

# Levonorgestrel decreases cilia beat frequency of human fallopian tubes and rat oviducts without changing morphological structure

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## SUMMARY

Levonorgestrel, a derivative of progesterone, effectively protects women against unwanted pregnancy as an emergency contraceptive. Previous studies have not been successful in determining the mechanism by which levonorgestrel acts. In the present study we analysed cilia beat action and cilia morphology following levonorgestrel exposure *in vitro* and *in vivo* using both light and electron microscopy. There was a significant decrease in the ciliary beat frequency (CBF) of human fallopian tubes between mucosal explants bathed in 5  $\mu\text{mol/L}$  levonorgestrel and those bathed in medium alone ( $P < 0.05$ ). There was a tendency for CBF to decrease more in the ampulla than in isthmus, but there were no differences between the proliferative and secretory phases. In rat oviducts, levonorgestrel produced a similar reduction in CBF (~10%) compared with the saline control group ( $P < 0.05$ ). Histological and ultrastructural analysis demonstrated no changes in the percentage of ciliated cells or in the classic '9 + 2' structure of cilia following levonorgestrel treatment in either system. Thus, levonorgestrel reduces CBF without damaging cilia morphology. Decreases in CBF may indicate a pathological role for levonorgestrel in the transportation of the ovum and zygote in the fallopian tube.

**Key words:** cilia beat frequency, human tubal epithelium, levonorgestrel, rat oviduct.

## INTRODUCTION

The oviduct is the part of the female reproductive tract that plays a crucial role in maternal interactions with gametes and embryos. It provides the appropriate environment for the maturation of spermatozoa, for fertilization and for the transport of ova and/or embryos to the site of implantation. Transit through the oviduct is driven by two major mechanisms: (i) smooth muscle contraction; and (ii) cilia motility.<sup>1</sup> Although the relative importance of each mechanism is not fully clear, several lines of evidence indicate that ciliary activity may be the dominant force that contributes to the transport of the gamete and embryo. Inhibition of muscular activity with isoprenaline has no effect on total transit time from the ampulla to the uterine cavity,<sup>2</sup> and women with immotile cilia syndrome are subfertile.<sup>3</sup> There is also a marked reduction in the number of ciliated cells and deciliation in the fallopian tubes of women with an ectopic pregnancy compared with women with an intrauterine pregnancy.<sup>4</sup> Several factors affect the beating of cilia in the fallopian tube, including progesterone (P4), cAMP, prostaglandins, adrenomedullin and  $\text{Ca}^{2+}$ .<sup>5–8</sup> Of these, P4 has been shown to regulate ciliary beat frequency (CBF) in the fallopian tubes of several mammalian species.<sup>8–12</sup>

Levonorgestrel (LNG), a second-generation progestin, differs from P4 in its structure and pharmacological properties, including effective dose, metabolism, pharmacokinetics, bioavailability and binding to serum binding proteins. It is an effective emergency contraceptive that protect against unwanted pregnancy when used as a 1.5 mg single dose or as a two-dose regimen of 0.75 mg LNG taken 12 h apart. Exposure to LNG during the preovulatory phase can either delay or inhibit ovulation, depending on the timing of administration.<sup>13</sup> Once the level of luteinizing hormone starts to rise, LNG cannot prevent ovulation, which may explain reported cases of its failure. A number of cases of ectopic pregnancy after LNG failure have been reported in the literature<sup>14–16</sup> and a high rate of ectopic pregnancy (4.1%) was observed after LNG failure based on a retrospective questionnaire given to users of LNG.<sup>17</sup> However, the multifactorial aetiological characteristics of ectopic pregnancy have thus far precluded a causal relationship being established between emergency contraception with LNG and ectopic pregnancy.

The effects of peri- and postovulatory administration of LNG are still not clear. Levonorgestrel has no direct effect on sperm

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function or endometrial receptivity markers, corpus luteum function, fallopian tube oestrogen and P4 receptor expression, fertilization or embryo implantation.<sup>18</sup> In addition, exposure to LNG also does not alter the expression of candidate molecules, such as oestrogen or P4 receptors, leukaemia inhibitory factor, vascular endothelial growth factor, inducible nitric oxide synthase and endocannabinoid receptor 1, which are potential regulators of the pathophysiological changes in tubal pregnancy in the fallopian tube and chorionic villi.<sup>19</sup> There is no reliable information regarding the effects of LNG effects on ciliary activity.

In the present study, we examined the effects of LNG on the CBF of normal human fallopian tube epithelium *in vitro* and on postovulatory rat oviduct *in vivo*. Moreover, we investigated possible modifications to ciliary morphological structure following LNG treatment using both light and transmission electron (TEM) microscopy.

