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Virulence shifts of Candida albicans biofilms induced by ethinylestradiol/drospirenone contraceptive

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ABSTRACT

It is estimated that approximately 167 million women worldwide use combined oral contraceptive (COC). In United States, products containing ethinylestradiol plus drospirenone (EE+DRSP) moved about US$ 616 million in 2008. There is evidence that contraceptives increase the virulence of *Candida albicans*. However, modulation of fungal virulence by drospirenone-containing COC remains unknown. The objective of this study was to evaluate whether virulence factors are increased when *C. albicans* is exposed to EE+DRSP. The ATCC®90028™, SC5314 and 15A2 strains were grown in the presence of EE+DRSP at 10 nM:10 μM, 50 nM:50 μM, and 100 nM:100 μM under continuous flow in a dedicated bioreactor for biofilm growth. 72-h biofilms were evaluated in relation to their proteolytic capacities, biomass production (MTT reduction assay), and induction of germ-tube formation and measurements of agar invasion. Only 50 nM:50 μM EE+DRSP caused reductions in biomass (p=0.023); the other did not promote any change (p>0.05). The contraceptive exposure was insufficient to cause any increase in germ tubes induction (p>0.05). There was a concentration-dependent increment in the hyphae invasiveness. On the other hand, the proteolytic activity increased in a concentration-dependent manner (p<0.01). When exposed to EE+DRSP, *Candida albicans* strains do not form greater amounts of biofilm, neither generate more germ tubes, nor become more invasive; however, they secrete greater quantities of proteases, which facilitate the adhesion and invasion of tissue.

Keywords: *Candida albicans*, biofilm, drospirenone, virulence.

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INTRODUCTION

It is estimated that approximately 167 million women aged 15-49 y.o. use combined oral contraceptives (COC) worldwide[1]. In the United States, it is the method chosen by at least 17.3% of women aged 15-44 y.o.[2] and the market of COC containing ethinylestradiol plus drospirenone (EE+DRSP) handled about US$ 616 million, in 2008[3]. In Great Britain, 16% of women aged 16-49 y.o. use COC[4]. In Japan, 1.3% of women in the same age group use such medicines[5]. In Brazil, 81% of women living under some form of union use COC for contraception purposes[6].

These drugs are used throughout fertile female life, which implies in quasi-continuous exposure to hormone analogs in concentrations higher than those considered physiological. Under the therapeutic point-of-view, maintaining such high concentrations ensures the temporary suspension of ovulation, but can also incur in side effects. One of the least prospected is the modulation of microbial virulence. Various microorganisms as *Trichomonas vaginalis*, Pseudomonas aeruginosa and Candida albicans have rudimentary receptors, called estrogen-binding proteins[7-10]. Regarding the latter organism, it is known that yeast to hyphae morphogenic transition is influenced by estrogen with increasing rate of germ tubes formation[11] as well as in the length of those[9].

Some steroids as alpha-estradiol, beta-estradiol, estriol, ethinylestradiol, cholesterol, and testosterone have been evaluated in relation to their ability to induce germ tube production[9,11]. Other steroids used as contraceptive agents need to be evaluated, amongst them, the drospirenone (DRSP).

The possibility of modulating fungal virulence by DRSP remains unknown. Therefore, the objective of this study was to evaluate whether virulence factors such as biofilm formation, secretion of proteases, induction of germ-tubes, and agar invasion are incremented when the fungus is exposed to EE+DRSP.

MATERIAL AND METHODS

Candida albicans strains

The reference strains ATCC®90028™ and SC5314 and the clinical strain 15A2 were used. Those strains were selected for this study once their virulent potential is well known and characterized[12,13].

Steroids

A stock solution of 1 μM ethinylestradiol (EE; CAS 57-63-6) and 1 mM drospirenone (DRSP; CAS 67392-87-4) was prepared in methanol. Working dilutions containing EE+DRSP were prepared at concentrations of 10 nM:10 μM (sub-pharmacological concentration), 50 nM:50 μM (pharmacological concentration) and 100 nM:100 μM (supra pharmacological concentration). These dilutions were prepared in YNB w/o amino acids (BD Co., Franklin Lakes, NJ). As control, YNB culture medium without steroid was used.

