

Short-Term Impact of Starch Particles on Endometriotic Cells *in vitro* and in a Xenograft Nude Mouse Model

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Abstract

Purpose: Investigating the impact of starch powder on human endometriotic cells *in vitro* and on the implantation and development of adhesions *in vivo*.

Basic procedure: *In vitro* - A human endometriotic cell line was incubated with different concentrations of starch particles and the effect on growth was measured by immunoassay. *In vivo* - Cells from a human endometriotic cell culture were injected with and without starch, into the peritoneal cavity of nude mice investigated 8, 15 respectively 22 days later.

Main findings: *In vitro* - A low dose of starch particles significantly inhibited the proliferation of endometriotic cells during incubation for 48 h. *In vivo* - There were significant differences in the development of adhesions when the endometriotic cells were injected intraperitoneally together with starch particles. The development of endometriotic implants was delayed but not significantly in the presence of starch particles.

Principal conclusions: Starch particles have a negative impact on the proliferation of endometriotic cells *in vitro* and affect the development of endometriotic implants *in vivo*.

Keywords: Nude mice; Mesothelium; Peritoneum; Starch; Endometriosis

Introduction

Tubal and pelvic adhesions are a major cause of female infertility in humans [1,2]. The development of adhesions is initiated by many factors such as peritoneal injury by mechanical trauma, fibrin depositions due to bleeding, leakage of fibrinogen during the inflammatory process post trauma [3], tissue ischemia [4,5], chemical agents [6], infections [7,8], foreign materials such as glove powder [9-12] and endometriosis [13].

It is well documented that starch-powdered gloves are not appropriate for abdominal surgery [14-16] since intraperitoneally-deposited starch particles can initiate an inflammatory reaction and the formation of adhesions [17,18]. However, the exact mechanism by which starch increases the propensity of tissue to form adhesions is not known [19,20]. Application of glove powder on a minimally or severely traumatized peritoneum facilitates tumour cell adhesion and growth [21], which raises the question whether this is also the case for endometriosis cells.

Previous studies have shown that starch particles in combination with a surgical trauma may induce the formation of adhesions [14] and granulomas. Case reports suggested that intra-abdominal granulomas or adhesions due to starch particles were caused by starch powder on medical gloves used during vaginal examination. The contents of the granulomata were investigated microscopically by polarized light [22]. Initially shown in animal and human studies [23]. The most abundant amount of particles is found after three days and reaches the peritoneal cavity in sufficient quantity to significantly increase the formation of post-operative adhesions after a standardized trauma [23].

Endometriosis is a disease with multidimensional etiology including hereditary, hormonal and immunological factors [24]. The reflux implantation theory postulates that retrograde menstruation into the peritoneal cavity contains viable endometrial cells with the capacity to adhere and grow on extra-uterine sites such as the

peritoneal surface and the ovaries [25]. Groothuis et al. [26] suggested that the mesothelial layer acts as a barrier preventing the attachment of ectopic endometrium and that the initiation of the attachment of refluxed endometrial cells in the peritoneal cavity depended on the condition of the mesothelial layer. Menstrual tissue easily adheres to the extracellular matrix of the peritoneum but not to an intact mesothelial layer [27]. The stromal cells in the endometrium have a crucial role in the attachment of endometriosis cells also to an intact mesothelium. The peritoneum consists of a mesothelial lining, basement membrane and extracellular matrix. Adhesion of endometrial fragments has been microscopically observed at locations where the mesothelium is absent or damaged and the extracellular matrix is exposed [28]. If the mesothelial lining is traumatized, this could be a prerequisite for endometrial cell adhesion. Starch particles present in the abdominal cavity might injure the mesothelium and thereby facilitate the attachment of these cells. After a gynaecological examination with powdered gloves, retrograde migrated starch particles could damage the mesothelial lining of the peritoneum and expose the extracellular matrix [23]. If menstrual tissue is present in the intra-abdominal cavity it is possible that the endometrial fragments could adhere.