## RESULTS

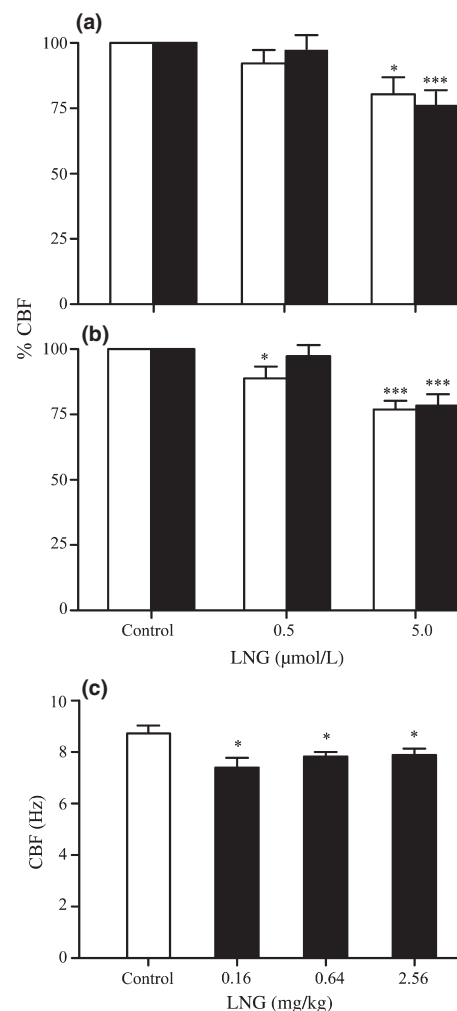
### Effects of LNG on CBF *in vitro* and *in vivo*

To explore the effects of LNG on CBF, we cultured tubal epithelium explants with different concentrations of LNG (0.5 and 5.0  $\mu\text{mol/L}$ ). The CBF of both the ampulla and isthmus regions from proliferative-phase human fallopian tubes decreased when mucosal explants were bathed in 5.0  $\mu\text{mol/L}$  LNG for 24 h compared with explants bathed in medium alone ( $P < 0.05$ ; Fig. 1a). This effect was concentration dependent, with 3–8% and 20–24% decreases in CBF in the presence of 0.5 and 5.0  $\mu\text{mol/L}$  LNG, respectively. Similar results were observed in secretory-stage fallopian tube explants (Fig. 1b). In addition, CBF was reduced in the ampulla compared with the isthmus under all three conditions in secretory-stage explants ( $P < 0.05$ ), but there were no differences among proliferative-stage samples (Fig. 1). For each treatment and for each region, there was no significant difference between explants from the proliferative and secretory stages (Table 1).

The effects of LNG *in vivo* were analysed by treating rats with LNG (0.16, 0.64 and 2.56 mg/kg) and evaluating the CBF in their oviducts. The CBF was reduced following treatment with 0.16, 0.64 and 2.56 mg/kg LNG compared with the saline-treated control ( $7.41 \pm 0.30$ ,  $7.82 \pm 0.19$  and  $7.89 \pm 0.24$  vs  $8.73 \pm 0.30$  Hz, respectively;  $P < 0.05$ ; Fig. 1c). The 10% decrease in CBF seen with 0.16 mg/kg LNG was similar to the 9.4% and 9.2% decreases seen with the two higher doses of LNG (0.64 and 2.56 mg/kg). Thus, treatment with 0.16 mg/kg LNG was sufficient to induce changes in CBF.

### Oviduct morphology in the presence of LNG

The mucosa of both the ampulla and isthmus of the human fallopian tube is lined with a pseudostratified epithelium that consists of two different cell types: ciliated and non-ciliated cells (Fig. 2). Ciliated cells were predominant in the oviduct and most of the non-ciliated cells showed an apical protrusion (Fig. 2). The percentage of ciliated cells in untreated human oviduct decreased from the ampulla to isthmus ( $63.78 \pm 2.75\%$  vs  $51.01 \pm 3.03\%$ , respectively;  $P < 0.05$ ; Fig. 3). There were no significant differences in the morphological structure and relative percentage of ciliated cells between the LNG-treated and control groups in the two regions



**Fig. 1** Effects of levonorgestrel (LNG) on the ciliary beat frequency (CBF). (a) Addition of 5.0  $\mu\text{mol/L}$  LNG to the culture medium significantly reduced the CBF of the human fallopian tube in the proliferative phase. (b) The CBF of human tubal epithelium in the secretory phase also decreased after treatment with 0.5 and 5.0  $\mu\text{mol/L}$  LNG, although this effect was not seen in the isthmus with the lower concentration of LNG. ( $\square$ , ampulla;  $\blacksquare$ , isthmus). (c) The CBF of the rat oviduct was reduced after treatment of rats with all three doses of LNG. Data are the mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with control (one-way ANOVA followed by the Newman–Keuls' test).

