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EFFECT OF PERITONEAL FLUID ON EARLY EMBRYONIC DEVELOPMENT

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GLOSSARY

Definition of terms

Embryotoxic: Substance(s) that can cause harmful to the embryo

Embryotoxicity: The ability of a substance to cause harm to the embryo which may result in death or abnormal development.

Endometriosis: A condition in which the endometrial tissue that composed of endometrial glands, stroma or both, outside the endometrial cavity (Tabibzadeh *et al.*, 2003).

Abbreviation

AFS	American Fertility Society
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
hCG	human chorionic gonadotrophin
HWM	modified Whitten's medium (with Hepes)
ICR	Institute Cancer of Research
IL	interleukin
IVF	in vitro fertilization
mWM	modified Whitten's medium
PF-E	peritoneal fluid with endometriosis

PF-NE	peritoneal fluid without endometriosis
PMSG	pregnant mare serum gonadotrophin
r-AFS	revised American Fertility Society
TNF	tumor necrosis factor
WM	Whitten's medium
zp	zona pellucida

ABSTRACT

The aetiology of endometriosis associated with infertility remains poorly understood. In recent years, the potential influence of peritoneal fluid (PF) or its cellular components have been proposed as possible mediators of infertility in endometriosis through it toxic effects on pre-implantation embryo. Several factors have been identified as embryotoxic factors, however, the mechanism of embryotoxicity have not well clarified. Studies were therefore undertaken to clarify the possible mechanisms of embryotoxicity in endometriosis and to determine the possible embryotoxic factor(s) that involved in the process. The role of exogenous nutrient (pyruvate) has also been examined in reducing or eliminating the embryotoxicity. Peritoneal fluid was collected from infertile women at reproductive age with 21 endometriosis (PF-E) (7 minimal or mild, 7 moderate, 7 severe) and 7 without endometriosis (PF-NE). Addition of PF-E to the culture medium from all stages of endometriosis significantly suppressed the mouse embryo growth at all stages of development, at 24, 48 and 72 hours, which correlated with the severity of the disease, as compared to control (no peritoneal fluid). Excessive pyruvate was able to reduce the embryotoxicity at all stages of development in minimal or mild and moderate PF-E, and only during the first 24 hours in severe PF-E. The level of IL-6 concentration was significantly higher in PF-E as compared to PF-NE and correlated with the severity of the disease. However, no significant difference was noted in the level of IL-8 between the two groups. Both interleukins were found to be embryotoxic with IL-6 is more potent. A positive correlation between the levels of IL-6 and its embryotoxicity was noted. These findings propose embryotoxicity to be a possible mechanism of infertility in endometriosis and the growth

promoting effects of pyruvate indicated the mechanism of embryotoxicity might involve functional disruption of the intermediary metabolism such that excessive pyruvate is required to maintain embryo growth.

KESAN CECAIR PERITONEUM KE ATAS PERKEMBANGAN AWAL EMBRIO (ABSTRAK)