Previous studies have shown that human tissue transplanted into nude mice generally retains its histological, chromosomal and biochemical properties [28]. Therefore the *in vivo* experiments in this

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study were carried out on immunodeficient nude mice with the purpose, in the presence of starch particles, of evaluating the intraperitoneal implantation of human endometriotic cells and any development of intra-abdominal adhesions. Starch particles have the potential to mechanically generate a non-intact mesothelial layer to which endometriotic cells could adhere. The *in vitro* experiments separately investigated the effect of starch on the growth of endometriotic cells in culture.

Materials and Method

Human endometriosis sample

Human tissue biopsies from endometriomas were obtained from four women during laparotomy. Two tissue biopsy samples, 10×10 mm, were aseptically taken from the capsule of each endometrioma, transported within the hospital without cryoprotectant directly to be frozen in liquid nitrogen. The biopsies were later verified histologically as endometriotic tissue. The local ethical committee approved the sampling procedure.

Cell culture

The frozen endometriotic tissues were thawed at +37°C, transferred to test tubes and prepared as follows: the tissue covering the inside of the endometriomas was detached from the encapsulating fibrous tissue by gentle scraping with a knife. The tissue was then exposed to 0.2% collagenase Type III (Worthington Biochemical Corporation Freehold, New Jersey, USA) for 2 hours. Thereafter the cell suspension was centrifuged at 1500 rpm for 10 minutes. The cells were resuspended in RPMI 1640 cell culture medium with L-glutamine and phenol red (Invitrogen Ltd), 20% Fetal Bovine serum (Invitrogen Ltd), sodium pyruvate, MEM 100 mM (Invitrogen Ltd), non-essential amino acids (Invitrogen Ltd), heparin 90 µg/ml (Sigma-Aldrich Fine Chemicals St Louis, MO, USA), penicillin-streptomycin with 10.000 Units/ml penicillin G sodium and 10.000 µg/ml streptomycin sulphate in 0.85% saline (Invitrogen Ltd), Hepes buffer solution 1M (Invitrogen Ltd) and seeded in T-75 cm² cell culture flasks (Nunc, Roskilde, Denmark) pre-coated with 0.2 % gelatine (Sigma-Aldrich).

The culture medium was changed the following day and thereafter once a week. From two of the four endometriomas biopsied, confluent monolayers were obtained after three weeks. The cultures were then trypsinized and subcultured on glass cover slips (Ø 13 mm, 2 mm thick, Bergman Labora, Stockholm, Sweden) pre-coated with 0.2 % gelatine (Sigma-Aldrich) placed in 24-well cell culture dishes (Nunc, Roskilde, Denmark) and maintained in a 5% CO₂/95% humidified air atmosphere at 37°C. Cultures from passages 4 and 6 were used for experiments [29]. The endometriosis cells were counted in a Burkert chamber and contained 0.29×10⁶ cells/ml. In the experiment, 0.6 ml cell suspension was injected intraperitoneally into each mouse.

Immunocytochemistry

To differentiate between endometriotic epithelial cells and stromal cells, an immunofluorescence staining procedure with monoclonal mouse-antihuman antibodies to vimentin and cytokeratin was used.

Human endometriotic cells on slides were rinsed with Hanks' buffered saline solution without phenol red (HBSS, Invitrogen Ltd), fixed with 4% formaldehyde in PBS Dulbeccos phosphate buffered saline w/o sodium bicarbonate (Invitrogen Ltd) for 15 minutes at 4°C. Without washing, monoclonal mouse-antihuman antibodies to vimentin (300 µl, 1:200, BioGenex Laboratories, Mainz, Germany) and cytokeratin (300 µl, 1:100, BioSite, Täby, Sweden) were added.

Following washings in PBS, primary antibodies were visualized with BioGenex Fast Red Detection kit® AA000-5M (BioSite Täby, Sweden).

Epithelial endometriotic cells stained positive for cytokeratin and negative for vimentin. Stromal cells were positive for vimentin and negative for cytokeratin [29]. The technique was used as a qualitative test to confirm that an endometriotic cell line with both epithelial and stromal cells had been obtained.

