High Concentrations of Progesterone and Mifepristone Mutually Reinforce Cell Cycle Retardation and Induction of Apoptosis

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Abstract. *High concentrations of progesterone (PG) and mifepristone (MF) reinforce the effect of each other, reducing the endometrial cancer (Ishikawa) cell densities in vitro. Whether this effect is caused by cell cycle retardation, induction of apoptosis, or a combination of both was questioned. During a 5 days period in the absence of PG, the G_1/G_0 fraction increased from 60% to 82%. After the first day of exposure with 32 and 95 μM PG the respective G_1/G_0 fractions increased to 79% and 82%. A similar pattern was evident after exposure to MF. After the first day, MF caused a clear increase in the G_1/G_0 fraction from 53% (control) to 72% (70 μM MF). In a third series of experiments, PG and MF were combined. After the first day, the G_1/G_0 fractions were 50% in absence of active agents, 67% in presence of 32 μM PG, 66% in presence of 23 μM MF and 76% when PG (32 μM) and MF (23μM) were combined. Both PG and MF induced apoptosis, which showed a time- and concentration-dependent pattern. In the control series the % apoptotic Ishikawa cells increased 2 to 3-fold during the experimental period and the effect of PG on apoptosis developed to a maximum after 4-5 days. On the fifth day, the trend was identical in three different assays commonly used to quantify different stages of apoptosis. The fractions of apoptotic cells, in the presence of 70 μM PG, increased from 2.5% to 28.2% (JC-1), from 1.4% to 13.2% (Annexin-FITC) and from 2.8% to 27.7% (DNA fragmentation assay), respectively. After five days, in a separate experiment, the fraction of apoptotic cells, as determined by JC-1 assay was 3.2% in the absence of active agents, 5.0% in the presence of 32 μM PG, 7.3% in the presence of 23 μM MF, and 12.4% in the presence of both PG (32 μM) and MF (23μM). The present study shows that supraphysiological PG concentrations and high pharmacological concentrations of MF cause cell cycle retardation and induce apoptosis. In combination, PG and MF mutually reinforce these effects.*

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A cell population represents a finely adjusted balance between cell proliferation (1) and cell death (2, 3). In fertile women, the endometrium undergoes hormone-regulated cyclic changes wherein proliferation and cell death is a part of the physiological process. However, disturbances in the normal pattern exist in both pre- and postmenopausal women. An example is endometrial hyperplasia which is a precursor lesion of endometrial carcinoma. In the treatment of proliferative disorders such as endometrial hyperplasias, progestins have been employed for many years (4).

The antiproliferative effects of progestins have been reviewed and were mainly attributed to progesterone receptor (PR) initiated signaling (5). However, a more complex picture has emerged during the last decade, with evidences of crosstalk between classical and new molecular targets (6-8). In a recent study, we found that progesterone (PG) at a concentration far above that needed for saturation of PR, caused a dramatic reduction of endometrial cancer (Ishikawa) cell density (9). Even if Ishikawa cells possess PR (10), mifepristone (MF), a potent blocker of PR-mediated effects (11), was unable to reverse the antiproliferative effect (9) and the evidence of extranuclear initiated signaling is convincing (12).

Progestins have been shown to retard the cell cycle and induce apoptosis. Two decades ago Sutherland and co-workers showed that progestin-induced growth inhibition of human breast cancer cell lines was associated with a significant decrease in the proportion of S-phase cells, with an accumulation of cells in the G_1/G_0 phase of the cell cycle (13). Cell culture studies have demonstrated that supraphysiological PG concentrations induced apoptosis in the breast cancer cell line T47-D (14) and in the endometrial cancer cell (Ishikawa) line (15).

In the present study we questioned whether the observed cellular pharmacological effects of PG, MF and their combination (9) were reflected in reduced input to (cell cycle retardation) and/or increased exit from (induction of apoptosis) the Ishikawa cell population.