from human fallopian tubes (Figs 2,3). There were no changes in the epithelial cell ultrastructure of the ampulla and isthmus of human fallopian tubes after exposure to LNG as viewed by TEM (Fig. 4). In treated tissues, the epithelial lining was made up of electron-lucent ciliated cells and non-ciliated (secretory) cells. The secretory cells were characterized by apical protrusions on which short microvilli could be seen. The non-ciliated cells also contained granules with a dark homogeneous matrix and some granules with a moderately electron-dense matrix. Typical '9 + 2' microtubules and mitochondria were observed in ciliated cells. There was no microtubular disarrangement, and extra tubules, single tubules or mitochondrial damage were not seen after LNG treatment.

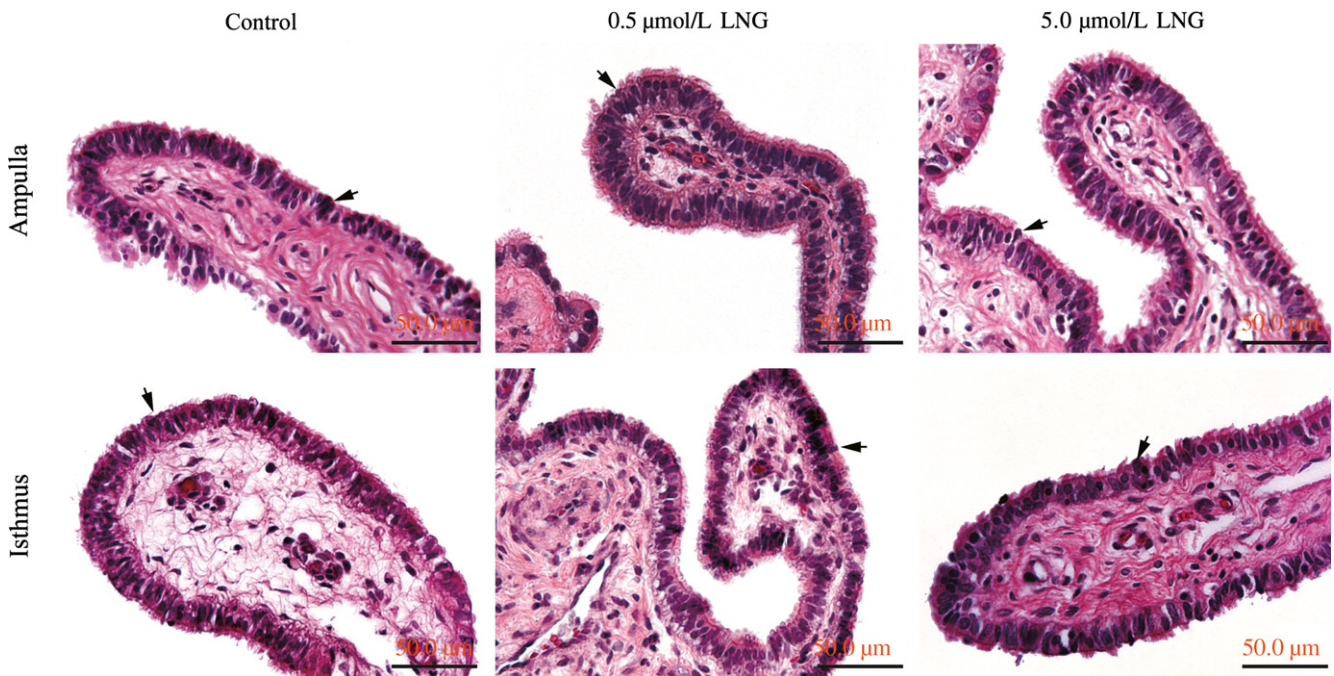
In rat oviducts, the ampulla region consisted of > 70% ciliated cells and few secretory cells (Fig. 5). As seen with the human explants, LNG exposure did not change the general structure and

**Table 1** Ciliary beat frequency of the ampulla and isthmus regions from human fallopian tubes cultured *in vitro* during different cycle stages