Cell adaptation

The three strains were grown in YNB (control) and YNB supplemented with EE+DRSP in different concentrations

(experimental groups). A loopful of each strain was transferred to 50 mL of media and incubated at 37°C, for 72 h, at 200 rpm and normoxia (80% N_2+20% O_2). Cells were harvested by centrifugation (5,000 x *g*) and washed three times with sterile 145 mM NaCl. Therefore, they were resuspended in sterile 145 mM NaCl until ca. 3×107 cells mL^{-1} (OD_{600} = 0.5) and used immediately.

Formation of biofilms under dynamic flow

A qualitative filter paper disc of 80 mm was sterilized in autoclave and oven dried. On it were dispensed 30 sterile 0.5mm antibiogram discs (Cefar Diagnostics Ltd, São Paulo, Brazil), to cover its periphery, but without any contact. In such antibiogram discs, 10 μL aliquots of suspensions grown in the presence of EE+DRSP or control were dispensed.

This set was inserted into a Paper Embed Biofilm Reactor (Figure 1) receiving continuous flow of YNB (control group) or YNB+EE+DRSP (experimental groups) in the flow ca. 100 μL min^{-1}. The disks were incubated at 37°C for 72 h[14].

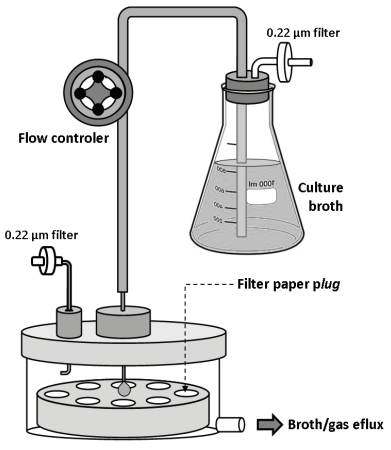


Figure 1. Paper Embed Biofilm Reactor (PEBR).

Secretion of proteases

After incubation, the systems were removed and 15 of each bioreactor discs were removed and inserted vertically into 1.5 mL microtubes. The wells were filled with 800 μL of a solution containing 0.2 mg mL^{-1} BSA, 10 nM sodium citrate and 10 nM citric acid buffer (pH 5.0) and incubated at 37°C, for 4 h and 100 rpm. After the digestion time, aliquots of 100 μL of supernatants were combined with 100 μL Coomassie solution (0.025% Coomassie brilliant G-250, 11.75% ethanol, 21.25% phosphoric acid) in 96 wells flat bottom microtiter plates. After 5 min, the absorbances were measures at 540 nm. As controls, there were used Coomassie plus saline (blank) and BSA-citrate plus Coomassie (basal concentration).

One enzyme unit was arbitrarily determined as the amount of enzyme able to promote digestion of 1 μg BSA.mL^{-1} min^{-1}. Proteolytic activity units by absorbances of MTT reduction (below described) were taken as specific proteolytic activities[15].

Estimative of biomass

After the incubation, with the disassembled systems, another 15 of each bioreactor discs were removed and placed vertically in 96 wells U bottom microplates. The wells were filled with 100 μL of 1 mg min^{-1} MTT. Control wells, containing sterile discs without biofilm, also received MTT. Plates were incubated in the dark at 37°C, for 4 h and 100 rpm. After the incubation, unreactive MTT was aspirated and 200 μL of isopropyl alcohol were added. After 5 min, 100 μL of the supernatant were transferred to 96 wells flat bottom microtiter plates and the absorbances were mea-

sured at 540 nm.

Induction germ tube formation

Fifteen-milliliter aliquots of fresh adapted cell suspensions were washed three times with sterile 145 mM NaCl. Cell numbers were determined using an improved Neubauer hemacytometer (Inlab Conf., São Paulo, Brazil). Cells were set at a density of 5×10^6 cells mL^{-1} in 15 mL of preheated (37°C) 1% BSA-YNB with or without EE+DRSP. The cultures were incubated for 5 h, at 37 °C and 200 rpm. Cells were fixed in 1% (vol vol^{-1}) glutaraldehyde and stained with lactophenol blue.

The percentage of germ tube formation was determined microscopically by evaluation of 100 cells per culture. Germ tubes with lengths equal to or greater than the parental cells were measured microscopically using a micrometer coupled to a Nikon LabOPhot-2 microscope eyepiece (Nikon Co., Tokyo, Japan).