Materials and Methods

Chemicals. PG and MF were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Cell culture. Ishikawa cells, originally derived from a well-differentiated endometrial adenocarcinoma (16), were obtained from the European Collection of Cell Cultures (Salisbury, UK). Monolayer cultures were established in RPMI 1640 medium (Sigma-Aldrich Chemical Company) with either 10% (v/v) dialyzed fetal calf serum (dFCS) or 10% (v/v) stripped FCS (sFCS) obtained from PAA-laboratories (Pasching, Austria) without phenol red to avoid potential steroid-mimicking effects (17) and without antibiotics. Cells were seeded at a density of approximately 1×10^5 cells/ml in 25 cm^2 culture flasks in a humidified atmosphere (5% CO_2) at 37˚C with daily changes of serum supplemented medium. PG and MF were added to the cultures after 5 days with cell expansion and medium with and without PG and/or MF was renewed daily for 5 days. Medium and nonadherent cells were aspirated and adherent cells were detached by trypsinization. PG and MF were dissolved in ethanol, but this did not affect cell growth (9). The Ishikawa cell densities were determined in a cytometer and viability was assayed by trypan blue dye exclusion.

Flow cytometry. Cell cycle analysis and quantification of apoptosis were performed by flow cytometry employing a FACScan flow cytometer equipped with an argon-ion laser (488 nm) (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Cell cycle analysis. After staining with propidium iodide (Cycle Test Plus DNA kit, Becton Dickinson Immunocytometry Systems), the DNA content of 2×10^4 cells was measured by flow cytometry and later processed by Multicycle-DNA cell cycle analysis software (Phoenix Flow Systems, San Diego, CA, USA). A singlet gate obtained from a FL2-width *versus* FL2-area dot plot was used to exclude doublets and aggregates in the samples.

Apoptosis detection by Annexin V-FITC binding. Apoptosis was detected by Annexin V-FITC binding with flow cytometry according to the manufacturer's recommendations (Oncogene Research Products, San Diego, CA, USA). Propidium iodide is excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Late apoptotic cells stain with both FITC and propidium iodide due to the final necrotic-like disintegration of the cells. Each sample contained 1×10^4 cells. The FITC signal was detected by FL1 (FITC detector, green fluorescence) at 518 nm and propidium iodide by FL2 (phycoerythrin fluorescence detector, red fluorescence) at 620 nm.

Apoptosis detection by DNA fragmentation. DNA fragmentation as a part of the apoptotic process was assayed according to the manufacturer's recommendations (DNA fragmentation detection kit, FragEL, Oncogene Research Products). For each sample 1×10^4 cells were analyzed by flow cytometry.

Apoptosis detection by mitochondrial membrane potential ($\Delta\psi$). The mitochondrial transmembrane potential ($\Delta\psi$) was determined using a potential-sensitive dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). Cells were stained with 2.5 μg/ml JC-1 (Molecular Probes, Eugene, OR, USA) and vortexed simultaneously to give a uniform red-violet color. After incubation in the dark at room temperature for 20 minutes, cells (1×10^4) were sedimented (500 × *g* for 5 min), washed twice with PBS and resuspended in 1ml phosphate buffered saline and kept on ice. The JC-1 monomer and J-aggregate were detected in FL1 and FL2 channels separately by flow cytometry.

Descriptive statistics. The results are presented as mean±SEM (if not stated otherwise).

Results

PG and cell cycle kinetics. The reduction in Ishikawa cell density obtained in the presence of supraphysiological concentrations of PG (9) may arise from cell cycle retardation. The present data show that the effect of PG develops time-dependently and in a concentration-dependent manner. The highest PG concentration caused an early and extensive cell cycle arrest, with accumulation of cells in the G_1/G_0 phase; in the control series such accumulation was seen after five days (Table I). Compared to the control, a small but distinct reduction of cell fraction in G_1/G_0 was seen with 0.32 μM PG after the first day. The fraction of Ishikawa cells in the S-phase gradually decreased from the first to the fifth day, both in the absence and presence of the lower PG concentrations (Table I). In contrast to this the highest PG concentrations caused a marked effect much earlier (Table I). For the G_2/M-phase a pattern similar to that in the S-phase was observed.

MF and cell cycle kinetics. High concentrations of MF caused a concentration-dependent cell density reduction with somewhat higher potency than PG (9). The possibility of an effect of cell cycle kinetics was considered. MF at 70 μM caused an early and extensive cell cycle retardation with accumulation of Ishikawa cells in G_1/G_0 phase; in the control series the accumulation in G_1/G_0 reached the same level after five days (Table II). However, for the last two days this MF concentration (70 μM) caused an extensive cytotoxic effect, so that the cell number became too low for a cell cycle assay. The lowest MF concentration showed similar values and pattern as observed in the control series. The fraction of Ishikawa control cells in the S-phase gradually decreased to approximately 30% from the first to the fifth day, whereas the G_2/M fraction was halved (Table II).