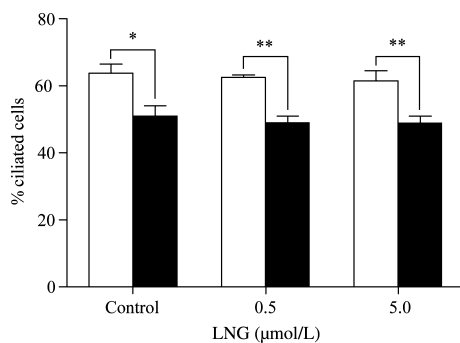
Cycle stage	Ampulla			Isthmus		
	0.5 $\mu\text{mol/L}$ LNG	5 $\mu\text{mol/L}$ LNG	Control	0.5 $\mu\text{mol/L}$ LNG	5 $\mu\text{mol/L}$ LNG	Control
Proliferative phase	4.57 $\pm$ 0.36	4.11 $\pm$ 0.57	5.13 $\pm$ 0.59	5.22 $\pm$ 0.48	4.21 $\pm$ 0.59	5.49 $\pm$ 0.65
Secretory phase	4.61 $\pm$ 0.34	4.08 $\pm$ 0.38	5.30 $\pm$ 0.47	5.59 $\pm$ 0.46	4.50 $\pm$ 0.36	5.88 $\pm$ 0.59
<i>P</i> -value	0.93	0.97	0.82	0.59	0.68	0.65

Data are the mean  $\pm$  SEM from nine and 10 measurements of proliferative- and secretory-phase explants, respectively. Data were analysed with unpaired Student's *t*-test.

LNG, levonorgestrel.



**Fig. 2** Histological analysis of human fallopian tubes after levonorgestrel (LNG) treatment *in vitro*. Light microscopy images showed no changes in the morphological structure of the tubal epithelium at the ampulla and isthmus region between control and LNG-treated groups. Arrows indicate apical protrusion.