Two observers conducted the scores. Intra-observer agreement (Obs_1, *kappa*=0.85; Obs_2, *kappa*=0.88) and inter-observer (*kappa*=0.87) values show good agreement. One-hundred cells were randomly measured for each treatment and three replicates were used for each culture condition. The arithmetic average of germ tube lengths was calculated and compared amongst the different treatments.

Agar invasion

Aliquots of 5 μL of appropriate suspensions of *C. albicans* strains were dispensed at 10 different points of a culture medium containing 2% glucose, 1.5% agar, 1% BSA and EE+DRSP (10 nM:10 μM, 50 nM:100 μM and 50 uM:100 μM), in triplicate. The volume of culture medium per plate was *ca.* 25 mL. Plates were sealed with plastic wrap to prevent drying and were incubated statically at 37°C in a normoxic atmosphere for 14 days. As control, we used the same medium without addition of EE+DRSP.

After the incubation/invasion time, the colonies were gently removed with tissue paper. Once clean, the invasion halos were measured with digital calipers using N-S and E-W cardinal orientation. The average values of six measurements were used for further calculations.

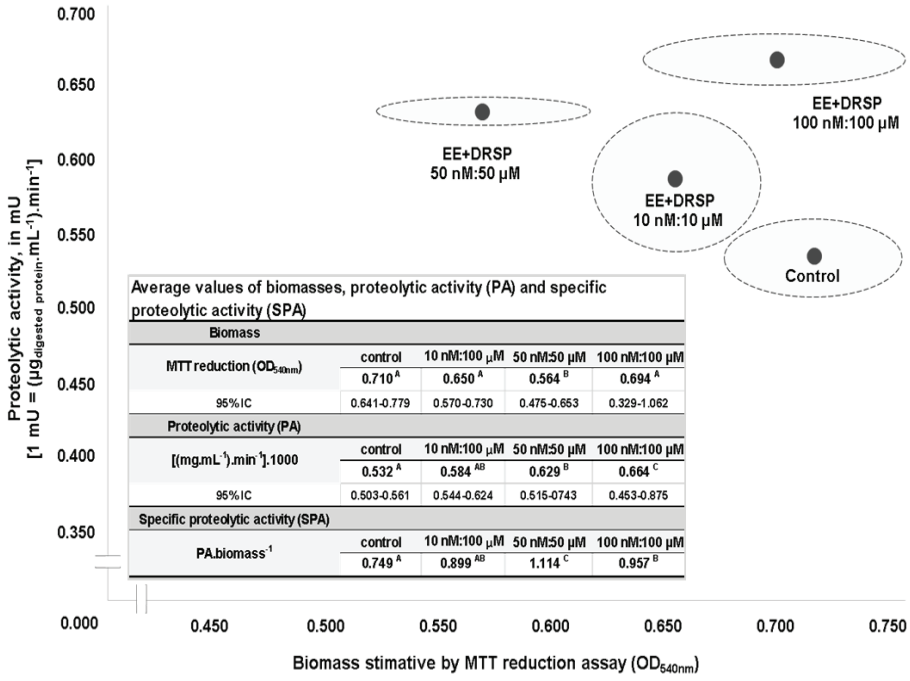
Statistics

Data from biomass measurement, proteolytic activity, germ tube formation and invasiveness were tested for normal distribution using the Kolmogorov-Smirnov test. As distributions were considered normal, the different treatments were compared by Tukey test. All tests were conducted with a 5% significance level.

It was used the statistical package SPSS 24.0 (IBM Co., Westchester, NY).

RESULTS

Figure 2 shows the dispersion of the grouped average biomasses of the three strains versus the sum of the grouped average proteolytic activity for each treatment and control.