Starch and endometriotic cell-proliferation *in vitro*

To determine the inhibitory or stimulatory effects of starch on endometriotic cells in a cell culture, three different concentrations of starch particles were added to cultures of two different endometriotic cell lines (E1 and E2) developed as described above. Each cell culture was incubated with 0.1 mg, 0.5 mg and 1 mg of starch particles. The cell cultures were titrated in flat-bottomed microtitre plate 100 µl/well culture medium at a concentration of 1×10³ cells/well in the culture media. Fetal Calf Serum (FCS 10%, 5% or 2%) was also used in the culture mediums. The incubations series were carried out in multiples of 10 for 24 hours and 48 hours respectively.

Replication of cellular DNA is a prerequisite for cellular proliferation. DNA synthesis has been the most common measure of mitosis and cell proliferation and a specific cell proliferation ELISA, BrdU (Bromodeoxyuridine, Roche Molecular Biochemical's, 10 µl/well) was used. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by an immunoassay. The result is quantified by measuring the absorbance at the wavelength 550 nm using a scanning multi-well spectrophotometer (ELISA reader, Dynatech MR5000). The developed color and thereby the absorbance values directly correlate with the amount of DNA synthesis and thus to the number of proliferating cells in the respective micro-cultures.

Animals

45 immunodeficient, athymic, nude nu/nu-Balb/c, 8 week old female mice (Bomholt, Denmark), weighing 25-35 g, were kept in micro-isolator cages and housed in a separate barrier facility. This well-controlled, pathogen-free environment had monitored ambient temperature and regulated cycles of light-dark cycles. The mice in this study were kept under identical conditions with food and water ad libitum. The local ethical committee approved the experiments.

Starch

An amount of 0.3 mg sterile Biosorb™ (Johnson and Johnson, Surgikos Ltd, Livingstone, Scotland) was suspended in 0.6 ml RPMI 1640 medium and injected intraperitoneally into each mouse. The injected amount of starch was calculated according to the weight of the animal and previous studies by van den Tol et al. [21].

Starch and endometriotic cells *in vivo* - operative procedure

The nude mice were divided into three groups.

Group A: 0.6 ml cell suspension of endometriotic cells (E1 & E2) in RPMI 1640 Medium was injected intraperitoneally into 14 mice.

Group B: 0.6 ml cell suspension of endometriotic cells (E1 & E2) in RPMI 1640 Medium and 0.6 ml of 0.3 mg sterile Biosorb™ in RPMI 1640 Medium were injected intraperitoneally into 17 mice.

Group C: 0.6 ml RPMI 1640 Medium only was injected intraperitoneally into 14 mice, i.e. controls.

A specially trained assistant in experiments on animals carried out the injections. Directly after the injection, the abdomen of each mouse was gently massaged for 20-30 s. with fingertips in order to distribute the fluid in the peritoneal cavity.

Eight days later 5 mice from groups A & B and 4 from group C were sacrificed. Laparotomy was performed non-blinded by the investigators and any finding of intraperitoneal adhesions and/or the development of endometriosis were assessed. Using an inverted microscope during the dissection, tissue samples were taken from the peritoneum where endometriosis or adhesions were present. The tissue samples were fixed in 4 % formaldehyde solution and subsequently embedded in paraffin for sectioning (5 µm) and routine staining with haematoxylin-eosin. The slides were studied with a Leica Wild M 32 microscope using polarized light (magnification x 100).

Fifteen days after the injections another 5 mice from each group were sacrificed and biopsies were taken in the same way during laparotomy. Finally, 22 days after the injections, 4 mice from group A, 7 from group B and 5 from group C were sacrificed. Again, the operative procedures were as previously described as well as the collection of biopsies.

Statistics

All results were gathered in a database and analyses were carried out with STATISTICA™ (Statsoft, USA). Non-parametric Mann-Whitney and Fisher's exact test, two-tailed, was used and differences were considered significant at $p < 0.05$.