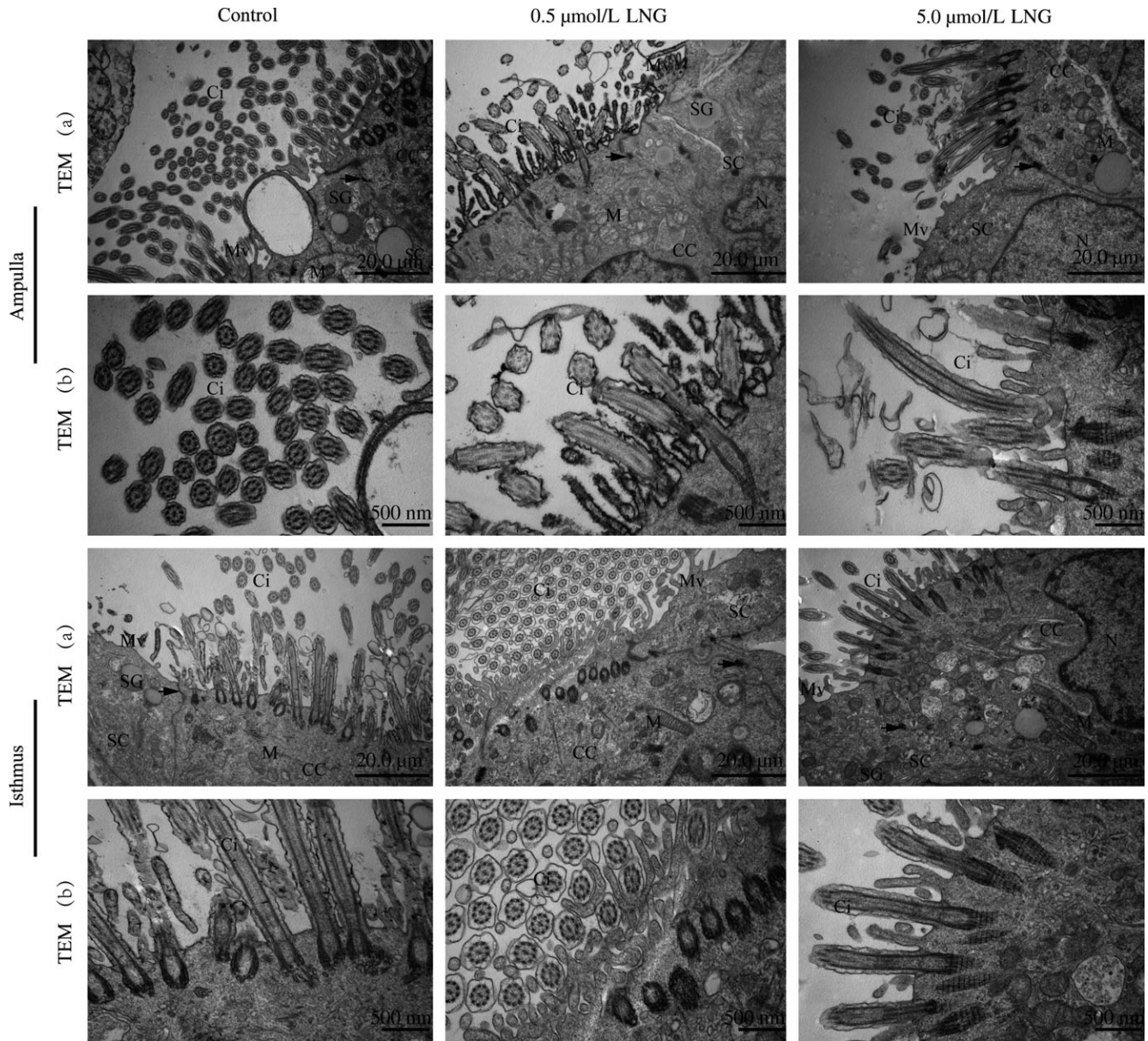


**Fig. 3** Percentage of ciliated cells in human fallopian tube epithelium after levonorgestrel (LNG) treatment *in vitro*. (□), ampulla; (■), isthmus. There was no difference in the percentage of ciliated cells (relative to non-ciliated cells) after LNG treatment. For all three groups, there was a lower percentage of ciliated cells in the isthmus than ampulla region. Data are the mean  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01.

ultrastructure of the oviduct tissue or relative percentage or number of ciliated cells (Fig. 5; Table 2).

## DISCUSSION

In the present study, we have demonstrated that LNG decreases the CBF in the human fallopian tube *in vitro* and in the rat oviduct *in vivo*. Similar observations were reported by Mahmood *et al.*,<sup>12</sup> who treated human fallopian tubes *in vitro* with P4 (10  $\mu\text{mol/L}$ ) and noted a reduction in the CBF of 40–50% 24 h after treatment. Wessel *et al.*<sup>10</sup> also demonstrated that 20  $\mu\text{mol/L}$  P4 reduced the CBF by 11% within 15 min in dissected fallopian tubes from cows. Paltieli *et al.*<sup>8</sup> proposed that the inhibitory effect of P4 on ciliary beating and the treatment of women with high levels of P4 during ovulation-induction cycles could lead to tubal pregnancies. Previous studies have shown no difference in the CBF between the isthmus and ampulla regions of the human fallopian tube during