Average values of biomasses, proteolytic activity (PA) and specific proteolytic activity (SPA)

Biomass				
MTT reduction (OD_{540nm})	control	10 nM:100 µM	50 nM:50 µM	100 nM:100 µM
	0.710 A	0.650 A	0.564 B	0.694 A
95%IC	0.641-0.779	0.570-0.730	0.475-0.653	0.329-1.062
Proteolytic activity (PA)				
$[(mg.mL^{-1}).min^{-1}].1000$	control	10 nM:100 µM	50 nM:50 µM	100 nM:100 µM
	0.532 A	0.584 AB	0.629 B	0.664 C
95%IC	0.503-0.561	0.544-0.624	0.515-0743	0.453-0.875
Specific proteolytic activity (SPA)				
$PA.biomass^{-1}$	control	10 nM:100 µM	50 nM:50 µM	100 nM:100 µM
	0.749 A	0.899 AB	1.114 C	0.957 B

Figure 2. Spatial distribution of biomasses and proteolytic activities of *C. albicans* biofilms grown in presence of EE+DRSP. In the embedded table, different upper-written letters denote statistical discrepancies ($p \leq 0.05$) amongst contraceptive concentrations.

In terms of biomass, it was observed that only at pharmacological concentration, EE+DRSP reduced biofilm formation compared to the control without any steroid ($p=0.023$). Moreover, the increased concentration led significant increases in virulence, in terms of proteolysis ($p<0.01$). The proteolytic activity was increased by EE+DRSP in a concentration-dependent manner.

The percentages of cells that showed germ tube without exposure to EE+DRSP were 23% (ATCC®90028™), 25% (SC5314), and 39% (15A2). With exposure to EE+DRSP, the values were 26% (ATCC®90028™), 24% (SC5314), and 37% (15A2), with no statistically significant differences between steroid presence and absence.

Regarding germ tube length, there were no changes resulting from exposure to xenobiotics, for any of the strains ($p \geq 0.670$). The germ tubes of controls had average sizes of 12.1±2.4 μm (ATCC®90028™), 13.4±2.8 μm (SC5314), and 11.0±1.9 μm (15A2). Exposure to EE+DRSP changed the length to 11.6±3.0 μm (ATCC®90028™), 14.5±2.8 μm (SC5314), and 12.9±3.2 μm (15A2).

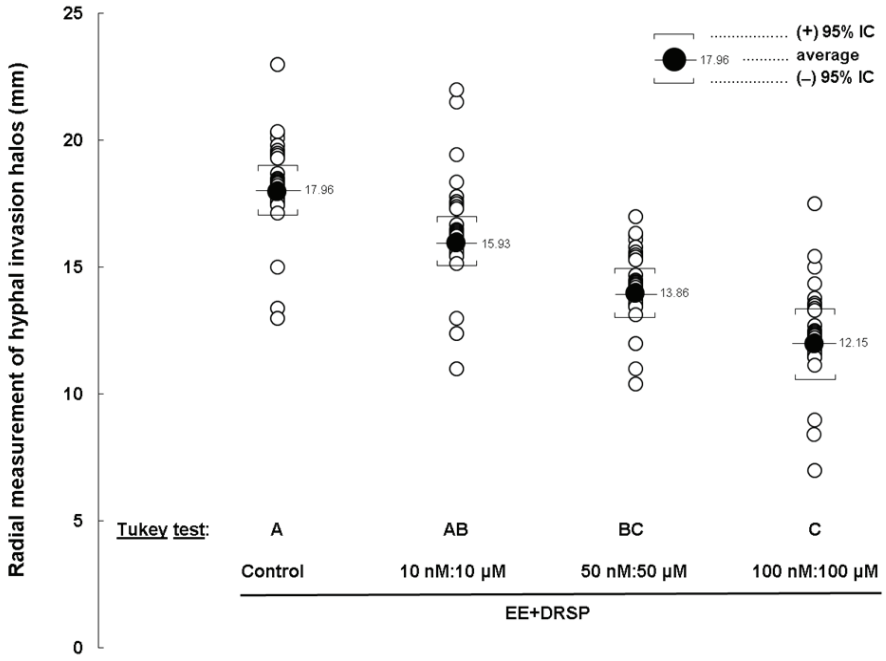


Figure 3. Multiple comparisons of hyphal invasiveness in presence of EE+DRSP.

The radial values of invasion halos of *C. albicans* hyphae ranged from 7.56 mm to 24.06 mm, for different EE+DRSP concentrations. The combination containing 10nM:10μM EE+DRSP did not differ from controls (p=0.406) (Figure 3). The experimental group containing five times more steroid (50 nM:50 μM EE+DRSP) showed lower potential invasiveness compared with the control (p=0.012). The tenfold increase in the concentration of steroids (100 nM:100 μM EE+DRSP) promoted a marked reduction in invasiveness (p<0.0001), compared to control. The concentrations 50 nM:50 μM and 10 nM:10 μM showed no significant differences between themselves (p=0.308).

