Cell culture of biopsied endometriomas after danazol/hormonal therapy: A study of growth features and fertility effects

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The growth features of cells from endometriomas biopsied from patients who had been treated with Danazol or with hormones have not been studied in vitro. Danazol is a versatile drug and despite its recognised efficacy in controlling endometriosis, little is known about its cytotoxicity and mechanism of action. Culture of the biopsied endometriomas permitted qualitative cytotoxic assessments by way of comparison with cultured normal uterine endometrial cells treated in vitro with Danazol. The growth characteristics were studied in monolayer and collagen gel cultures. Cytopathology was characterised by light and electron microscopy. Since endometriosis is associated with infertility in women, data from in vitro fertilisation (IVF) were analysed with respect to treatment modalities as compared with cases suffering fallopian occlusion. Danazol reduced pregnancy chances. Two factors may be responsible: (a) altered follicle development resulting in poor oocyte quality (b) reduced nidation because of long-lasting endometrial cytotoxicity. The experimental findings reported here support the latter explanation. Consequently, Danazol therapy should be conditional; patients wishing to achieve pregnancy should preferably receive hormonal therapy.

Endometriosis is a disease affecting women of reproductive age and is associated with infertility. Clinics offering assisted reproduction are often confronted with the problem of treating the disease. In addition to surgical interventions, medical treatment is based on (a) combined oestrogen-progesterone regimens to provoke a pseudopregnancy effect (b) gestrinone (ethyl-norgestrinone) which is a steroid having androgenic, anti-progestogenic and anti-oestrogenic action (c) lynesterol (d) analogues of gonadotrophin releasing hormone particularly agonists (GnRH-a) to down-regulate pituitary function (e) danazol, an isoxazol derivative, is a versatile pharmacologic agent which induces inhibition of pituitary and gonadal function combined with androgenic effects, leading to suppressed steroidogenic activity. As in vitro fertilisation and embryo transfer (IVF-ET) has been regarded as an adequate method to overcome infertility among endometriotic patients, it has been integrated in the therapy. Although the efficacy of Danazol is well documented its mechanism of action in causing endometriotic dysplasia remains unclear. The pharmacology of Danazol is very complicated and its effects are not tissue specific. Hence, Danazol therapy is used in some completely unrelated disorders. The fact that endometriotic foci do not disappear despite successful control after Danazol application implies long-lasting inhibitory effects, which are not as yet understood. As there are no published reports on the culture of biopsies from Danazol treated endometriomas, the presented observations provide the first hints as to the possible mode of action. Biopsies from hormonally treated endometriomas were also cultured for the purpose of comparison. Further, endometrial tissue from normal endometria were cultured and treated with Danazol in vitro.

The non-specificity of Danazol together with its long-lasting effects on cell growth and function also raised the question as to whether Danazol might cause detrimental effects on follicle development and oocyte maturation, since the primordial follicles may not be spared of adverse effects during the course of a protracted therapeutic regimen. As an attempt to
address this issue too, a retrospective analysis of IVF-ET data was carried out with respect to endometriosis and mode of therapy, in comparison with patients who only suffered from fallopian occlusion.

**Material and Methods**

**Cell culture**

Tissues obtained from biopsied endometriomas of treated patients or from abraded normal endometria were minced, washed and placed into an enzyme mixture of approximately 20 x the tissue volume. The enzyme mixture contained about 0.25 U/ml each of collagenase and dispase in Ham's F10 medium. Enzymatic digestion under slow agitation was done overnight at room temperature. This procedure gave superior yields of both epithelial and stromal cells compared to digestion with 0.25% trypsin at 37°C.

The digested material was pipetted a few times to facilitate cell dispersion and then let to sediment a few minutes when aggregates consisting mainly of epithelial cells sedimented. The supernatant was enriched in stromal cells. Both fractions were freed of debris and red blood corpuscles (RBC) by placing each on a Percoll (Pharmacia, Sweden) double gradient consisting of 45 and 22.5% Percoll. Cell debris remained on the 22.5% layer while cells settled at the interphase of the two layers. RBCs settled at the tip of the tube.