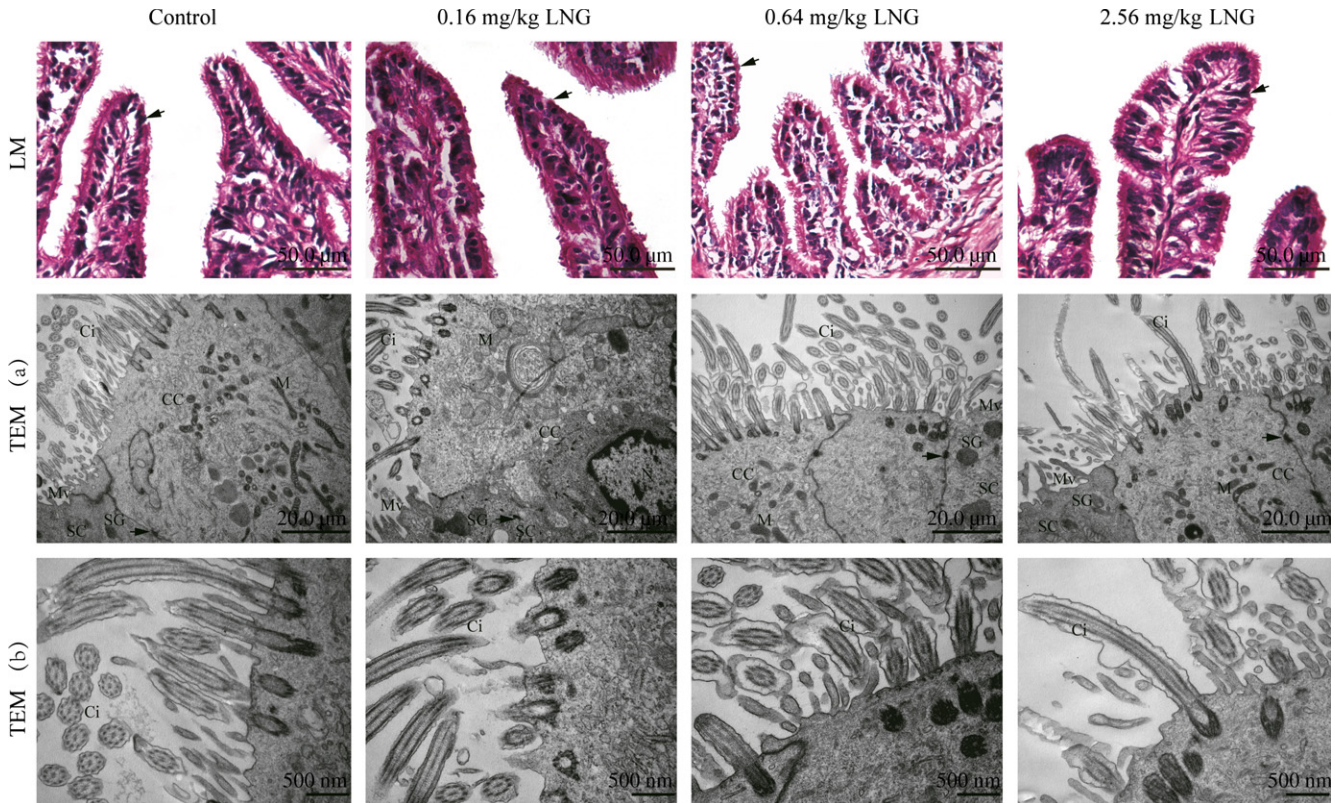


**Fig. 4** Ultrastructural analysis of human fallopian tube epithelium after levonorgestrel (LNG) treatment *in vitro*. Transmission electron microscopy (TEM) images showed no change in the ultrastructure of the tubal epithelium at the ampulla and isthmus regions between the control and LNG-treated groups (TEM (a)). Similarly, typical '9 + 2' microtubules of cilia were shown by higher-magnification images (TEM (b)). Arrows indicate desmosomes. CC, ciliated cell; SC, secretory cell; Ci, cilia; M, mitochondria; Mv, microvilli; SG, secretory granule; N, nucleus.

any stage of the menstrual cycle.<sup>12,20</sup> The findings of the present study indicate that, in the presence of LNG, the CBF tended to be lower in the ampulla than in the isthmus during both the proliferative and luteal phase, although the differences did not reach statistical significance for either region between the two stages.

In the present study we used a minimal dose of LNG in the rat experiments that is equivalent to nearly sevenfold the dose used in women (1.5 mg orally). Given that in rats the bioavailability of orally administered LNG (9%) is much lower than that in humans (100%),<sup>21</sup> separate groups of animals were also administered two higher doses. Previous studies have indicated that P4 reduces the CBF during the mid luteal phase in guinea-pigs,<sup>9</sup> which supports our findings. Levonorgestrel, an orally active P4 derivative, has a higher affinity for the P4 receptor than P4 ( $K_d = 1\text{--}5$  nmol/L).<sup>22</sup>

Thus, the inhibition of ciliary activity in the presence of LNG may be attributed to P4-like activity of LNG acting via the progesterone receptor. A serum concentration of 40 nmol/L LNG, which was measured 2 h after oral administration of 10  $\mu\text{g}$  LNG in rats,<sup>23</sup> is substantially higher than the physical concentration of P4 after ovulation and the therapeutic range of the drug (active therapeutic range 10–20 nmol/L). This may explain the similar effects of higher doses of LNG on CBF in rats compared with the low dose and the various adverse effects of LNG. Although acute post-coital administration of LNG after ovulation did affect the ciliary action *in vivo* in the present study, the post-coital administration of LNG has been shown previously to have no effect on fertilization and subsequent processes in both a rodent and primate study.<sup>24,25</sup> However, we note that the occurrence of ectopic



**Fig. 5** Histological and ultrastructural analysis of rat oviduct after levonorgestrel (LNG) treatment *in vivo*. Light microscopy (LM) images showed no difference in morphological structure of oviductal epithelium between the control and LNG-treated groups. Transmission electron microscopy (TEM) images (TEM (a)) and higher-magnification images (TEM (b)) of cilia structures also seemed to be normal. Arrows indicate apical protrusions in LM images and desmosomes in TEM images. CC, ciliated cell; SC, secretory cell; Ci, cilia; M, mitochondria; Mv, microvilli; SG, secretory granule; N, nucleus.

**Table 2** Percentage of ciliated cells at the ampulla of rat oviduct after levonorgestrel exposure

	<i>n</i>	% Ciliated cells
Control	12	69.75 ± 1.82
LNG (mg/kg)		
0.16	13	67.54 ± 2.19
0.64	10	70.01 ± 1.74
2.56	11	68.38 ± 3.01
<i>P</i> -value		0.78

Data are the mean ± SEM. Data were analysed with one-way analysis of variance.