Cell cycle and the combination of PG and MF. A mutual reinforcement between PG and MF was observed in the Ishikawa cell density studies (9). In separate experiments, the cell cycle kinetics were characterized for the single

Table I. *The effect of PG on Ishikawa cell cycle kinetics is presented. After expanding the Ishikawa cells in medium with 10% (v/v) dFCS, the cells were exposed to 0.32-95 μM PG. The culture medium including PG was renewed every day. The results are presented as percentage distribution of cells in G_1/G_0, S and G_2/M after 1, 3 and 5 days' exposure to PG (n=4).*

	Cell cycle phase (% of cells)		
	G_1/G_0	S	G_2/M
Control			
Day 1	60±0.6	29±2.5	11±1.2
Day 3	78±0.7	15±0.3	7±0.2
Day 5	82±0.3	11±0.2	7±0.7
PG (0.32 μM)			
Day 1	56±0.2	33±0.2	11±2.0
Day 3	78±0.5	16±0.5	6±0.1
Day 5	81±0.3	13±0.2	6±0.2
PG (3.2 μM)			
Day 1	56±0.2	33±1.9	11±1.9
Day 3	77±0.3	16±0.2	7±0.2
Day 5	81±0.2	13±0.4	6±0.2
PG (32 μM)			
Day 1	73±0.7	19±0.4	8±0.8
Day 3	77±0.5	15±0.4	8±0.2
Day 5	81±0.6	11±0.6	8±0.5
PG (95 μM)			
Day 1	79±0.4	12±0.4	9±0.6
Day 3	82±0.3	10±0.2	8±0.2
Day 5	82±1.5	9±1.5	9±0.1

Table II. *The effect of MF on Ishikawa cell cycle kinetics is presented. After expanding the Ishikawa cells in medium with 10% (v/v) dFCS, the cells were exposed to 0.23-70 μM MF. The culture medium including MF was renewed every day. The results are presented as percentage distribution of cells in G_1/G_0, S and G_2/M after 1, 3 and 5 days' exposure to MF (n=3).*

	Cell cycle phase (% of cells)		
	G_1/G_0	S	G_2/M
Control			
Day 1	53±0.1	33±0.2	14±0.2
Day 3	76±0.5	17±0.4	7±0.2
Day 5	79±0.5	14±0.4	7±0.1
MF (0.23 μM)			
Day 1	53±0.5	34±0.4	13±0.5
Day 3	76±0.2	17±0.3	7±0.2
Day 5	79±0.3	14±0.3	7±0.2
MF (2.3 μM)			
Day 1	53±0.4	35±0.4	11±1.9
Day 3	76±1.4	17±1.3	7±0.1
Day 5	81±0.2	12±0.1	7±0.2
MF (70 μM)			
Day 1	72±0.1	18±0.1	10±0.1
Day 3	82±0.5	11±0.7	7±0.1
Day 5	n.d.	n.d.	n.d

n.d. Not possible to determine due to complete cell disintegration.

Table III. *Induction of Ishikawa cell apoptosis by PG as determined by JC-1 assay. After expanding the Ishikawa cells in medium with 10% (v/v) dFCS, the cells were exposed to PG (0.32-95 μM). The culture medium including PG was renewed every day. The results are presented as % apoptotic cells after exposure to PG (n=4).*

	Apoptosis (% of cells)		
PG (μM)	Day 1	Day 3	Day 5
0	1.8±0.1	2.1±0.2	2.5±0.2
0.32	1.6±0.1	2.1±0.3	3.0±0.6
3.2	1.6±0.2	2.2±0.1	3.1±0.3
32	2.1±0.2	3.6±0.5	4.9±0.8
95	13.1±2.0	21.2±1.3	28.2±2.8

substances and compared with the combination of PG and MF. The expected MF antagonism of the PG effect was absent. Figure 1 shows that after the first day a reinforcement of the effect existed when the two compounds were combined. The effect developed from the first to the fifth day with an increase in G_1/G_0 fraction from 67% to 76% with 32 μM PG, 66% to 75% with 23 μM MF and 76% to 78% when 32 μM PG was combined with 23 μM MF as compared to 50% to 76% in the control. The S and G_2/M fractions were reduced correspondingly (data not shown).