After washing, the cell fractions were counted in cell chambers (Neubauer) and plated either as monolayers or in collagen gel. The culture medium was Ham's F10 containing 15% foetal calf serum (FCS). Collagen gel cultures were prepared by adding cells suspended in FCS to a mixture of 7 parts collagen solution (2 g/l in 0.1% acetic acid from Serva, Heidelberg, Germany), 2 parts NaHCO3 (11.76 g/l in H2O) and one part 10x concentrated Ham's F10. The mixture was kept in an ice-bath. Upon addition of the cells and dispensing into culture plates, the temperature was quickly raised to 37°C. Incubation at 37°C was in 5% CO2 in air at saturated humidity.

**Danazol exposure in vitro**

Pure Danazol was obtained as a gift from Winthrop Labs., Newcastle upon Tyne, U.K. Danazol was dissolved in absolute ethanol to make a stock solution of 5 mg/ml. Owing to its extremely low solubility in water, the stock solution was diluted to 0.5 mg/ml in 50% ethanol before addition to the cultures to give a final test concentration of 2.5 μg/ml. The ethanol concentration in the culture medium was 0.2%.

**Electron microscopy**

Cultures were processed for electron microscopy by fixation in 2.5% glutaraldehyde in cacodylate buffer. Post-fixation in 2% osmic acid was followed by dehydration in ethanol and embedment in araldite. Thin sections were contrasted with lead citrate and uranyl acetate and examined in a Zeiss electron microscope at 75 kV.

**Selection IVF-ET data**

A cohort of 32 endometriotic patients (24 to 40 years old) who opted for IVF were selected from records of the University Clinics for Obstetrics and Gynaecology. In all cases endometriosis had been confirmed endoscopically and staging was according to the Revised American Fertility Society classification, 1985. Cases were included only when the partners’ spermiograms were normal. An equal number of aged-matched controls were chosen from cases who opted for IVF because of fallopian occlusion.

The cohort of endometriotic patients comprised two groups receiving different treatments. The first group of 21 cases (11 stages I/II and 10 stages III/IV, as per classification) received 800 mg Danazol daily. The second group of 11 cases (5 stages I/II and 6 stages III/IV) were hormonally treated with either Lynestrenol (4 cases, 5 mg daily) or with a GnRH-a (7 cases, 0.5 mg Triptorelin per 28 days with 1.2 mg Buserelin nasal spray daily). A lapse of about 6 months post-treatment was observed before the patients could participate in the IVF programme.

Statistical significance was calculated by using the t test for comparison of means.

**Results**

**Primary cultures of endometrioma biopsies**

Endometrioma biopsies were taken during endoscopic examination of consenting patients who either had received treatment or who were being examined for staging of the disease prior to treatment. Plate 1 shows representative growth characteristics of primary cultures which were obtained from several biopsies. Fig 1 shows a typical 2-3 week culture of endometriotic tissue from a patient who had completed Danazol therapy. These cultures were
characterised by poor growth accompanied by gradual degeneration. The inset in Fig. 1 shows an individual colony. Such occasional colonies were observed after maintaining the cultures 2-3 weeks and since these colonies consisted of morphologically apparently normal cells, it is reasonable to assume that they grew from intact clonogens. In contrast, Fig. 2 shows a typical 24 hr culture of material from either untreated or hormonally treated patients. All cultures were always plated at the same defined density. Tissues from untreated and hormonally treated patients are characterised by cell proliferation or growth as demonstrated in Figs 3 and 4, respectively. Figs. 1 to 4 are observations from monolayer cultures.