Results

In vitro: Immunoassay of endometriotic cells when starch was added

Proliferation of the endometrial cells was affected when starch was added. Addition of 0.1 mg, 0.5 mg or 1 mg starch had overall an inhibitory but non-significant effect ($p = 0.06$) on the cell proliferation when analyzing all incubations with and without starch. In the E1 cell line there was a significantly inhibitory effect in all the starch incubations compared to the controls ($p < 0.01$). The same general effect was not seen in the E2 cell line ($p < 0.8$). There was no difference in the cell cultures in the inhibition of cell proliferation with the different Fetal Calf Serum (FCS) concentrations of 10% FCS, 5% FCS or 2 % FCS (Figures 1A,1B,2A and 2B) in the 24-hour culture. The greatest inhibitory effect from starch in E1 as well as in E2 was found in the 48-hour incubation and with the lowest amount of 0.1 mg starch added ($p < 0.01$).

In vivo: Development of adhesions and endometriotic implants

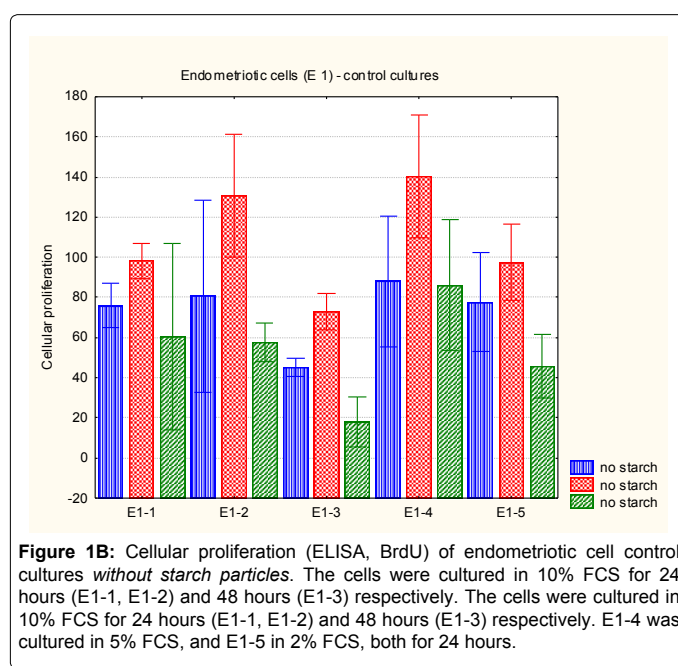
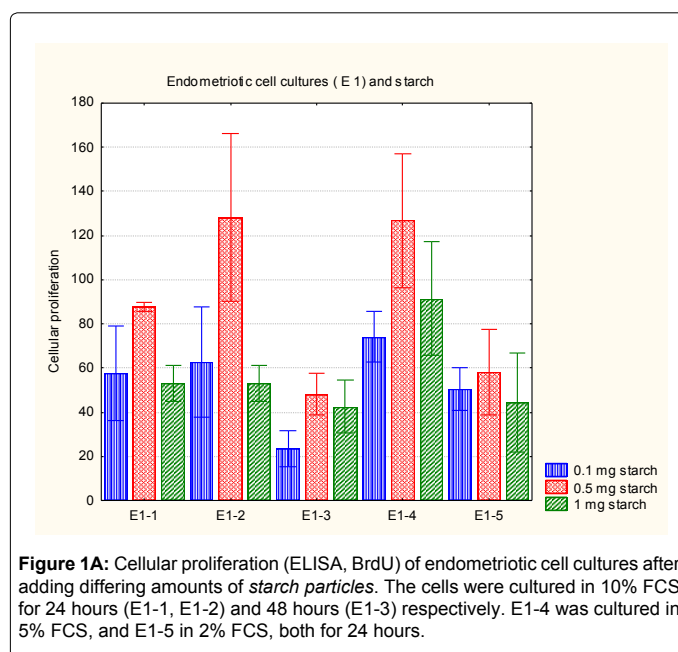
All the mice survived the operative procedure without any clinical signs of complications. Implants of endometriosis were observed in four mice. Two of these had been injected with endometriotic cells 15 days previously. The other two had had injections of endometriotic cells and starch 22 days previously (Tables 1-3).

Filmy adhesions between the uterine horn and the peritoneum were seen in four mice injected with endometriotic cells and starch 15 days previously. The surrounding tissue was inflamed with thick fibrosis. Microscopically, haematoxylin-eosin stained peritoneal implants sections showed the presence of endometrial acinar glands. They were few and varying in size and forms. The inner lining of the glands consisted of glandular columnar epithelium (Figure 3).