PG and apoptosis. The possibility that PG induced programmed cell death (apoptosis) was investigated since PG reduced Ishikawa cell density in a concentration-dependent manner and the highest concentrations caused cytotoxicity. The JC-1 assay detects changes in mitochondrial membrane potential ($\Delta\psi$) which are considered as an early event in the apoptotic process. The apoptosis in the control was low, but showed a markedly increase over the experimental period (Table III). The two lowest PG concentrations resulted in similar values as observed for the control, whereas the two highest concentrations caused a marked increase in apoptosis (Table III).

The second assay (Annexin V-FITC) verified the observations described above. In the Annexin V-FITC assay, late apoptotic cells stain with both FITC and propidium iodide and showed a time-dependent increase in apoptotic Ishikawa cells (Table IV). The two lowest PG concentrations gave a small but marked induction of apoptosis. A steep response was seen in the interval between 0.32 and 95 μM.

The third assay (DNA fragmentation) confirmed the results described above. The DNA fragmentation assay detects nucleosomal fragments from DNA degradation, which is considered a late event of the apoptotic process. Table V shows a time-dependent increase in the DNA fragmentation for control cells as well as for the PG-exposed cells. The lowest PG concentrations appeared to give slightly

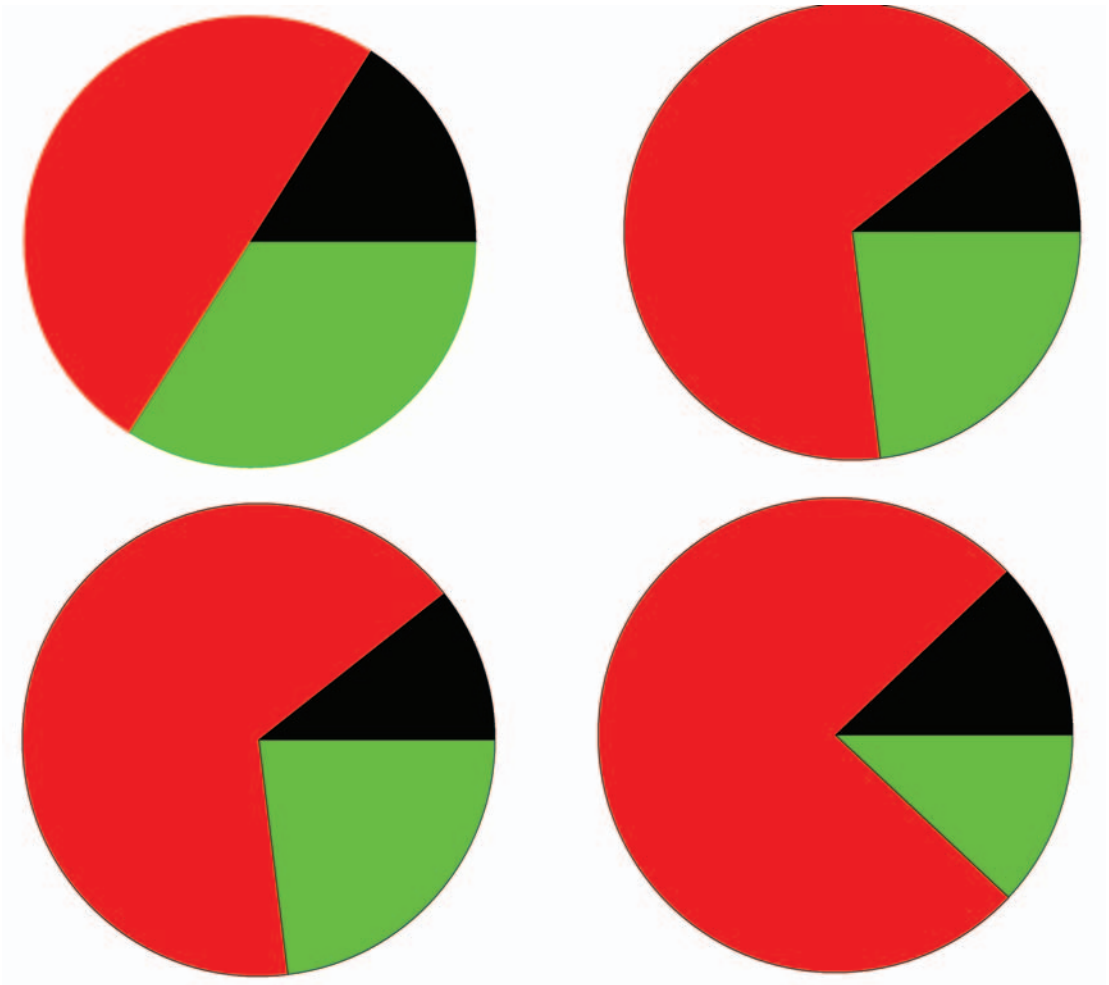

Figure 1. *Early effects (day 1) of 32 μM PG, 23 μM MF and their combination on the cell cycle distribution are shown. Cell cycle kinetics was analyzed by flow cytometry as described in the Materials and Methods. The distribution (G_1/G_0 = red, S = green and G_2/M = black) of Ishikawa cells in the absence of PG/MF (upper left panel), in the presence of 32 μM PG (lower left panel), in the presence of 23 μM MF (upper right panel) and with the combination of 32 μM PG and of 23 μM MF (lower right panel) is presented as mean value (n=4).*

higher fragmentation compared to the control series, whereas the response increased drastically between 0.32 and 95 μM PG (Table V).