Etiologi penyakit endometriosis berkaitan dengan masalah kesuburan masih kurang difahami. Sejak kebelakangan ini, cecair peritoneum (CP) atau komponen sel-sel di dalamnya telah dicadangkan sebagai perantara yang berupaya mempengaruhi masalah kesuburan di dalam penyakit endometriosis melalui kesan toksiknya ke atas embrio di peringkat pra-implantasi. Beberapa faktor telah dikenalpasti sebagai embriotoksin, walaubagaimanapun, mekanisme ketoksikan embrio ini masih belum dijelaskan dengan sempurna. Kajian ini telah dikendalikan untuk menjelaskan mekanisme ketoksikan embrio pada pesakit endometriosis dan mengenalpasti faktor-faktor embriotoksin yang mungkin terlibat. Peranan nutrien eksogenus (piruvat) didalam mengurangkan atau menghilangkan ketoksikan embrio juga telah dijalankan. Cecair peritoneum diperolehi daripada wanita diperingkat umur reproduktif yang mempunyai masalah kesuburan, iaitu 21 Endometriosis (CP-E) (7 ringan, 7 sederhana, 7 teruk) dan 7 tiada endometriosis (CP-TE). Tambahan CP-E ke dalam media kultur, dari semua peringkat endometriosis, didapati merencat perkembangan embrio pada setiap peringkat perkembangan, iaitu pada 24, 48 dan 72 jam selepas kultur, dan didapati kolerasi terhadap keterukan penyakit endometriosis, berbanding dengan kumpulan kawalan (tiada cecair peritoneum). Tambahan piruvat yang berlebihan didapati mampu mengurangkan ketoksikan embrio di setiap peringkat perkembangan, pada CP-E ringan dan CP-E sederhana, dan bagi CP-E teruk, kesan ini hanya dilihat pada 24 jam yang pertama sahaja. Paras IL-6 pada CP-E adalah tinggi secara signifikan berbanding dengan CP-TE, dan didapati kolerasi terhadap keterukan

penyakit. Tiada perbezaan yang signifikan pada paras IL-8 dari kedua-dua kumpulan. Kesan ketoksikan embrio dapat dilihat pada kedua-dua interleukin dengan IL-6 mempunyai keupayaan yang lebih tinggi. Didapati juga kolerasi positif yang signifikan di antara paras IL-6 dan kesan ketoksikan embrio. Penemuan ini mencadangkan ketoksikan embrio sebagai mekanisme terhadap masalah kesuburan pada pesakit endometriosis dan peranan piruvat didalam menggalakkan pertumbuhan embrio menunjukkan mekanisme ketoksikan embrio ini mungkin melibatkan gangguan terhadap fungsi metabolisme perantara yang dibuktikan dengan kehadiran piruvat yang berlebihan diperlukan untuk mengekalkan pertumbuhan embrio.

1. BACKGROUND

The peritoneal fluid is the physiologic environment of the fallopian tube and the oocyte after ovulation, and believed to be an exudation product of the ovary, at least in part (Bouckaert *et al.*, 1986). This fluid has been shown to contain a plethora of substances, including macrophages, cytokines, growth factors, enzymes, proteins and prostaglandins, which subject to change with pathological conditions (Harada *et al.*, 2001). The direct communication of the peritoneal fluid with the lumen of the fallopian tube (Seli & Arici, 2000), suggests that it may serve as a medium for fertilization and early embryonic development, but whether it exerts a regulatory role on them is unknown.

In recent years, many investigators have focused on this fluid to study various aspects of endometriosis, one of the most frequently encountered gynecologic diseases (Al-Fozan & Tulandi, 2003; Tabibzadeh *et al.*, 2003). To date, the aetiology of endometriosis associated with infertility remains poorly understood. A number of postulates have been proposed to explain the possible aetiology of endometriosis associated with infertility ranging from alterations in the peritoneal fluid (Ryan & Taylor, 1997; Gomez-Torres *et al.*, 2002), ovulatory dysfunction (Dmowski *et al.*, 1986; Ronnberg, 1990), sperm phagocytosis (Soldati *et al.*, 1989; Jha *et al.*, 1996), impaired fertilization (Cahill *et al.*, 1997; Azem *et al.*, 1998) and implantation defects (Pellicer *et al.*, 1998; Illera *et al.*, 2000).

Many authors (Harada *et al.*, 2001; Gomez-Torres *et al.*, 2002) suggested that the factors that cause the infertility in endometriosis might be hidden in the

peritoneal fluid especially because this fluid is a major controlling the peritoneal microenvironment (Taketani *et al.*, 1992; Tabibzadeh *et al.*, 2003) where most of the reproductive organs located. The fluid is in contact with peritoneal endometrial implants as well as the tubal microenvironment in which fertilization occurs, hence subtle alterations of this fluid and /or cellular constituents might adversely influence reproduction. Despite continuously bathes the pelvic cavity, uterus, fallopian tubes and ovaries (Seli & Arici, 2000) and host the processes of ovulation, gamete transportation, fertilization and early embryonic development (Syrop & Halme, 1987), this fluid is away from routine site of investigations.

