3 ml of human tubal fluid medium (HTF; GIBCO, BRL, Life
154 Technologies, Inc., Grand Island, NY, USA). The cervix was cleaned with saline solution and
155 its diameter was estimated. A cervical adaptor (Wisap, Munich, Germany) of appropriate
156 size was fixed to the cervix by vacuum. The uterus was then flushed by gently
157 introducing 5 ml of HTF medium (GIBCO) and recovering it by aspiration with a
158 syringe. The "dead space" in the cannula was estimated to be about 2.5 ml. This
159 procedure was repeated 5 times using the same fluid, to ensure removal of a maximal
160 number of spermatozoa. The pressure applied to the vacuum syringe and to the
161 aspiration syringe of the cervix adapter used for flushing was moderate to prevent
162 bleeding, which could have contaminated the material.

163 Sonographic monitoring of uterine flushing was performed using a 3.5 Mhz
164 abdominal probe (Justavision 400, Toshiba) to assure that the fluid had gone through the
165 uterine cavity and that it had been totally removed. Whenever we observed that some
166 amount of fluid had been retained in the uterus, a neonatal feeding tube # 6 (Embramed,
167 São Paulo, Brazil) was gently inserted through the cervix to remove the fluid. The
168 recovered fluid was placed in a sterile tube. The tubes containing the cervical mucus and
169 fluid recovered after flushing were placed at 37ºC under CO_2 for 1 h to stabilize the
170 temperature of the HTF medium and to allow the migration of spermatozoa into the

171 medium (Gould *et al*., 1984; Zinaman *et al*., 1989). Following this procedure, two
172 centrifugation cycles were performed using PBS (Dulbecco's, GIBCO, BRL, Life
173 Technologies, Inc, Grand island, NY, USA) and the pellet was diluted in 1 ml of the
174 PBS. Ten µl were placed into a Makler counting chamber (Sefi Medical Instruments,
175 Haifa, Israel) to assess the sperm concentration. When the material was very thick
176 because of the condition of the mucus, it was passed several times through a syringe
177 with a fine needle to break the mucus filaments before centrifugation and no enzymatic
178 treatment was used to solubilize the cervical mucus (World Health Organization, 1999).
179
180 ***Endometrial biopsy***
181 After the uterine flushing, endometrial specimens were obtained by biopsy
182 (Pipelle de Cornier®, Prodimed, Neuilly-en-Thelle, France), trying to obtain a representative
183 specimen from the uterine cavity. The specimens were fixed in 10% buffered neutral
184 formalin, dehydrated, embedded in paraffin and cut into 4 µm blocks, deparaffinized,
185 rehydrated, stained with hematoxylin and eosin (HE) according to standard protocols
186 and submitted to histology evaluation and immunohistochemical analysis.
187
188 ***Glycodelin-A immunohistochemical staining***
189 Paraffin-embedded tissue sections were deparaffinized and hydrated in graded
190 ethanol. Then, processed as described, including microwave heat treatment (Kamarainen
191 *et al.,* 1996). Endometrial expression of glycodelin-A was evaluated in sections of 5 µm
192 of endometrial tissue. Rabbit anti-glycodelin IgG was used as the first antibody, and
193 biotinylated swine antirabbit IgG (Dako, Glostrup, Denmark) and normal rabbit serum

194 was used as the second and the control antibody, respectively. Another negative control
195 was added using the first antibody, but immunoabsorbed with purified glycodelin-
196 A (16 μg/mL). Endogenous peroxidase activity was blocked by treatment with 0.6%
197 perhydrol in methanol. The staining was carried out using EnVision®+ System-HRP
198 (DakoCytomation, Carpinteria, CA, USA) and rabbit anti –glycodelin IgG (1.2 μg/ml,
199 20 hours, + 4°). Preimmune IgG from the same rabbit was also used as a negative
200 control. The tissue sections were counterstained with haematoxylin (blue). Staining
201 intensity was recorded using a semiquantitative scale of 0, 1, 2 or 3 (none, weak,
202 moderate or strong, respectively).
203
204 ***Assessment of spermatozoa AR***
205 After the sperm concentration had been evaluated, the fluorescent probe
206 fluorescein isothiocyanate-labelled *Pisum sativum lectin* (FITC-PSA) was used to evaluate the
207 AR status. Two slides were prepared from the sperm suspension, air-dried at room
208 temperature and protected from light. After drying, they were immersed in cool absolute
209 methanol for 30 seconds. After that, the slides were stained by immersing them in FITC-
210 PSA for 30 min at a concentration of 40 μg/ml in PBS and protected from light at room
211 temperature. After incubation, the slides were washed in PBS and stored in the dark until
212 evaluation for AR and vitality. Evaluation was done using a fluorescent microscope (Zeiss,
213 Axioplan II, Jena, Germany) equipped with a specific filter for the FITC-PSA method
214 (with 494-blue excitation, 520 emission, 510–514 barrier). Two hundred cells were evaluated
215 in the fields chosen at random. The only spermatozoa that were considered acrosome-reacted
216 were those with the following patterns: (a) patchy fluorescence of the acrosomal region
217 (partially acrosome-reacted); (b) fluorescence of the equatorial band only (acrosome-reacted).

218

219 ***Levonorgestrel and progesterone assay***

220 The concentration of serum and uterine LNG was determined by using a
221 validated method of high performance liquid chromatography coupled to a tandem mass
222 spectrometry (LC-MS/MS). All procedures were carried out in compliance with Good
223 Laboratory Practices approved by the Brazilian National Authority on Sanitary Surveillance.
224 The standards of LNG used in the experiments were USP (Rockville, MD, USA).
225 Biological specificity of the method was checked by processing independent plasma
226 samples and blank samples obtained from women not using any kind of hormones and
227 with the using of pure HTF medium.