Cytochemical characterization confirmed the lesions as endometriosis and immunoreactivity was assessed. The expression of cytokeratin as an epithelial marker was positive. There were significant differences (Fischer's exact test) in the development of adhesions on Day 15 compared to Day 22 when endometriotic cells were injected intraperitoneally together with starch particles ($p < 0.01$). There were also significant differences on Days 8 and 15 in the adhesion development ($p < 0.05$). No statistical differences in the development of endometriosis implants were found between groups A and B, i.e. without or with starch (Table 1 and 2).

Discussion

The most widely accepted theory on the pathogenesis of endometriosis, postulates that retrograde menstrual flow transports



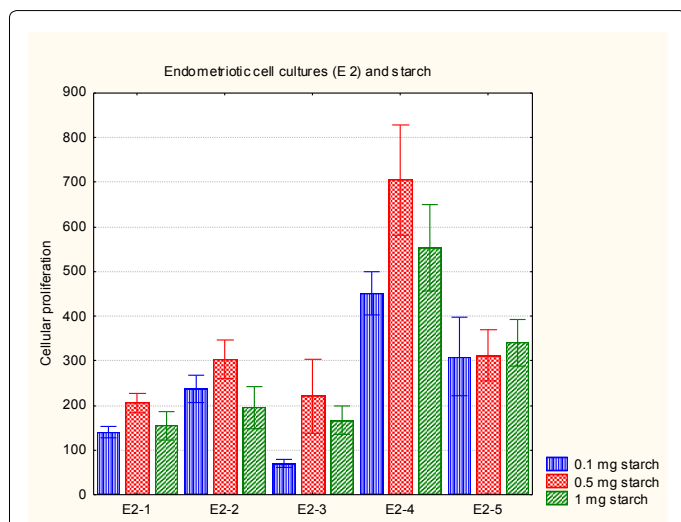


Figure 2A: Cellular proliferation (ELISA, BrdU) of an endometriotic cell culture after adding differing amounts of starch particles. The cells were cultured in 10% FCS for 24 hours (E2-1, E2-2) and 48 hours (E2-3) respectively. E2-4 was cultured in 5% FCS, and E2-5 in 2% FCS, both for 24 hours.

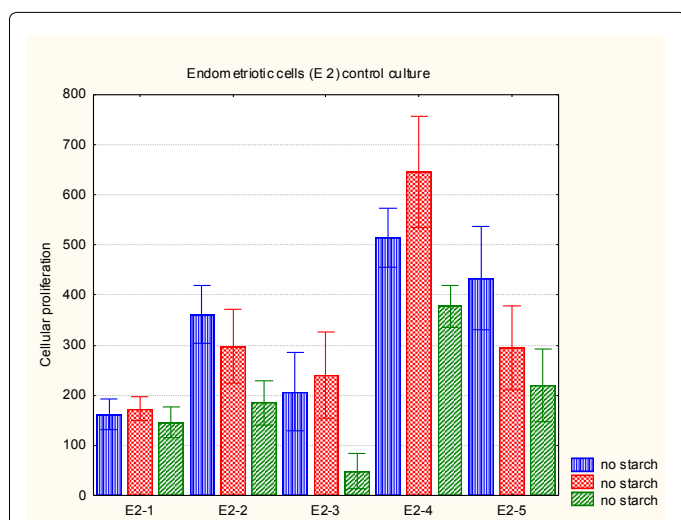


Figure 2B: Cellular proliferation (ELISA, BrdU) of endometriotic cell control cultures without starch particles. The cells were cultured in 10% FCS for 24 hours (E2-1, E2-2) and 48 hours (E2-3) respectively. E2-4 was cultured in 5% FCS, and E2-5 in 2% FCS, both for 24 hours.

desquamated endometrial cells through the Fallopian tubes into the peritoneal cavity and that the remaining viable cells subsequently become implanted and grow [25]. The mechanism of adherence of regurgitated endometrial cells to the peritoneum is still unclear. Nude mice represent a well-known model of experimental endometriosis [29]. In a previous animal model, the transplantation of proliferative or secretory human endometrium had an implantation rate varying from 33 % to 87 % if Estradiol and Progesterone were added [29,30]. In the present study, a 13 % implantation rate was obtained without hormonal supplementation.