A recent focus of attention concerning this fluid is to determine the factor(s) within it that is believed can cause harmful to the embryo or known as embryotoxic factors, as a mediator of infertility in endometriosis and to determine whether this factor(s) can be reduced or eliminated from the fluid.

There is thus a need to re-define the role of peritoneal fluid especially in endometriosis, on early embryonic development.

2. GENERAL OBJECTIVES

1. To determine the effects of peritoneal fluid (embryotoxicity) from women with endometriosis (PF-E) on *in vitro* development of early mouse embryos and to relate with the severity of the disease.

2. To determine whether the embryotoxicity can be reduced or eliminated from the PF-E.
3. To determine the embryotoxic factor(s) in the PF-E as a possible mediator of infertility in endometriosis.
4. To determine the embryotoxicity of the embryotoxic factor(s) determined on *in vitro* development of early mouse embryos.

3. GENERAL METHODS AND MATERIALS

3.1 Subject selection

The diagnosis of endometriosis was based solely upon direct laparoscopic visualization of endometriotic implants or during laparotomy. Disease was staged as minimal or mild, moderate and severe, according to the classification of revised American Fertility Society (r-AFS), 1985. Despite using the same classification, many researchers (Damewood *et al.*, 1990; Rier *et al.*, 1994; Polak *et al.*, 2003) combined the minimal and mild endometriosis as one group.

Peritoneal fluid samples were obtained from 21 infertile women of reproductive age who underwent laparoscopy examination or laparotomy. They were including 7 women with minimal or mild, 7 with moderate and 7 with severe endometriosis. The subjects were from Hospital Universiti Sains Malaysia and Hospital Kota Bharu, Kelantan. The mean age of these patients is 30.88 $\pm$ 0.87 (mean $\pm$ S.E.M), (range: 26 - 36) with the duration of infertility was at least 18 months. They received general infertility work ups and non-received hormonal treatment or had intrauterine device insertion within 6 months before the

procedure. The inclusion criteria included: infertile, either primary or secondary whereas the exclusion criteria included: age above 40 years and presence of other pathological condition which observed during laparoscopy or laparotomy, such as uterine fibroid or pelvic tumor. The study was approved by the Universiti Sains Malaysia Ethical Committee.

3.2 Peritoneal fluid collection and preparation

Peritoneal fluid samples were collected by the gynaecologic surgeon from the anterior and posterior cul-de-sacs by Veress needle during laparoscopy either for evaluation for infertility or for treatment of infertility, or during laparotomy. The fluid was obtained before any manipulative procedures done. All patients were placed in a supine position to standardize the method of collection. The fluid was placed in a sterile heparinzed tube; the volume was recorded and transported to the laboratory immediately. Those samples with heavily blood stained were discarded. The collected peritoneal fluid volume varied from 2 – 10 mls, (4.94 $\pm$ 0.61, mean + S.E.M).

Samples were prepared as described by Tan *et al.*, (1989). The fluid was centrifuged at 600-x g for 10 minutes at 4°C. The cell-free supernatants were heated inactivation in a water bath (56°C) for 30 minutes to inactivate the complement protein. Then, it was separated into aliquots and stored at –80°C till the time of assay.

3.3 Animal preparation

In the present study, female and male mice of ICR strain, 8 – 10 weeks of age and weighing 15 – 20 gram were used. The mice were provided by Laboratory

Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur. Animals had free access to food pellets and drinking water.