Parallel to monolayers, cells were also grown in collagen gels. After enrichment of the stromal and epithelial fractions by sedimentation followed by removal of debris on Percoll gradients,stromal cells were plated entrapped in collagen gel while epithelial cells were plated on the gel surface as a monolayer. Fig. 5 shows the stromal elements from a non-treated patient after one week culture. The stromal cells growing in the three-dimensional gel culture and those growing in monolayer were phenotypically different. The growth of cells from hormonally treated patients did not differ from that of non-treated controls. To compare the morphology with cells from normal endometria,stromal and epithelial cells from uterine endometria obtained from aborntional curettages were also grown. Fig. 6 shows such a culture in gel. The arrow head points to the epithelial component grown on the gel. No differences could be noticed between the growth behaviour and phenotypes of the uterine and ectopic tissues obtained from non-treated and hormonally treated patients. The inset in Fig. 6, on the other hand, shows a photomicrograph of a gel culture with cells from a patient treated with danazol. After about 2 to 3 weeks of culture, clonal growth produced a few clumps showing devious phenotype as well as growth behaviour.

**In vitro exposure to danazol**

To study the effects of Danazol in vitro, only normal uterine endometria obtained from curettages were used. Plate 2 summarises the results in a series of photomicrographs. Fig. 7A indicates the general appearance of monolayer cultures 24 hr after the cells were plated. A corresponding culture plated at the same density in collagen gel is shown in Fig. 9A. The three-dimensional dispersion of the cells in the gel shows a seemingly lower density at any single focal plane. Danazol (2.5 μg/ml) was added at 24hr culture when the cells had attached. At the plated density confluent or dense cultures were obtained within 5 days. Such cultures are shown in Figs 8A and 10A for monolayers and gels, respectively. The corresponding Danazol treated cultures are seen in Figs 7B and 9B wherein growth retardation is obvious. As Danazol was added in alcoholic solution, the corresponding amounts of ethanol were added to both culture types. The presence of 0.28% ethanol caused no discernible effect, as seen in Figs. 8B and 10B for monolayers and gels, respectively.

As sub-culturing is a way of provoking cell proliferation and, therefore, growth, cells from both culture systems (monolayer and gel) were enzymatically detached and passaged. Fig 11A shows a 4 to 5 day culture of Danazol exposed cells. The non-treated control is shown in Fig. 11B. Upon repeated exposure of the secondary cultures to Danazol, the cells eventually manifested cytopathological features as demonstrated in Fig. 12A which shows a 2 week secondary culture. The control (which received a corresponding quantity of ethanol) is shown in Fig. 12B.

**Danazol cytopathology**

The salient cytopathological features after in vitro exposure to Danazol are documented in electron photomicrographs in Plate 3. Both gel and monolayer cultures were examined. The degenerative features were essentially the same under either culture.
condition. Fig 13 shows the cytoplasmic remnant of a
cell which was exposed to Danazol during 10 days
primary followed by 6 days secondary culture in
collagen gel. The abundance of lipid droplets seen in
the electron photomicrograph is comparable to what
was seen during phase contrast observation of the
cultures (Plate 2, Fig. 12A) as highly refractile cells.
The vacuolated or annular form of the lipid droplets
may indicate lipid inhomogeneity, reflecting
perturbed cellular lipid metabolism. The lipid content
in viable cells in gel culture is usually homogenous and
does not get lost during processing, as
exemplified in Fig. 15 (L). The correlate out of
monolayer culture is shown in Fig. 14 which shows
under low magnification a couple of degenerated and
intact cells. The arrow head points to a large
autophagic vacoule. Some of the crystalline particles
in autophagic vacoules could be minute Danazol
crystals which had precipitated. The crystaline of
these particles was confirmed by polarised light
microscopy.

Figs 15-18 show only cells from primary gel
cultures after 10 days exposure to danazol. A
prominent feature of many viable cells was the highly
irregular nucleus. A striking example of invagination
is given in Fig. 15. In view of the increased surface
area of the nuclei in relation to the cytoplasm, it is
possible that the nuclear to cytoplasmic ratio shifts in
favour of the nucleus as reported by Schweppe
et al. Large primary (Ly) and secondary (S)
lysosomes were often observed, as demonstrated in
Figs. 16 and 17 respectively. The earliest signs of
distension of the endoplasmic reticulum which can be
seen in Figs. 16 and 18, are followed later by the
disorganisation of the crista and finally condensation
of the mitochondria. Autophagic vacoules of different
stages and content was a constant prominent feature.