The focus in this study was on the effect on endometriotic cells of starch particles from glove powder *in vitro* and if starch injected intraperitoneally *in vivo* into nude athymic mice together with endometriotic cells could affect implantation at the peritoneum. The hypothesis was that starch particles injected intraperitoneally would

damage the mesothelial cells and in this way provide conditions for endometriotic cells to attach to the peritoneum.

An association between intraperitoneal starch and the growth of endometriotic cells was found. *In vitro* starch had an overall negative impact on the endometriotic cell proliferation, which was significant when the two cell lines studied were exposed to the lowest dose tested. *In vivo*, there were no significant differences in implantation rate but there was a delay when starch was added. This raises the question whether the negative impact of starch on endometriotic cells *in vitro* is a reason for the delayed development of endometriosis implants *in vivo*. There were more adhesions in the latter group, i.e. when starch had been added, than in the endometriotic cell group without starch. This was an expected finding since the development of adhesions due to foreign material such as starch particles has previously been thoroughly investigated [4,10,14,16,23].

In a rabbit model and in humans, it takes approximately 10 days for adhesions to develop [18,23]. After a peritoneal injury with fibrin deposition, the subsequent fibrinolysis is an important process determining whether normal peritoneal healing will occur or adhesions will be formed [31,32]. Previous data has shown that adhesion formation is an ongoing inflammatory activity, with the possibility of an increased fibrinolytic process during endometriosis and/or adhesions to restrict the formation of adhesions [33]. Immune deficient nude mice are homozygous mutants with congenital thymus aplasia resulting in a deficient T-lymphocyte system but still competent for inflammatory reaction with plasma cells, lymphocytes, leukocytes, macrophages and foreign-body giant cells [34]. Several studies have demonstrated ectopic growth of both normal human endometrium and endometriotic tissue in athymic nude mice [35]. However, a normal functioning T-cell system is essential for the inflammatory reaction in the development of persistent adhesions and might contribute to the lack of findings in the Day 22 group [36].

The etiology of endometriosis, whether the disease involves genetics or the immune system or is due to retrograde menstruation, is

	Mice sacrificed day 8	Mice sacrificed day 15	Mice sacrificed day 22
No. of mice	5	5	4
No. of mice with endometriotic glands	0	2 *	0
No. of mice with adhesions	1 **	0	0

*not related to injection site, ** filmy adhesion

Table 1: Group A: Endometriotic cells injected intraperitoneally.

	Mice sacrificed day 8	Mice sacrificed day 15	Mice sacrificed day 22
No. of mice	5	5	7
No. of mice with endometriotic glands	0	0	2 *
No. of mice with adhesions	0	4 (filmy adhesions)	0

*not related to injection site

Table 2: Group B: Endometriotic cells and starch injected intraperitoneally.

	Mice sacrificed day 8	Mice sacrificed day 15	Mice sacrificed day 22
No. of mice	4	5	5
Presence of endometriotic glands	0	0	0
No. of mice with adhesions	0	0	0

Table 3: Group C: RPMI Medium injected intraperitoneally.

still an unsolved problem. There are worldwide geographic variations as well as variations in the use of vaginally deposited starch particles from examination gloves, condoms or vaginal transducer covers.

The question whether starch, together with retrogradely shed endometrial tissue, could contribute to the destroying of the peritoneal mesothelium and to subsequent implantation of endometriosis has to be further investigated.

Conclusion

Starch has an overall negative impact on the growth of endometriotic cells *in vitro* which was significant when the lowest dose of starch was used. In this experimental model with immune deficient nude mice, human endometriotic cells and starch particles can cause intraperitoneal adhesions *in vivo*. Endometriotic implants might develop with a delay *in vivo* when starch particles are present. Further investigations are needed to study the interaction between starch particles and endometriotic cells.

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