Females were superovulated by injected intraperitoneally with 5 IU of pregnant mare serum gonadotrophin (PMSG: Folligon, Intervet International B.V, Holand) (Appendix A) to stimulate the follicular growth and 46 hours later with 5 IU of human chorionic gonadotrophin (hCG: Chorulon, Intervet International B.V, Holand) (Appendix B) to trigger ovulation. Immediately after hCG injection, the female mice were caged at a 1: 1 ratio with males for overnight. Mating was confirmed after 18 to 19 hours by the presence of vaginal plug or sperm-positive smear. The protocol was used as described by Tan *et al.*, (1989).

3.4 Mouse embryo collection

Twenty-four hours after mating confirmed, which was about 42 to 44 hours after hCG injection, the female mice were sacrificed by cervical dislocation and the oviducts were dissected and transferred to a Petri dish (NUNC, Denmark) contained few drops of flushing solution, modified Whitten's medium with Hepes (HWM) (Appendix C). With one ml syringe attached to a blunt 32-gauge needle, the oviduct was flushed with HWM to separate the 2-cell mouse embryos under the dissecting microscope at room temperature. Only morphologically normal embryos were used in the experiment and they were pooled in a Petri dish contained HWM. Therefore, the medium used for oviduct flushing, embryo collection and holding before culture was HWM.

3.5 Mouse embryo culture

Before culture, each sample of the peritoneal fluid supernatant from all stages of endometriosis (study group) was thawed and filtered (0.2 μm filter, Whatman)

and added to the culture well (culture well plate: NUNC, Denmark) whereas in control group, the culture well only contained the 2-cell embryos in culture medium, without present of peritoneal fluid.

The collected normal 2-cell embryos were rinsed with HWM to remove any foreign materials. Then, they were distributed randomly between the study and control groups, which have been designed according to the respective experiments. Embryos were cultured in groups of 10-12 per well, which contained modified Whitten's medium (mWM) (Appendix D).

The embryos were cultured at different time of experiments based on the number of 2-cell mouse embryos collected. All the experiments were done in the same culture environment. Embryos were cultured in an incubator at 37°C, which was gassed with 5%CO_2 in air. The pH of culture medium was maintained at 7.3-7.4.

3.6 Embryo evaluation

Viability assessment is an essential part of embryo culture study. Different terms have been proposed such as 'embryo quality, 'embryo viability' and 'developmental competence'. Overstrom, (1996) has proposed five approaches to evaluate embryo viability including:

1. morphology
2. development *in vitro* (culture)
3. differential (live/dead) cell staining
4. fluorescent metabolic probes and
5. micro assays of embryo metabolism

In this study, the first two approaches were used. Several characteristics including embryo color/darkness, homogeneity of blastomere size, cytoplasmic granulation and degree of blastomere fragmentation were observed. The embryonic stages at 24, 48 and 72 hours were observed under Inverted microscope (Axiovert S100 : Carl Zeiss Company, Germany).

The control range for normal growth was defined as described by Tan *et al.*, (1989).

At 24 hours: 4 cells or greater

At 48 hours: morulae or greater

At 72 hours: blastocysts or greater

For the degenerated embryos, they were defined as described by Morcos *et al.*, (1985) including dark granular cytoplasm, fragmentation and cell mass retracted from the zona pellucida.

The flow chart of mice preparation and embryo culture is outline in Figure 3.1.

3.7 Statistical analysis

All analysis was done using the Instat Programme. To evaluate the development of mouse embryos, results from individual culture well were pooled according to their groups. Data were analyzed by chi-square test and expressed in percentage. p values < 0.05, were considered significant.

Female mice (ICR strain: 8 to 10 weeks)

Ovarian hyperstimulation

i. Injection of PMSG (5 IU)

ii. Injection of hCG (5 IU), 46 hours later

Mating with male mice

(ICR strain: 8 to 10 weeks)

Mating confirmed by presence of vaginal plug/sperm-positive smear

(18-19 hours after mating)

Embryo collection

Flushing of oviducts with HWM

(42-44 hours after hCG injection)

Embryo culture

2-cell mouse embryos with

i. Control group (no PF-E)

ii. Study group (with PF-E)