DISCUSSION

Although there are reports of virulence increments triggered by contraceptives[16], to our knowledge, this study is the first in relation to biofilm formation and secretion of fungal proteases.

This pioneering study revealed that when exposed to EE+DRSP, C. albicans cells do not increase the biomass of resulting biofilms and, when there was a change, involved a reduction of such biomass. In addition, exposure to contraceptives was not sufficient to cause any increase in the induction of germ tubes. This, in principle, was considered unexpected since other groups published earlier that female estrogenic hormones may induce increases in the number of cells with these structures[9,11]. However, DRSP is a modern drug[17], with which it seeks a better contraceptive coverage with minimum side effects[18]. Its structure and activity make it different from other contraceptives used previously[19]. Unlike other contraceptives, DRSP has a significant anti-androgenic action[20]. Preliminary data from our laboratory show that

androgenic agents greatly increase the formation of hyphae arising from germ tubes (unpublished data). Here, it has been shown that the EE+DRSP association exerts no enhancer effect on virulence related to mycelial.

Moreover, there was a higher proteolytic activity when the cells were challenged with contraceptives and this ratio was dose-dependent. When *C. albicans* strains are exposed to suprapharmacological concentrations, it may occur the expression of genes encoding the transcription factors EFG1, CPH1, and TUP1 which upregulate yeast dimorphic transition to filamentous growth[21]. The same factors co-induce transcription of various proteases[22]. Further studies should be conducted to evaluate these transcription factors when exposed to EE+DRSP.

As important as the increase in the global proteolytic activity is the specific protease activity (SPA), which shows the extent of the action of xenobiotics in the cells. All challenges with EE+DRSP positively modulate the secretion of proteases. Of note, the concentration 50 nM:50 μM incurred in higher values for SPA. This higher proportional secretion certainly confers a worse prognosis in terms of tissue destruction.

The obtained results showed that with the increase in the concentrations of steroids of five and ten times order, there is a tendency to reduce the hyphal invasiveness. This reduction in invasiveness was totally unexpected because previously published studies predicted for the possibility of increasing the production rate of hyphae in the presence of steroid molecules[9,11]. However, when analyzed carefully, some differences in experimental driving can be decisive for such discrepancies. First, the cited studies[9,11] evaluated the formation of germ tubes (first stage of hyphae formation) in a liquid environment while here it was done on semi-solid substrate, which refers to the reality regarding the tissue invasion. Second, they employed both 17-β-estradiol, a molecule with chemical, pharmacokinetic, and pharmacodynamic characteristics diverse from the ones tested here[19,23].

The clinical impact of a concentration-dependent reduction in hyphae formation is interesting. We obtained a significant reduction for 50 nM:50 μM EE+DRSP in relation to control. Such concentration is close to the plasma concentration found in women under contraceptive therapy[19,24]. The association of steroids in concentrations close to those of tissue distribution may interfere with fungal metabolism, providing a retarding in the rate of mycelial growth.

Concluding, when exposed combined oral contraceptives containing EE+DRSP *C. albicans* does not form greater amounts of biofilm, either generates more germ tubes or becomes invasive. However, it secretes higher amounts of proteases, which may facilitate fungal adhesion and tissue invasion.

Disclosure of Conflict of Interest

No conflict of interest.

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BIBLIOGRAPHIC REFERENCES

1. United Nations. Department of economic and social affairs. Population division 2018. World Contraceptive Use 2018. New York: United Nations. [Consulted in 2022 September 5]. Available at:https://www.un.org/en/development/desa/population/publications/dataset/contraception/wcu2018.asp

2. Mosher WD, Jones J. Use of contraception in the United States: 1982-2008. Vital Health Stat 23. 2010;29:1-44.

3. Singer N. A birth control pill that promised too much. The New York Times. [Consulted in 2022 September 5]. Available at: https://www.nytimes.com/2009/02/11/business/worldbusiness/11iht-11pill.20100508.html

4. Lader D. Contraception and sexual health, 2008/09. Office for National Statistics, London; 2009 (Opinions survey report N° 41).