228 Briefly, the bioanalytical assay for quantification of LNG was developed using
229 an online SPE method (Spark Holland model Symbiosis Generic, Emmen, Netherlands).
230 The mass spectrometer (Sciex/Applied Biosystems, model API5000, Toronto, Canada)
231 was equipped with a photoionization source (APPI) running in positive ion mode was set
232 up in Multiple Reaction Monitoring (MRM) for the transition *m/z* 313.3→245.1 for
233 LNG. Toluene was used as solvent at the flow rate of 0.15 ml/min. The lowest limit of
234 quantification (LLOQ) of the method was 20 pg/ml and it was linear over the range 20 –
235 5000 pg/ml. The run time was 5.5 min and the retention time of the LNG was 3.8 (± 0.3) min.

236 The determination of progesterone was performed in duplicate using commercial
237 kits of electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics GmbH,
238 Mannheim, Germany) with a measurement range of 0.030 to 60.0 ng/ml and interassay
239 variation of 2.4%.

240

241 ***Statistical analysis***

242 The total number of spermatozoa recovered from the uterine cavity and from the
243 cervical mucus was compared using ANOVA and Tukey-Kramer test for paired
244 samples. The level of significance was established at $p < 0.05$. All values are shown as
245 mean ± standard error of the mean (SEM).

246

247 **Results**

248 ***Spermatozoa recovery from uterine cavity***

249 A total of 86 cycles were initiated but 38 experiments were canceled because
250 there was inadequate cervical mucus (11 cycles), an inadequate post coital test (12
251 cycles), premature ovulation (9 cycles) or personal problems with the couple (6 cycles).
252 Only 48 (56%) experiments were successfully completed. No adverse events were
253 reported. The procedures were initiated when the greatest follicular diameter was 14.3 ±
254 0.7; 13.1 ± 0.5; 14.0 ± 0.7, and 13.0 ± 0.7 mm for the experiments performed in groups
255 I, II, III, and IV, respectively.

256 The number of recovered spermatozoa was $14.5 \pm 3.9 \times 10^6$ cells/ml (range $0.2 \times$
257 10^6 to 104.9×10^6 motile spermatozoa/ml) and $17.3 \pm 6.8 \times 10^6$ cells/ml (range 0.1 to
258 66.4×10^6 motile spermatozoa/ml) in the group in which uterine flushing was performed
259 24 or 48 hours after the pill intake, respectively, without differences between the cycles
260 treated with LNG or placebo. The characteristics of cervical mucus after the LNG or
261 placebo administration were not changed significantly.

262

263

Acrosome reaction status

The AR status was evaluated in the spermatozoa separately in the samples recovered from the cervical mucus and the uterine cavity after uterine flushing. Table I shows the values in each treatment group. The AR percentage in cervical mucus ranged from 9.3% to 10.2% in those spermatozoa obtained in the placebo group and from 8.0% to 12.5% in the LNG treated groups. The AR percentage obtained from the spermatozoa in uterine flushing ranged from 6.2% to 12.7% and 7.8% to 13.0% in placebo- and LNG-treated groups, respectively. There were no significant differences between the AR rates in LNG or placebo treated cycles at different times of LNG exposure after sexual intercourse, or after artificial insemination.

Endometrial biopsies and glycodelin-A staining

All the biopsies showed a proliferative pattern. Glandular and stromal elements were considered separately and given equal importance. Treatment with LNG did not change the secretory pattern of glycodelin-A. There were no differences between the glycodelin-A staining intensity on single dose of 1.5 mg of LNG or placebo treated cycles when the biopsies were taken at 24 or 48 hours after treatment. However, when the biopsies were obtained at 48h after pill intake, the results were affected by the small number of cases available for the analysis (Figure 1).

Levonorgestrel concentrations

The LNG concentration was measured only on the day of the experiment. The LNG concentration in the uterine flushing medium after 24 hours of LNG administration was 1.38% of the total value observed in serum (47.9 vs. 3,462.9 pg/ml). At 48 hours

after the LNG intake the hormone was no longer detected in uterine flushings (< 20 pg/ml). In serum, the value at 48 hours after LNG intake was almost the 50% of those observed at 24 hours after the LNG intake (Table II).

Discussion

Levonorgestrel is a progestin that has been widely used for EC although its mechanism of action is still unclear. In a randomised clinical trial comparing LNG with the Yuzpe regimen, LNG was shown to prevent significantly more pregnancies than the Yuzpe regimen and its effectiveness increased the closer the drug was administered to the time of coitus (WHO, 1998). Some studies have shown that LNG affects the preovulatory events (Swahn *et al*., 1996; Durand *et al*., 2001; Hapangama *et al*., 2001; Croxatto *et al*., 2001; Marions *et al*., 2002), and it was postulated that probably it affects also ovulation, thickening of the cervical mucus, sperm migration, penetration or transport, fertilization, and endometrial function (Swahn *et al*., 1996; Trussell J and Raymond,1999; Croxatto *et al*., 2001; Durand *et al*., 2001; Hapangama *et al*., 2001, Marions *et al*., 2002; Gemzell-Danielsson and Marions, 2004).

In order to contribute to the understanding of the mechanisms of action involved in the contraceptive effect of LNG as an EC our primary objective was to explore if the drug could modify the in vivo status of spermatozoa retained in the endocervix and in the uterine cavity after sexual intercourse or artificial insemination. The results showed that a single dose of 1.5 mg of LNG given before the follicular rupture did not influence the AR status of spermatozoa after different time spans between coitus and the pill intake (12, 24 or 36 hours). In addition, the AR rate was similar when it was evaluated at different times between LNG or placebo administration and the recovery of spermatozoa (24 or 48 hours).