LNG, levonorgestrel.

implantation in animals is rare,<sup>26</sup> although the reason for this is unknown. Most likely, there are different mechanisms underlying the transition of gametes to embryos in humans and other mammals. We propose that a high plasma concentration of LNG, which leads to decreased CBF, is responsible, in part, for ectopic implantation of embryos in the oviduct.

Histological and ultrastructural analysis provided direct evidence of the effect of LNG on the composition and morphology of epithelial cells. Total cell numbers remained constant and the percentage of ciliated cells (relative to secretory cells) did not change in response to LNG treatment. Oestradiol and P4 control

the transformation between ciliated and secretory cells, as well as the induction of ciliogenesis and deciliation in multiple mammalian oviduct epithelial cells, including those from horses, dogs and humans.<sup>27–29</sup> However, there are no marked changes in the relative number of ciliated and secretory cells in any oviductal segment during the oestrous cycle in rats because of its short duration.<sup>30</sup> Thus, it follows that the timing of hormonal stimulation was related to changes in the number and morphology of cells. Chen *et al.*<sup>31</sup> found that the ratio of ciliated to non-ciliated cells changed significantly when porcine oviduct epithelial cells were cultured *in vitro* to mimic dioestrus simulation (35 ng/mL P4 and 10 pg/mL oestradiol) for 10 days and were subsequently treated with 50 pg/mL oestradiol and 0.5 ng/mL P4 for 2.5 days. The duration of treatment in the present study was much shorter than that used by other authors. A lower number of ciliated cells in the isthmus compared with the ampulla has been reported in the oviduct of mammals such as the horse,<sup>29</sup> Chinese Meishan pig<sup>32</sup> and the mouse,<sup>33</sup> as well as in humans.<sup>34</sup> Cilia are primarily responsible for picking up the ovum and transporting the zygote;<sup>35</sup> however, the well-developed muscle layer and relatively few cilia in the isthmus region produce a tubal microenvironment that favours the early development of the embryo.<sup>35</sup>

The role of ciliated cells in the transport of germinal cells in the mammalian oviduct and in mucociliary clearance of bacteria or particles in the respiratory epithelium depends on the function of healthy, beating cilia with a normal morphological structure.

Numerous ultrastructural defects or anomalies of the classic '9 + 2' structure of epithelial cilia are associated with ciliary dyskinesia, resulting in respiratory diseases, infertility and ectopic pregnancy.<sup>36</sup> In the present study, administration of LNG *in vitro* and *in vivo* led to a rapid decrease in CBF independent of changes in cilia morphology. A non-genomic signalling mechanism or signalling via the classical P4 receptor have been suggested to be involved in the rapid reduction in CBF that occurs within 30 min of P4 administration in both cows and mice.<sup>10,37,38</sup> Further studies are needed to determine the exact mechanism of action of LNG and the signalling pathways involved.

In conclusion, we are the first to demonstrate the effect of LNG on the CBF of human fallopian tube *in vitro* and of rat oviduct *in vivo*. We propose that a reduction in the CBF by LNG may delay the precisely timed transport events that occur in the oviduct.

## METHODS

### *In vitro* experiments

#### *Preparation of LNG solutions*

Crystalline LNG obtained from Sigma (St Louis, MO, USA) was dissolved in 1 mL dimethylsulphoxide (DMSO) and was further diluted with Dulbecco's modified Eagle's medium (DMEM) mixed 1 : 1 with Ham's F12 medium (Invitrogen, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum, 0.5 µmol/L streptomycin (Evans Medical, Surrey, UK; 100 µg/mL) and 5 µmol/L penicillin (100 U/mL; Glaxo Laboratories, Middlesex, UK).

#### *Collection and incubation of human fallopian tubes*

Normal fallopian tubes were collected from 19 patients (38–48 years of age) undergoing hysterectomy for benign conditions, after obtaining written consent and local ethics committee approval. All women had regular menstrual cycles and no woman had used hormonal medication within 3 months of surgery. As assessed by the time since the last menstrual period, endocrine profile and histological assessment of the endometrium, nine women were in the proliferative stage and 10 were in the secretory stage of the menstrual cycle. Salpinx samples from each individual were placed in 50 mL tubes with the culture medium described above and immediately transported to the laboratory. Fallopian tubes were rinsed several times to remove all visible evidence of blood and the muscularis and serosa were then removed. Small (1–2 mm<sup>2</sup>) pieces of tissue were dissected free from the ampulla and isthmus portions of the fallopian tube and individually transferred to wells of a 24-well plate (three plates were used for each individual). Each well contained 1 mL culture medium with LNG (at different concentrations) or culture medium alone. Samples were incubated at 37°C, 100% humidity and 5% CO<sub>2</sub>. After 24 h, one plate was used to the CBF and the remaining two were fixed and processed for light microscopy and TEM.