5. Hayashi A (2009) Japanese women shun the pill. 2009. [Consulted in 2022 September 5]. CBS News. Available at: http://www.cbsnews.com/stories/2004/08/20/health/main637523.shtml

6. Brazil Ministry of Health. National survey of demography and health of children and women (PNDS). Sexual activity and contraception. 2006. [Consulted in 2022 September 5]. Available at http://bvsms.saude.gov.br/bvs/pnds/atividade_sexual.php

7. Ford LC, Hammill HA, DeLange RJ, Bruckner DA, Suzuki-Chavez F, Mickus KL, et al. Determination of estrogen and androgen receptors in Trichomonas vaginalis and the effects of antihormones. Am J Obstet Gynecol. 1987;156(5):1119-21.

8. Rowland SS, Falkler WA Jr, Bashirelahi N. Identification of an estrogen-binding protein in Pseudomonas aeruginosa. J Steroid Biochem Mol Biol. 1992;42:721-727.

9. Cheng G, Yeater KM, Hoyer LL. Cellular and molecular biology of Candida albicans estrogen response. Eukaryot Cell. 2006;5:180-191.

10. Chelikani V, Rawson FJ, Downard AJ, Gooneratne R, Kunze G, Pasco N, et al. Electrochemical detection of oestrogen binding protein interaction with oestrogen in *Candida albicans* cell lysate. Biosens Bioelectron. 2011;15;26(9):3737-41.

11. White S, Larsen B. *Candida albicans* morphogenesis is influenced by estrogen. Cell Mol Life Sci.1997;53:744-749.

12. Thien ZM, Samaranayake YH, Samaranayake LP. In vitro biofilm formation of *Candida albicans* and non-albicans Candida species under dynamic and anaerobic conditions. Arch Oral Biol. 2007;52:761-767.

13. Rosa EAR, Rached RN, Ignácio SA, Rosa RT, José da Silva W, Yau JYY, et al. Phenotypic evaluation of the effect of anaerobiosis on some virulence attributes of *Candida albicans*. J Med Microbiol. 2008;57(10):1277-1281.

14. Selow ML, Rymovicz AU, Ribas CR, Saad RS, Rosa RT, Rosa EA. Growing *Candida albicans* biofilms on paper support and dynamic conditions. Mycopathologia. 2015;180(1-2):27-33.

15. Carvalho AP, Gursky LC, Rosa RT, Rymovicz AU, Campelo PM, Grégio AM, et al. Non-steroidal anti-inflammatory drugs may modulate the protease activity of *Candida albicans*. Microb Pathog. 2010;49(6):315-22.

16. Güzel AB, Küçükgöz-Güleç U, Aydin M, Gümral R, Kalkanci A, Ilkit M. Can-

dida vaginitis during contraceptive use: the influence of methods, antifungal susceptibility and virulence patterns. J Obstet Gynaecol. 2013;33(8):850-6.

17. Fuhrmann U, Krattenmacher R, Slater EP, Fritzemeier KH. The novel progestin drospirenone and its natural counterpart progesterone: biochemical profile and antiandrogenic potential. Contraception. 1996;54:243-251.

18. Schindler AE. The "newer" progestogens and postmenopausal hormone therapy (HRT). J Steroid Biochem Mol Biol. 2014;42:48-51.

19. Krattenmacher R. Drospirenone: pharmacology and pharmacokinetics of a unique progestogen. Contraception. 2000;62:29-38.

20. Schindler AE. Antiandrogenic progestins for treatment of signs of androgenisation and hormonal contraception. Eur J Obstet Gynecol Reprod Biol. 2004;112:36-41.

21. Banerjee D, Martin N, Nandi S, Shukla S, Dominguez A, Mukhopadhyay G, et al. A genome-wide steroid response study of the major human fungal pathogen *Candida albicans*. Mycopathologia. 2007;164(1):1-17.

22. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microb Mol Biol Rev. 2003;67:400-428.

23. Stanczyk FZ, Hapgood JP, Winer S, Mishell DR Jr. Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. Endocr Rev. 2013;34:171-208.

24. Brenner PF, Goebelsmann U, Stanczyk FZ, Mishell DR Jr. Serum levels of ethinylestradiol following its ingestion alone or in oral contraceptive formulations. Contraception. 1980;22:85-95.