312 Although the LNG concentration had reached 3463 ± 276 pg/ml and 48 ± 11
313 pg/ml in serum and uterine flushing, respectively, after 24 hours of LNG intake, no
314 change in the AR status was detected in the recovered spermatozoa. Also, the AR rate
315 was similar to previous results with human spermatozoa recovered from the cervix at 72
316 hours after coitus without any drug treatment (Bielfeld *et al.,* 1992). The present results
317 are in agreement with results from the previous *in vitro* experiments showing that LNG
318 at 1 ng/ml is unable to induce AR (Brito *et al.,* 2005; Munuce *et al*., 2005).
319 The speculation that LNG as EC could affect the cervical mucus and sperm
320 penetration was based on a well documented effect of progestin and the main mechanism
321 of action of progestin-only contraceptive pill (Moghissi *et al.,* 1973). It was also based
322 on a previous study by Kesseru *et al.* (1974) showing that, after a single dose of LNG
323 intake as an EC, there is a decrease of spermatozoa recovered from the uterus. This has
324 been observed at 3 hours after coitus and more significantly at 7 hours. Immobilization
325 of spermatozoa due to alkalinization of the uterine fluid, and increased the viscosity of the
326 cervical mucus after 9 hours of coitus may account for this change. Those mechanisms
327 may have contributed to the lack of spermatozoa penetration.
328 Our findings contradict those results because we observed that a single 1.5 mg
329 dose of LNG did not impair the quality of cervical mucus and spermatozoa penetration.
330 It was possible to recover an adequate number of viable and motile human spermatozoa
331 both from the cervix and the uterine cavity at 36, 48 or 60 hours after coitus. Probably,
332 the differences between our results and those of Kesseru and his coworkers, (1974)
333 could result from different doses of LNG used, the times between coitus and evaluation,
334 and the methodology of spermatozoa recovery used more than 30 years ago. Therefore,

335 the initial hypothesis that LNG could interfere with sperm function and penetration and
336 could contribute to the mechanism of action in EC was not confirmed in our *in vivo* and
337 *in vitro* studies (Brito *et al.,* 2005; Munuce *et al*., 2005).

338 Additionally, LNG given before the follicular rupture did not influence the expression
339 of glycodelin-A in endometrial biopsies taken at 24 or 48 hours after pill intake. The
340 administration of LNG for EC prior to the LH surge does not appear to affect endometrial
341 histology or chronological dating of endometrial maturation (Durand *et al*., 2001).

342 A second study from the same group (Durand *et al*., 2005) evaluated only
343 ovulatory women and observed an early rise in serum glycodelin-A concentration and its
344 expression in the endometrium when the drug was administered before the LH peak.
345 They observed that a maximum glycodelin-A endometrial expression was significantly
346 lower when LNG was administered at the time of the LH peak compared to drug intake
347 before the LH surge.

348 We studied the pattern of glycodelin-A expression due to its antifertility activity
349 and because the study from Durand *et al.* (2005) showed an effect when LNG was
350 administered before the LH surge. However, our results did not show any effect. It may
351 be significant that, in the study by Durand and her colleagues (2005), endometrial
352 biopsies were taken under native conditions, whereas in this study, the biopsies were taken
353 after endometrial flushing that obviously decreases the amount of secreted glycodelin in
354 the uterine cavity. The effect of endometrial flushing on immunolocalization of
355 glycodelin in endometrial tissue has not been determined. Another explanation for the
356 difference is that, although LNG was administered before the follicular rupture, the drug
357 could have been administered close to the LH peak.

358 Finally, because the biopsies were taken 24 or 48 hours after LNG intake, the exposure

time could have been too short for any significant effect to be observed in the glycodelin synthesis. Nevertheless, for the purposes of the present study, the effects of prior endometrial flushing were not likely to cause any bias because comparison was made from the biopsies taken after flushing in the same way from women who had taken either LNG or placebo.

The previous results on glycodelin-A expression in the endometrium of users of an LNG-releasing intrauterine system (Mandelin *et al*., 1997) or LNG-releasing contraceptive implants (Mandelin *et al*., 2001) cannot be extrapolated to users of LNG as EC because the length of LNG exposure was different. The lack of an association between AR and glycodelin-A expression in the endometrium was not surprising because, unlike glycodelin-F from follicular fluid, glycodelin-A does not interfere with AR (Yeung *et al*., 2006), and the spermatozoa recovered from uterine flushings are not likely to have been in contact with the inhibitory glycodelin isoform F.

In conclusion, LNG administered as EC at a single dose of 1.5 mg after 24 or 48 hours after sexual intercourse or artificial insemination did not influence the AR status or endometrial expression of glycodelin-A and probably they were not part of the mechanism of action of this kind of EC. Additionally, the administration of the drug has not effect on the quality of cervical mucus or in the penetration of spermatozoa to the uterine cavity. Therefore, the mechanism of action of LNG as an EC remains an enigma.

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383

384 **References**

385 Bahamondes L, Nascimento JA, Munuce MJ, Fazano F and Faundes A (2003) The in
386 vitro effect of levonorgestrel on the acrosome reaction of human spermatozoa from
387 fertile men. Contraception 68,55-59.

388 Bielfeld P, Graf M, Jeyendran RS and Zaneveld LJ (1992) No change in acrosome
389 reaction of human spermatozoa during storage in cervical mucus. Andrologia 24,83-86.