Analysis of IVF Data
The Table 1 presents a summary of the pertinent
parameters which were compared. The differences
between the oestradiol values and frequencies of
fertilisation for the Danazol and non-treated groups
(tubal disorder) were statistically significant at the

<table>
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<th>Parameters</th>
<th>Patient Category</th>
<th>Fallopian occlusion</th>
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<tbody>
<tr>
<td></td>
<td>Danazol</td>
<td>Hormone</td>
</tr>
<tr>
<td>E₂ at Ovulation (pg/ml)</td>
<td>1228 ± 642</td>
<td>1474 ± 747</td>
</tr>
<tr>
<td>Oocytes recovered</td>
<td>4.9 ± 2.5</td>
<td>5.9 ± 4.4</td>
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<tr>
<td>Fertilisation (39%) (45%)</td>
<td>105/188</td>
<td>56%</td>
</tr>
<tr>
<td>Embryos transferred (3.0 ± 1.6)</td>
<td>2.4 ± 1.7</td>
<td>4.8 ± 1.8</td>
</tr>
<tr>
<td>Pregnancies per Transfer (4/19 (21%), 4/18 (22%)</td>
<td>8/33 (24%)</td>
<td>8/21 (38%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.7 4.8</td>
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P = 0.01 and P = 0.05 confidence levels, respectively.
When the mean E₂ values in serum were calculated
per oocyte, it was found that both endometriotic
groups had practically identical mean values which
was significantly lower than that of the reference
group. The lower numbers of transferable embryos
which the two endometriotic groups yielded was
probably not only attributable to the reduced
frequency of fertilisations but also to intrinsically
poorer oocyte quality as reflected by the E₂ values per
oocyte. When the incidence of pregnancies was
calculated on the basis of individual patients, not on
the basis numbers of transfers as commonly done, it is
seen that patients who received Danazol therapy had
their chances of getting pregnant compromised by
about 50%.

Discussion
The enzymatic dispersion and culture subjects cells
to proliferative stress, which is a simple but yet
reliable means of gauging cell function. As the system of monolayer cell culture on plastic surfaces suffers inherent defects, cells were also grown either in or on collagen gels, according to histological type. The resulting combined culture is an in vitro construct of an endometrium. This system is an attempt to mimic a natural microenvironment by providing primary components of the extracellular matrix (ECM) viz. collagen fibrils and fibronectin. Thus, the stromal elements grew under three-dimensional conditions while the epithelial cells grew under two-dimensional condition on the gel surface. Under these conditions, the possibility for cell-cell interaction is also fostered. This system demonstrates that cells show considerable disparity in their reactions towards toxic stresses, as compared to common indiscriminate culture of cells as monolayers on plastic surfaces. Thus although the cytopathological effects of danazol treatment were essentially the same in monolayers and gel cultures, the temporal course of events differed.

Endometriotic cells which were exposed to a regimen of Danazol during therapy showed reproducibly poor growth performance in vitro, whereas non-treated and hormonally treated cells showed growth characteristics which could not be distinguished from those of normal uterine endometrial cells. Danazol exposure reduced attachment and inhibited proliferation. Occasional intact clonogens produced colonies after prolonged incubation. Such clonogens were invariably of stromal origin. Since epithelial cell colonies were not observed, this finding could reflect differential sensitivity to Danazol. Attached non-clonogenic cells typically developed an overload of lipid droplets and degenerated. As biopsies were performed several months after Danazol therapy had been completed, the observed effects in vitro may be regarded as long-term effects.

The direct action of Danazol was studied by using cultures of uterine endometrial cells. The inhibitory effects on the growth characteristics observed in primary and secondary cultures for monolayers and gels, respectively, were similar to what was observed for endometriotic cells from therapied patients, notwithstanding the fact that the concentration used in vitro may not be comparable to exposure in vivo. Rose et al., have also reported on the inhibitory effects of Danazol on the growth of endometrial cells in culture. Cells manifesting cytopathological effects generally degenerated by lysis. The sequence of events leading to degeneration was faster in monolayer cells. The cells in gel cultures were in this respect comparatively refractory. It is for this reason that the described effects for gel cultures did not always temporally correspond to those for monolayers.