#### *Measurement of CBF*

Measurement of CBF was performed at 37°C. To control for inherent variations in CBF between individual tubes, each LNG-

treated sample from both the ampulla and isthmus portions was tested on every tube. The CBF was measured using an inverted bright-field microscope (TE2000; Nikon Instruments, New York, NY, USA) equipped with a × 40 objective. Images of moving cilia were acquired with a 12 bit high-speed camera (EC1020; Prosilica, Burnaby, Canada) that recorded a 50 × 50 pixel region with a speed of 30 frames/s for 10 s. Single ciliated cells and small groups of ciliated cells were not used because readings from such areas were inconsistent. Five regions were randomly imaged from each treated sample from the same individual, and five pieces were measured. The CBF was then determined by manually reviewing the digital images.

#### *Sample preparation for light microscopy and TEM*

After 24 h incubation, samples were washed gently in phosphate-buffered saline (PBS; pH 7.2). For haematoxylin and eosin (H&E) staining, specimens were fixed in paraformaldehyde solution for 48 h at 4°C, dehydrated in a graded ethanol series and embedded in paraffin. Sections (5 µm) were cut on a microtome (Leica, Wetzlar, Germany). The morphology of the oviductal epithelium was analysed under a microscope (DSM 2500; Leica Stereozoom, Leica Microsystems, Heerbrugg, Switzerland). The number of cells per treated sample was determined using 10 light microscopy fields captured at × 400 magnification and digitized with ImageJ (Rasband, W.S., National Institutes of Health). For TEM, samples were immersed in 2% glutaraldehyde (Sigma) at 4°C, with additional post-fixation in 1% OsO<sub>4</sub> (Merck, Darmstadt, Germany) for 2 h. After fixation, the tissues were dehydrated in a graded ethanol series and embedded in Epon 618. Ultrathin sections were cut with an ultramicrotome and stained with saturated uranyl acetate and lead citrate. Sections were assessed at 80 kV using a Philips CM-120 transmission electron microscope (Amsterdam, The Netherlands).

### *In vivo* experiments

#### *Animals*

Adult female Sprague–Dawley rats (10–12 weeks old) and adult male rats (12–16 weeks old) were used. All rats were kept in the same room with water and pet chow available *ad libitum*, lights on from 0730 to 2130 hours and a temperature of 24°C. Animal care and experimental procedures were in accordance with the guidelines of the Institutional Ethics Committee.

Female rats were underwent daily to vaginal smears to determine the stage of the oestrous cycle. Each pro-oestrous female rat was caged individually with two fertile males for one entire night. At 0800 hours on the day of oestrus, the presence of a vaginal plug and the presence of spermatozoa in the vaginal smear verified mating. Levonorgestrel was administered within 6 h of mating.

#### *Preparation of LNG solutions and treatments*

Mated female rats were divided into four groups: 0.16, 0.64 and 2.56 mg/kg LNG-treated groups and a vehicle (saline)-treated control group. Each group included more than 10 rats. Levonorgestrel was weighed and transferred to a 2 mL volumetric

centrifuge tube, to which 200  $\mu$ L saline was added, and the resulting suspension was vortexed. Drug suspension or vehicle was administered orally using a 20 G blunt needle. After 2 h (the time to peak LNG levels in rats<sup>23</sup>), rats were anaesthetized with an intraperitoneal injection of 40 mg/kg of 1% pentobarbital sodium.

#### Measurement of CBF

Oviducts were collected from anaesthetized rats into a Petri dish filled with DMEM/F12 medium (1 : 1) and cut open longitudinally with fine scissors under a dissecting microscope. The rats were then killed. Small (1–2 mm<sup>2</sup>) pieces of tissue were dissected from the ampulla of each oviduct. Some samples from each rat were used for measurement of CBF, whereas others were fixed and processed for light microscopy and TEM as described above for the *in vitro* experiments.

#### Data analysis

Results are given as the mean  $\pm$  SEM. The CBF is given as the percentage of that in the control group *in vitro*. Student's *t*-test or one-way ANOVA (PRISM software version 5.0; GraphPad Software, San Diego, CA, USA), followed by the Newman–Keuls' post comparison test, were used to determine the significance of difference between different groups. Statistical significance was set at two-tailed  $P < 0.05$ .

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#### DISCLOSURE

The authors declare that there are no conflicts of interest.

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