390 Brito KS, Bahamondes L, Nascimento JA, de Santis L and Munuce MJ (2005). The in
391 vitro effect of emergency contraception doses of levonorgestrel on the acrosome reaction
392 of human spermatozoa. Contraception 72,225-228.

393 Cheng L, Gulmezoglu AM, Oel CJ, Piaggio G, Ezcurra E and van Look PF (2004)
394 Interventions for emergency contraception. *Cochrane Database Syst Rev* CD001324.

395 Croxatto HB (1996) Gamete transport. In: E.Y. Adashi, J.A. Rock and Z. Rosenwaks,
396 Editors, *Reproductive endocrinology, surgery, and technology*, Lippincott-Raven,
397 Philadelphia, pp. 385–402.

398 Croxatto HB, Devoto L, Durand M, Ezcurra E, Larrea F, Nagle C, Ortiz ME, Vantman
399 D, Vega M and von Hertzen (2001) Mechanism of action of hormonal preparations used
400 for emergency contraception: a review of the literature. Contraception 63,111–121.

401 Croxatto HB, Ortiz ME and Muller AL (2003) Mechanism of action of emergency
402 contraception. Steroids 68,1095-1098.

403 Devoto L, Fuentes A, Palomino A, Espinoza A, Kohen P, Ranta S and Hertzen H (2005)
404 Pharmacokinetics and endometrial tissue levels of levonorgestrel after administration of

405 a single 1.5-mg dose by the oral and vaginal route. Fertil Steril 84,46-51.

406 Durand M, Cravioto CM, Raymond EG, Duran-Sanchez O, De la Luz Cruz-Hinojosa M,
407 Castell-Rodriguez A, Schiavon R, and Larrea F (2001) On the mechanism of action of
408 short-term levonorgestrel administration in emergency contraception. Contraception
409 64,227–234.

410 Durand M, Seppala M, Cravioto M del C, Koistinen H, Koistinen R, Gonzalez-Macedo J
411 and Larrea F (2005) Late follicular phase administration of levonorgestrel as an
412 emergency contraceptive changes the secretory pattern of glycodelin in serum and
413 endometrium during the luteal phase of the menstrual cycle. Contraception 71,451-457.

414 Gainer E, Kenfack B, Mboudou E, Doh AS and Bouyer J (2006) Menstrual bleeding
415 patterns following levonorgestrel emergency contraception. Contraception 74,118-124.

416 Gemzell-Danielsson K and Marions L (2004) Mechanisms of action of mifepristone and
417 levonorgestrel when used for emergency contraception. Hum Reprod Update 10,341–348.

418 Gould JE, Overstreet JW and Hanson FW (1984) Assessment of human sperm function
419 after recovery from the female reproductive tract. Biol Reprod 31,888-894. Hapangama D,
420 Glasier AF and Baird DT (2001) The effects of peri-ovulatory administration of
421 levonorgestrel on the menstrual cycle. Contraception 63,123–129.

422 Israel R, Mishell DR Jr, Stone SC, Thorneycroft IH, Moyer DL (1972). Single luteal
423 phase serum progesterone assay as an indicator of ovulation. Am J Obstet Gynecol
424 112, 1043-1046.

425 Kamarainen M, Leivo I, Koistinen R, Julkunen M, Karvonen U, Rutanen EM and
426 Seppala M (1996) Normal human ovary and ovarian tumors express glycodelin, a

427 glycoprotein with immunosuppressive and contraceptive properties. Am J Pathol
428 148,1435–1443.

429 Kesseru E, Garmendia F, Westphal N and Parada J (1974) The hormonal and peripheral
430 effects of dl-norgestrel in postcoital contraception. Contraception 10,411-424.

431 Mandelin E, Koistinen H, Koistinen R, Affandi B and Seppala M (1997)
432 Levonorgestrel-releasing intrauterine device-wearing women express contraceptive
433 glycodelin A in endometrium during the midcycle: another contraceptive mechanism?
434 Hum Reprod 12,2671–2675.

435 Mandelin E, Koistinen H, Koistinen R, Arola J, Affandi B and Seppala M (2001)
436 Endometrial expression of glycodelin in women with levonorgestrel-releasing subdermal
437 implants. Fertil Steril 76, 474–478.

438 Marions L, Hultenby K, Lindell I, Sun X, Stabi B and Gemzell Danielsson K (2002)
439 Emergency contraception with mifepristone and levonorgestrel: mechanism of action.
440 Obstet Gynecol 100,65–71.

441 Moghissi KS, Syner FN and McBride LC (1973) Contraceptive mechanism of
442 microdose norethindrone. Obstet Gynecol 41,585-594.

443 Munuce MJ, Nascimento JA, Rosano G, Faundes A, Saboya-Brito K and Bahamondes L
444 (2005) In vitro effect of levonorgestrel on sperm fertilizing capacity and mouse embryo
445 development. Contraception 72,71-76.

446 Nikkanen V, Söderström K, Tuusa S and Jaakkola UM (2000) Effect of local epididymal
447 Levonorgestrel on the fertilizing ability of male rat, a model for post-testicular
448 contraception. Contraception 61,401-406.

449 Perloff WH and Steinberger E (1964) In vivo survival of spermatozoa in cervical mucus.
450 Am J Obstet Gynecol 88,439-442.

451 Seppala M, Taylor RN, Koistinen H, Koistinen R and Milgrom E (2002) Glycodelin: a
452 major lipocalin protein of the reproductive axis with diverse actions in cell recognition
453 and differentiation. Endocr Rev 23,401–430.

454 Swahn ML, Westlund P, Johannisson E and Bygdeman M (1996) Effect of post-coital
455 contraceptive methods on the endometrium and the menstrual cycle. Acta Obstet
456 Gynecol Scand 75,738–744.