The producers of Danazol (Winthrop Laboratories) kindly provided 1g of Danazol as a gift without any information about the pharmacological application of the substance, which has an extremely poor solubility in aqueous media. The fact that Danazol is taken orally by patients - and the substance obviously gets resorbed - raises questions as to how the substance is made pharmacologically available. This aspect determines the pharmacokinetics, incorporation and effects in target tissues.

The ultrastructural features described here after in vitro treatment of normal uterine endometrial cultures with Danazol are similar to the observations of Schorppe et a1, on patient biopsies of endometriotic foci during the course of therapy. The salient ultrastructural features are suggestive of cytoplasmic toxicity involving two processes: (a) perturbed lipid metabolism (b) intracellular deposition/accumulation of the substance (with augmentation of cytotoxic effects). Disorders in cellular lipid metabolism can lead to a whole array of detrimental effects on physiological processes leading to the ultimate destruction of cells. The proposed change in nuclear/cytoplasmic ratio of affected cells is a general adaptive response of cells subjected to cytotoxic effects.

Figs 13 - 18—Electron microphotographs revealing the ultrastructural cytopathology of uterine endometrial cells exposed to Danazol in vitro. Fig. 13: A degenerating cell having an overload of annular lipid droplets (L). The cell received Danazol exposure during primary (1 week) and secondary (1 week) culture in monolayer. 5400x. Fig. 14: Another cell from the same monolayer culture as in Fig. 13, which was still viable. The arrow points to a large autophagic vacuole containing crystalline particles - possibly Danazol. 2700x. Fig. 15: A viable cell in collagen gel culture after 10d Danazol exposure. The nucleus is highly segmentated. As cells in collagen gel develop extensive filopodia, the amount of cytoplasm around the nuclei may be reduced to a narrow rim. The lipid droplets (L) appear normal. 7500x. Fig. 16: Another viable cell in collagen gel culture after 10d Danazol exposure, showing a large autophagic vacuole. Distention of endoplasmic reticulum and large lysosomes (Ly) are seen. 7500x. Fig. 17: A typical degenerating cell in collagen gel after 10d Danazol. Arrow heads point to the autophagic vacuoles. Secondary lysomes (S) of various sizes are seen. Lipid droplets are annular. Filopodia (F) from neighboring cells which were in contact with the degenerating cell are seen. 7500x. Fig. 18: Filopodium of a viable cell in the vicinity of a degenerating cell, Swollen mitochondria with raffined cristae, autophagic vacuoles and irregularly shaped lysosomes are seen in the filopodium. 12000x.
stress. Remanent endometrial foci after Danazol therapy probably consist largely of cells which incorporated sublethal amounts of the substance. As the substance may precipitate in the form of microscopic crystals either within the cells, or within the extracellular matrix and thence internalised by phagocytosis, these depositions could be responsible for the long-term action.

The common evaluation of IVF success according to pregnancies calculated on the basis of embryo transfers could be deceptive. The reduced chances of pregnancy after Danazol therapy became overt only when the incidence of pregnancies was individualised per patient. The cause for the lowered incidence cannot be unequivocally proven as it can always be argued that reduced fertility is idiopathic to the disease itself. This is supported by earlier reports on decreased follicle numbers, retrievable oocytes and fertilisation frequency. To what extent Danazol may be culpable cannot be decided from these studies.

As the cases included in this study were age-matched and in all instances susceptible partner dysspermia eliminated, the cohort of cases qualifying for evaluation became reduced in number. These results are, therefore, regarded as being preliminary. Two known clinical facts (a) the effective control of disease itself. This is supported by earlier reports on decreased follicle numbers, retrievable oocytes and fertilisation frequency and (b) remanent foci despite successful control, provoke thought on the mode of action of this drug.

Conclusion

The efficacy of Danazol in the control of endometriosis is affirmed. Its application in women of reproductive age should be subject to careful consideration of the patients' wish to bear children. In cases desiring pregnancy, an alternative hormonal therapy may be preferred since Danazol reduces pregnancy chances.

References