MF and apoptosis, and the combination of PG and MF. Separate experiments with the JC-1 assay were performed to test whether MF was able to antagonize the effect of PG. As observed earlier, the apoptotic fractions increased steadily during the experimental period (Table 6). The presence of MF (23 μM) caused an increase in apoptosis from 2.0% to 7.3% during the exposure period. None of the concentrations of MF antagonized the effect of PG. The percentage of apoptotic cells was similar to that induced by PG alone. However, Table VI shows clearly that 23 μM MF clearly enhanced the effect of 32 μM PG.

Discussion

We found that PG, in concentrations much higher than that needed to saturate PR, reduced Ishikawa cell density in a concentration-dependent manner (9). Furthermore, PG and MF, in such concentrations, acted in synergy to inhibit proliferation and/or stimulate cell death. The present work demonstrates that PG reduced the Ishikawa cell population concentration-dependently by two mechanisms: cell cycle retardation and induction of apoptosis.

Progestins cause an initial transient acceleration through the G_1-phase of the cell cycle that is followed by cell cycle arrest and growth inhibition (18). In the present study, the effect of PG showed a time-dependent pattern. The highest PG concentration caused maximal arrest in the G_1/G_0-phase

Table IV. *Induction of Ishikawa cell apoptosis by PG as determined by Annexin V-FITC. After expanding the Ishikawa cells in medium with 10% (v/v) sFCS, the cells were exposed to PG. The culture medium including PG was renewed every day. The results are presented as % apoptotic cells, mean value with range (n=2).*

	Apoptosis (% of cells)		
PG (μM)	Day 1	Day 3	Day 5
0	1.0 (1.0-1.0)	1.1 (1.0-1.1)	1.4 (1.4-1.5)
0.32	1.3 (1.3-1.3)	1.5 (1.3-1.6)	2.2 (2.1-2.3)
3.2	1.9 (1.9-1.9)	2.1 (1.9-2.3)	2.9 (2.7-3.0)
32	2.8 (2.3-3.2)	2.9 (2.9-3.0)	6.6 (6.6-6.6)
95	5.9 (5.9-5.9)	6.6 (5.8-7.3)	13.2 (12.8-13.5)

Table V. *Induction of apoptosis by PG as determined by DNA fragmentation assay. After expanding the Ishikawa cells in medium with 10% (v/v) dFCS, the cells were exposed to PG. The culture medium including PG was renewed every day. The results are presented as % apoptotic cells (n=4).*

	Apoptosis (% of cells)		
PG (μM)	Day 1	Day 3	Day 5
0	1.2±0.2	1.6±0.3	2.8±0.6
0.32	1.5±0.2	1.8±0.1	3.3±0.2
3.2	1.5±0.1	2.2±0.1	3.3±0.1
32	2.4±0.2	6.0±0.2	10.3±0.5
95	6.3±0.4	20.8±0.8	25.7±0.4