457 Trussell J and Raymond EG (1999) Statistical evidence about the mechanism of action
458 of the Yuzpe regimen of emergency contraception. Obstet Gynecol 93,872–876.

459 von Hertzen H, Piaggio G, Ding J, Chen J, Song S, Bartfai G, Ng E, Gemzell-
460 Danielsson K, Oyunbileg A, Wu S, et al. (2002) Low doses of mifepristone and two
461 regimens of levonorgestrel for emergency contraception: a WHO randomised trial.
462 Lancet 360,1803–1810.

463 Williams M, Thompson LA, Li TC, Mackenna A, Barratt CL and Cooke ID (1993)
464 Uterine flushing: a method to recover spermatozoa and leukocytes. Hum Reprod 8:925-928.

465 World Health Organization Task Force on Postovulatory Methods of Fertility Regulation
466 (1998) Randomized controlled trial of levonorgestrel versus the Yuzpe regimen of
467 combined oral contraceptives for emergency contraception. Lancet 352,428–438.

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

471 Yeung WSB, Lee K-F, Koistinen R, Koistinen H, Seppälä M, Ho PC and Chiu PCN
472 (2006) Roles of glycodelin in modulating sperm function. Mol Cell Endocrinol 250,149-156.

473 Zinaman M, Drobnis EZ, Morales P, Brazil C, Kiel M, Cross NL, Hanson FW and
474 Overstreet JW (1989) The physiology of sperm recovered from the human cervix:
475 acrosomal status and response to inducers of the acrosome reaction. Biol Reprod 41,790-797.

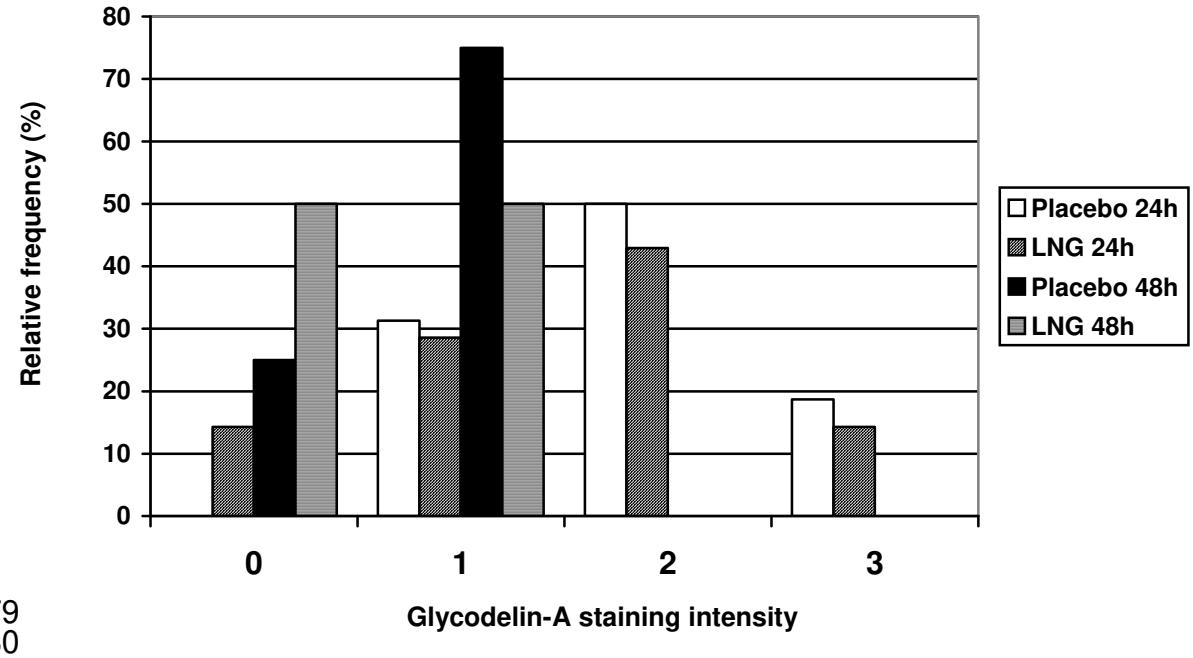

Figure 1. Glycodelin-A immunostaining intensities of glandular endometrial cells (in percentage) in LNG and placebo treated groups at different interval between treatment and biopsy: 0=no staining, 1=weak, 2=moderate, and 3=strong.

Table I. Acrosomal reaction status of spermatozoa recovered from uterine cavity and cervical mucus in the different groups.

Group		Treatment	Spermatozoa from	AR (Mean ± SEM)
I	Treatment 12 hours after coitus and uterine flushing performed 24 hours after pill administration.	Placebo	Mucus	11.0 ± 2.1
			Flushing	10.3 ± 2.7
		LNG	Mucus	11.0 ± 0.9
			Flushing	12.7 ± 2.6
II	Treatment 12 hours after coitus and uterine flushing performed 48 hours after pill administration.	Placebo	Mucus	10.2 ± 2.0
			Flushing	6.2 ± 1.5
		LNG	Mucus	10.0 ± 1.8
			Flushing	13.0 ± 3.0
III	Treatment 36 hours after coitus and uterine flushing performed 24 hours after pill administration.	Placebo	Mucus	9.8 ± 3.1
			Flushing	7.0 ± 0.8
		LNG	Mucus	12.5 ± 2.5
			Flushing	7.8 ± 1.1
IV	Treatment 24 hours after artificial insemination and uterine flushing performed 24 hours after pill administration.	Placebo	Mucus	9.3 ± 3.3
			Flushing	7.3 ± 2.3
		LNG	Mucus	8.0 ± 0.7
			Flushing	10.0 ± 1.5

Table II. Levonorgestrel concentrations in serum and uterine flushing at different time after 1.5 mg of LNG intake

	Samples after 24 h of LNG administration		Samples after 48 h of LNG administration
	Serum (n=18)	Uterus flushing (n=8)	Serum (n=6)
Mean	3,462.9 pg/ml	47.9 pg/ml	1,458.8 pg/ml
SEM	275.5	10.6	88.6
Range	(1790 - 5495)	(9.4 – 112.1)	(1123 - 1760)

4. Conclusões

- A administração oral de 1,5 mg de LNG para AE não alterou a taxa de RA dos espermatozóides humanos e não prejudicou a qualidade do muco cervical após 36-60 horas do coito e 24-48 horas após a ingestão do LNG.

- A concentração do LNG no fluido de lavagem da cavidade uterina foi 1,38% dos valores de LNG observados no soro após 24 horas da ingestão de 1,5 mg de LNG para AE.

- Não houve mudança no padrão de expressão de glicodelina-A no endométrio humano nos ciclos tratados com 1,5 mg de LNG para AE após 24 ou 48 horas da administração do progestágeno.

5. Referências Bibliográficas

Alvarez-Sanchez F, Montes de Oca VML, Brache de Mejia V *et al.* Migración espermática en diferentes etapas del ciclo menstrual de la mujer. **Ciencia y Sociedad 1978; 3:35-50.**

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; 68:55-59.**

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

Baldi E, Luconi M, Bonaccorsi L, Forti G. Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. **Front in Biosc 1998; 3:1051-59.**

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. **Front in Biosc 2000; 5:110-23.**

Bielfeld P, Graf M, Jeyendran RS, Zaneveld LJ. No change in acrosome reaction of human spermatozoa during storage in cervical mucus. **Andrologia 1992; 24: 83-86.**

Blackmore B, Beebe S, Danford D, Alexander N. Progesterone and 17-alpha hydroxyprogesterone: novel stimulators of calcium influx in human sperm. **J Biol Chem 1990; 265:1376-80.**

Bray C, Brown J C K, Publicover S, Barratt C L R. Progesterone interaction with sperm plasma membrane, calcium influx and induction of the acrosome reaction. **Reprod Med Rev 1999; 7:81-93.**

Brito KS, Bahamondes L, Nascimento JA, de Santis L, Munuce MJ. The in vitro effect of emergency contraception doses of levonorgestrel on the acrosome reaction of human spermatozoa. **Contraception 2005; 72:225-228.**

Brown S.E., E. Mandelin, S. Oehninger, J.P. Torner, M. Seppala, Jones H.W. Jr., Endometrial glycodelin-A expression in the luteal phase of stimulated ovarian cycles. **Fertil Steril 2000; 74:130–133.**

Canzler E, Ahrendt H J, Ahrendt S. Experiences with levonorgestrel in postcoital contraception. **Zentralbl Gynäkol 1984; 106:1182-91.**

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. **Biol Reprod 1998; 59:733-42.**

Cheng L, Gulmezoglu AM, Oel CJ, Piaggio G, Ezcurra E, van Look PF. Interventions for emergency contraception. ***Cochrane Database Syst Rev* 2004; CD001324.**

Clark, G.F., Oehninger, S., Patankar, M.S., Koistinen, R., Dell, A., Morris, H.R., Koistinen, H., Seppala, M. A role for glycoconjugates in human development: the human feto-embryonic defence system hypothesis. **Hum Reprod 1996; 11:467–473.**

Croxatto HB. Gamete transport. In: E.Y. Adashi, J.A. Rock, Z. Rosenwaks, Editors, *Reproductive endocrinology, surgery, and technology*, Lippincott-Raven, Philadelphia, **1996: 385–402**.

Croxatto HB, Devoto L, Durand M, Ezcurra E, Larrea F, Nagle C, Ortiz ME, Vantman D, Vega M, von Hertzen. Mechanism of action of hormonal preparations used for emergency contraception: a review of the literature. **Contraception 2001; 63:111–121.**

Croxatto HB, Ortiz ME, Muller AL. Mechanism of action of emergency contraception. **Steroids 2003; 68:1095-1098.**

Dell, A., Morris, H.R., Easton, R.L., Panico, M., Patankar, M., Oehninger, S., Koistinen, R., Koistenen, H., Seppala, M., Clark, G.F. Structural analysis of the oligosaccharides derived from glycodelin, a human glycoprotein with potent immunosuppressive and contraceptive activities. ***J Biol Chem*** **1995; 270:24116–24126.**

Devoto L, Fuentes A, Palomino A, Espinoza A, Kohen P, Ranta S, 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.**

Durand M, Cravioto CM, Raymond EG, Duran-Sanchez O, De la Luz Cruz-Hinojosa M, Castell-Rodriguez A, Schiavon R, Larrea F. On the mechanism of action of short-term levonorgestrel administration in emergency contraception. **Contraception 2001; 64:227–234.**

Flesch F M e Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. **Biochim et Biophys Act 2000; 1469: 197-235.**

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

Gainer E, Kenfack B, Mboudou E, Doh AS, Bouyer J. Menstrual bleeding patterns following levonorgestrel emergency contraception. **Contraception 2006; 74:118-124.**

Gemzell-Danielsson K e Marions L. Mechanisms of action of mifepristone and levonorgestrel when used for emergency contraception. **Hum Reprod Update 2004; 10:341–348.**