Table VI. *The effect on apoptosis by combining PG and MF as determined by JC-1 assay is shown. After expanding the Ishikawa cells in medium with 10% (v/v) dFCS, the cells were exposed to PG (32 μM) and MF in the given concentrations. The culture medium including PG and/or MF was renewed every day. The results are presented as % apoptotic cells (n=3).*

	Apoptosis (% of cells)		
PG (μM)/ MF (μM)	Day 1	Day 3	Day 5
0/0	1.4±0.1	2.8±0.1	3.2±0.1
32/0	2.1±0.1	3.3±0.2	5.0±0.1
32/0.23	2.0±0.1	3.5±0.1	4.9±0.3
32/2.3	2.1±0.1	3.8±0.3	5.4±0.2
32/23	3.1±0.2	6.8±0.1	12.4±0.2
0/23	2.0±0.1	6.5±0.6	7.3±0.2

after 24 h, whereas lower concentrations appeared to reach their maximal effect after 4-5 days. After the first day, the two lowest PG concentrations produced a small but distinct reduction of Ishikawa cells in the G_1/G_0-phase after 24 h exposure, an observation compatible with the report by Musgrove and co-workers in 1991 (18). The observation that the PR-negative cell line C4-I showed a concentration-dependent accumulation in the G_1/G_0-phase (19) supports the idea that extranuclear initiated signaling is involved in the effect of supraphysiological concentrations of PG.

In agreement with the present observations, PG has been reported to induce apoptosis in endometrial hyperplastic specimens and Ishikawa cells (15), various breast cancer cell lines (20), the human ovarian cancer cell line SNU-840 (21) and C4-I cells, a human uterine cancer cell line (19). In mammary cancer cell lines, PG in concentrations of 1.0 and 10 μM caused a concentration-dependent cell density reduction in the PG-positive cell line T47-D, but not in the PR-negative cell line MDA-231 (14). In human endometrium levonorgestrel in an intrauterine device (IUD) caused induction of apoptosis (22, 23). In Ishikawa cells treated with 10 μM PG, an increase in apoptotic activity started at 24 h and continued up to 72 h (15), in agreement with the present observations. A similar time-dependency was reported for an ovarian cell line, SNU-840 cells, where PG (10 and 100 μM) inhibited proliferation and induced apoptosis after 24 and 48 h (21). All these studies show that the effect of PG on apoptosis is concentration dependent and needs several days to develop maximally.

MF is a potent antiprogestin (24). We found that MF, in concentrations above those needed for saturation of PR, exhibited a PG-mimetic antiproliferative effect (9). Even if MF mainly has been classified as an antiprogestin, a number of additional effects have been described (25, 26). MF was somewhat more potent (IC_{50}: 15-20 μM) in reducing Ishikawa cell density compared with PG (IC_{50}: 50-60 μM), but together they showed a mutual reinforcement (9). Like PG, MF also caused cell cycle arrest in the G_1/G_0-phase with high concentrations. Previous studies of MF on breast cancer (T-47D and MCF-7) cells showed an arrest in the G_1/G_0-phase (27, 28). In contrast a previous study on Ishikawa cells reported an arrest in the S-phase, the fraction increasing from 24% to 58% in the absence and presence of 100 μM MF, respectively (29). This disagrees with the present finding of 33% and 18% of cells in the S-phase in the absence and presence of 70 μM MF, respectively. The reason for this discrepancy is unclear. In agreement with the present observations, a recent study reported that Ishikawa cells accumulated in the G_0/G_1 phase after exposure to MF (30).

In addition to cell cycle arrest, we found that MF induced apoptosis in a concentration-dependent manner, but needed 4-5 days to be fully developed. Our observation of MF-induced apoptosis is in agreement with more recent studies on endometrial cell lines such as Ishikawa cells (29, 30), EM42 cells (31) and HEC1A cells (30).

As far as we know, the additive effect of PG and MF has not previously been reported. This interaction caused an increased cell density reduction (9) and cell cycle arrest, as well as an induction of apoptosis. Recent studies of gene expression employing microarray technology, showed that supraphysiological concentrations of PG (32) and high

pharmacological concentrations of MF (33) changed the expression of a large number genes, among them genes which are involved the regulation of the cell cycle and apoptosis. Whether this extranuclear initiated signaling may be utilized in a therapeutic setting remains unanswered, but is, at least, a possibility for local administration such as in IUD.

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