Gould JE, Overstreet JW, Hanson FW. Assessment of human sperm function after recovery from the female reproductive tract. **Biol Reprod 1984; 31: 888-894.**

Grimes D A, Raymond E G. Emergency contraception. **Ann of Inter Med 2002; 137:180-189.**

Hapangama D, Glasier AF, Baird DT. The effects of peri-ovulatory administration of levonorgestrel on the menstrual cycle. **Contraception 2001; 63:123–129.**

Johansson E, Brache V, Alvarez F, Faundes A, Cochon L, Ranta S, *et al.* Pharmacokinetic study of different dosing regimens of levonorgestrel for emergency contraception in healthy women. **Hum Reprod 2002; 17:1472-6.**

Julkunen M, Apter D, Seppala M, Stenman U, Bohn H. Serum levels of placental protein 14 reflect ovulation in nonconceptional menstrual cycles. **Fertil Steril 1986a; 45: 47–50.**

Julkunen M, Koistinen R, Sjoberg J, Rutan M, Wahlstrom T, Seppala M. Secretory endometrium synthesis placental protein 14. **Endocrinology 1986b; 118:1782–1786.**

Julkunen M, Rutanen E-M, Koskimies A, Ranta T, Bohn T, Seppala M. Distribution of placental protein 14 in tissues and body fluids during pregnancy. **Br J Obstet Gynaecol 1985; 11:1145–1151.**

Julkunen M, Seppala M, Janne O A. Complete amino acid sequence of human placental protein 14: a progesterone-associated uterine protein homologous to ß-lactoglobulins. **Proc Natl Acad Sci USA, 1988; 85:8845–8849.**

Kesseru E, Garmendia F, Westphal N, Parada J. The hormonal and peripheral effects of dl-norgestrel in postcoital contraception. **Contraception 1974; 10:411-424.**

Kesserü E, Larrañaga A, Parada J. Post-coital contraception with d-norgestrel. **Contraception 1973; 7:367-79.**

Kubba A A, White J O, Guillebaud J, Elder M G. The biochemistry of human endometrium after two regimens of postcoital contraception: a dl-norgestrel/ ethinylestradiol combination or danazol. **Fertil Steril 1986; 45:512-16.**

Landgren B M, Johannisson E, Aedo A R, Kumar A, Yong-en S. The effects of levonorgestrel administered in large doses at different stages of the cycle on ovarian function and endometrial morphology. **Contraception 1989; 39:275–289.**

Li TC, Ling E, Dalton C, Bolton A E, Cooke I D. Concentration of endometrial PP14 in uterine flushings throughout the menstrual cycle in normal, fertile women. **Br J Obstet Gynaecol 1993;100: 460–464.**

Ling W Y, Robichaud A, Zayid I, Wrixon W, Macleod SC. Mode of action of DL-norgestrel and ethinylestradiol combination in postcoital contraception. **Fertil Steril 1979; 32:297-302.**

Ling W Y, Wrixon W, Zayid I, Acorn T, Popat R, Wilson E. Mode of action of dl-norgestrel and ethinylestradiol combination in postcoital contraception. II. Effect of postovulatory administration on ovarian function and endometrium. **Fertil Steril 1983; 39:292-97.**

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 Clin Endocrinol Metab 1998; 83:877-85.**

Mandelin E, Koistinen H, Koistinen R, Affandi B, Seppala M. Levonorgestrel-releasing intrauterine device-wearing women express contraceptive glycodelin-A in endometrium during the midcycle: another contraceptive mechanism? **Hum Reprod 1997; 12:2671–2675.**

Mandelin E, Koistinen H, Koistinen R, Arola J, Affandi B, Seppala M. Endometrial expression of glycodelin in women with levonorgestrel-releasing subdermal implants. **Fertil Steril 2001; 76:474–478.**

Marions L, Hultenby K, Lindell I, Sun X, Stabi B, Gemzell Danielsson K. Emergency contraception with mifepristone and levonorgestrel: mechanism of action. **Obstet Gynecol 2002;** 100**:65–71.**

Meizel S e Turner K O. Chloride efflux during the progesterone-initiated human sperm acrosome reaction is inhibited by lavendustin A, a tyrosine kinase inhibitor. **J Androl 1996; 17:327-30.**

Melendrez C S, Meizel S, Berger T. Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction in vitro. **Mol Reprod Dev 1994; 39:433-38.**

Mortimer D, Templeton AA. Sperm transport in the human female reproductive tract in relation to semen analysis characteristics and time of ovulation. **J Reprod Fertil 1982; 64:401-8.**

Moyer DL, Rimdusit S, Mishell DR Jr. Sperm distribution and degradation in the human female reproductive tract. **Obstet Gynecol 1970; 35:831-40.**

Muller AL, Llados CM, Croxatto HB. Postcoital treatment with levonorgestrel does not disrupt postfertilization events in the rat. **Contraception 2003; 675:415-9.**

Munuce MJ, Nascimento JA, 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 2006; 731:97-101.**

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; 72:71-76.**

Nikkanen V, Söderström 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; 61:401-406.**

Nilsson C G, Luukkainen T. Improvement of a d-norgestrel-releasing IUD **Contraception 1977; 15:295-306.**

Nilsson C G, Lähteenmäki P, Luukkainen T. Levonorgestrel plasma concentrations and hormone profiles after insertion and after one year of treatment with a levonorgestrel-IUD. **Contraception 1980c, 21:225-33.**

Nilsson C G, Luukkainen T, Arko H. Endometrial morphology of women using a d-norgestrel-releasing intrauterine device. **Fertil Steril 1978; 29:397-401.**

Oehninger S, Coddington C C, Hodgen G D, Seppala M. Factors affecting fertilization: endometrial placental protein 14 reduces the capacity of human spermatozoa to bind to the human zona pellucida. **Fertil Steril 1995; 63:377–383.**

Oehninger S, Sueldo C, Lanzendorf S, Mahony M, Burkman L J, Alexander N J, *et.al.* A sequential analysis of the effect of progesterone of specific sperm functions crucial to fertilization in vitro in infertile patients. **Hum Reprod 1994; 9:1322-27.**

Okamoto N A , Uchida A, Takakmura K, Kariya Y, Kanzaki H, Riittinen L, Koistinin R, Seppala M, Mori T. Suppression by human placenta protein 14 of natural killer cell activity. **Am J Reprod Immunol 1991; 26:137-142.**

Osman R A, Andria M L, Jones A D, Meizel S. Steroid induced exocytosis: the human sperm acrosome reaction. **Biochem Biophys Res Commun 1989; 160:828-33.**

Perloff WH, Steinberger E. In vivo survival of spermatozoa in cervical mucus. **Am J Obstet Gynecol 1964; 88:439-442.**

Richlin S S, Ramachandran S, Shanti A, Murphy A A, Parthasarathy S. Glycodelin levels in uterine flushings and in plasma of patients with leiomyomas and polyps: implications for implantation. **Hum Reprod 2002; 17(10):2742-2747.**

Roblero L, Guadarrama A, Ortiz M E, Fernandez E, Zegers-Hochschild F. High potassium concentration improves the rate of acrosome reaction in human spermatozoa. **Fertil Steril 1988; 49:676-79.**

Seppala M, Taylor RN, Koistinen H, Koistinen R, Milgrom E. Glycodelin: a major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. **Endocr Rev 2002; 23: 401–430.**

Seppala M. Advances in uterine protein research: reproduction and cancer. **Int J Gynaecol Obstet 2004; 852: 105-18.**

Sirivaidyapong S, Bevers M M, Colenbrander B. Acrosome reaction in dog sperm is induced by a membrane-localized progesterone receptor. **J Androl 1999; 20:537-44.**

Sueldo C, Oehninger S, Subia E, Mahony M, Alexander N, Burkman L J, *et al.* Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. **Fertil Steril 1993; 60:136-40.**

Swahn ML, Westlund P, Johannisson E, Bygdeman M. Effect of post-coital contraceptive methods on the endometrium and the menstrual cycle. **Acta Obstet Gynecol Scand 1996; 75:738–744.**

Tesarik J, Mendoza C, Moss J, Carreras A. Selective expression of progesterone receptor on the human sperm surface. **Fertil Steril 1992; 58:784-92.**

Tremblay D, Gainer E, Ulmann A. The pharmacokinetics of 750μg levonorgestrel following administration of one single dose or two doses at 12- or 24 interval. **Contraception 2001; 64:327-31.**

Ugocasi G, Rózsa M, Ugocasi P. Scanning electron microscopic SEM; changes of the endometrium in women taking high doses of levonorgestrel as emergency postcoital contraception. **Contraception 2002; 66:433–437.**

Van Cong N, Vaisse C, Gross M, Slim R, Milgrom E, Bernheim A. The human placental protein 14 PP14; gene is localized on chromosome 9q34. **Hum Genet 1991; 86:515–518.**

Von Hertzen H, Piaggio G, Ding J, Chen J, Song S, Bartfai G, *et al.* Low doses of mifepristone and two regimens of levonorgestrel for emergency contraception: a WHO randomised trial**. Lancet 2002; 360:1803–1810.**

Von Hertzen H, Van Look P.F.A. Randomised controlled trial of levonorgestrel versus the Yuspe regimen of combined oral contraceptives for emergency contraception. Task Force on postovulatory methods of Fertility Regulation. **Lancet 1998; 353:428-433.**

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.**

Williams M, Thompson LA, Li TC, Mackenna A, Barratt CL, Cooke ID. Uterine flushing: a method to recover spermatozoa and leukocytes. **Hum Reprod 1993; 8:925-928.**

World Health Organization Task Force on Postovulatory Methods of Fertility Regulation. Randomized controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. **Lancet 1998; 352:428–438**.

Yanagimachi R. Mammalian fertilization. In: E. Knobil and J.D. Neill, Editors, *The physiology of reproduction*, Raven Press, **New York 1988; pp. 135–185.**

Yeung WSB, Chiu PCN, Wang CH, Yao YQ, Ho PC. The effects of levonorgestrel on various sperm functions. **Contraception 2002; 66:453-457.**

Young D C, Wiehie R D, Joshi S G, Poindexter A N. Emergency contraception alters progesterone-associated endometrial protein in serum and uterine luminal fluid. **Obstet Gynecol 1994; 84:266-271.**

Yuzpe A A, Thurlow H J, Ramzy I, Leyshon J I. Post coital contraception- a pilot study. **J Reprod Med 1974; 13:53-58.**

Zinaman M, Drobnis EZ, Morales P, Brazil C, Kiel M, Cross NL, *et al.* The physiology of sperm recovered from the human cervix: acrosomal status and response to inducers of the acrosome reaction. **Biol Reprod 1989; 41:790-797.**

6. Bibliografia de Normatizações

FRANÇA, J.L.; BORGES, S.M.; VASCONCELLOS, A.C.; MAGALHÃES, M.H.A. – **Manual para normatização de publicações técnico-científicas**. 4ª ed., Editora UFMG, Belo Horizonte, 1998. 213p.

Normas e procedimentos para publicação de dissertações e teses. Faculdade de Ciências Médicas, UNICAMP. Ed. SAD – Deliberação CCPG-001/98 (alterada 2005).