Medium : modified Whitten's medium

Atmosphere : CO_2 incubator, with 5% CO_2 in air

pH : 7.3 - 7.4

Temperature : 37°C

Figure 3.1: Flow chart showing mouse preparation and embryo culture

4. EMBRYOTOXICITY OF PERITONEAL FLUID WITH ENDOMETRIOSIS (PF-E)

4.1 Introduction

Embryotoxicity is defined as the ability of a substance to cause harm to an embryo, which may result in death or abnormal development. There is increasing evidence that macrophages, cytokines and other local products present in PF-E may be the mediators for infertility by causing alterations in the peritoneal environment, subsequently generate embryotoxic activity (Gomez-Torres *et al.*, 2002). This is supported by the facts that peritoneal fluid surrounds the ovary and proximal part of the fallopian tubes and passes easily into the tubal lumen in which fertilization takes place (Seli & Arici, 2000). Early embryonic growth is therefore exposed to this fluid with its cellular and soluble components.

The embryotoxic factors can be an antibody, a cytokine or may be nutritional (Fein *et al.*, 1998). There is a large array of factors that have been reported to have a potential in the inhibition of early embryo growth such as IL-1 (Fakih *et al.*, 1987; Taketani *et al.*, 1992), TNF-α (Taketani *et al.*, 1992), IL-6 (Gomez-Torrez *et al.*, 2002), interferon (IFN)-σ (Seli & Arici, 2000), ovum capture inhibitor (OCI) (Suginami & Yano, 1988) and various types of autoantibodies such as phospholipid, ribonucleoprotein and double stranded DNA (Gleicher *et al.*, 1987).

Peritoneal fluid embryotoxicity has been studied in women with and without endometriosis. Although several investigators (Morcos *et al.*, 1985; Tan *et al.*, 1989; Illera *et al.*, 2000; Gomez-Torres *et al.*, 2002) report that peritoneal fluid obtained from infertile patients with endometriosis adversely affect mouse embryo cleavage and viability *in vitro*, others (Dodds *et al.*, 1992; Awadalla *et al.*, 1997) have found no harmful effects of this fluid on embryo growth, when compared to peritoneal fluid from infertile women without endometriosis (PF-NE).

To this end, the relationship between infertility and embryotoxicity in endometriosis is still not clear. The reason for these equivocal studies is unclear, although PF-E has frequently been shown to be toxic to the pre-implantation embryo (Harada *et al.*, 2001).

Specific objectives:

1. To determine the embryotoxicity of PF-E on the development of early mouse embryos.
2. To determine the effects of different stages of PF-E on the development of early mouse embryos.

4.2 Methods and materials

Mouse embryo culture:

- mWM was added to each culture well.

Study group:

50 μl of each sample of PF-E (prepared as previously described in the General Methodology: Chapter 3) was added to each culture well containing mWM.

Control group:

Each culture well was prepared with 1 ml mWM (control without the peritoneal fluid)

- The collected 2-cell mouse embryos were distributed randomly
- The total volume in each culture well was 1 ml.
- There were 7 wells used for each stage of PF-E and the control group.
- The embryos were incubated and observed at 24, 48 and 72 hours.

4.3 Results

Addition of 5% (50μl) heat-inactivated PF-E to the culture medium from all stages of endometriosis significantly ($p<0.001$ versus control) suppressed the embryo growth at all stages of embryo development, which are shown in Figure 4.1 (minimal or mild PF-E), Figure 4.2 (moderate PF-E) and Figure 4.3 (severe-PFE). A positive relationship between embryotoxicity and the severity of endometriosis was also observed, as shown in Figure 4.4.

The morphology of the embryos is shown in Figure 4.5 (normal embryos) and Figures 4.6 and 4.7 (degenerated embryos). Normal embryos consist of blastomeres of even in size and shape and no cytoplasmic fragments whereas the degenerated embryos either contain dark granular cytoplasm, are fragmented or have cell mass retracted from the zona pellucida.

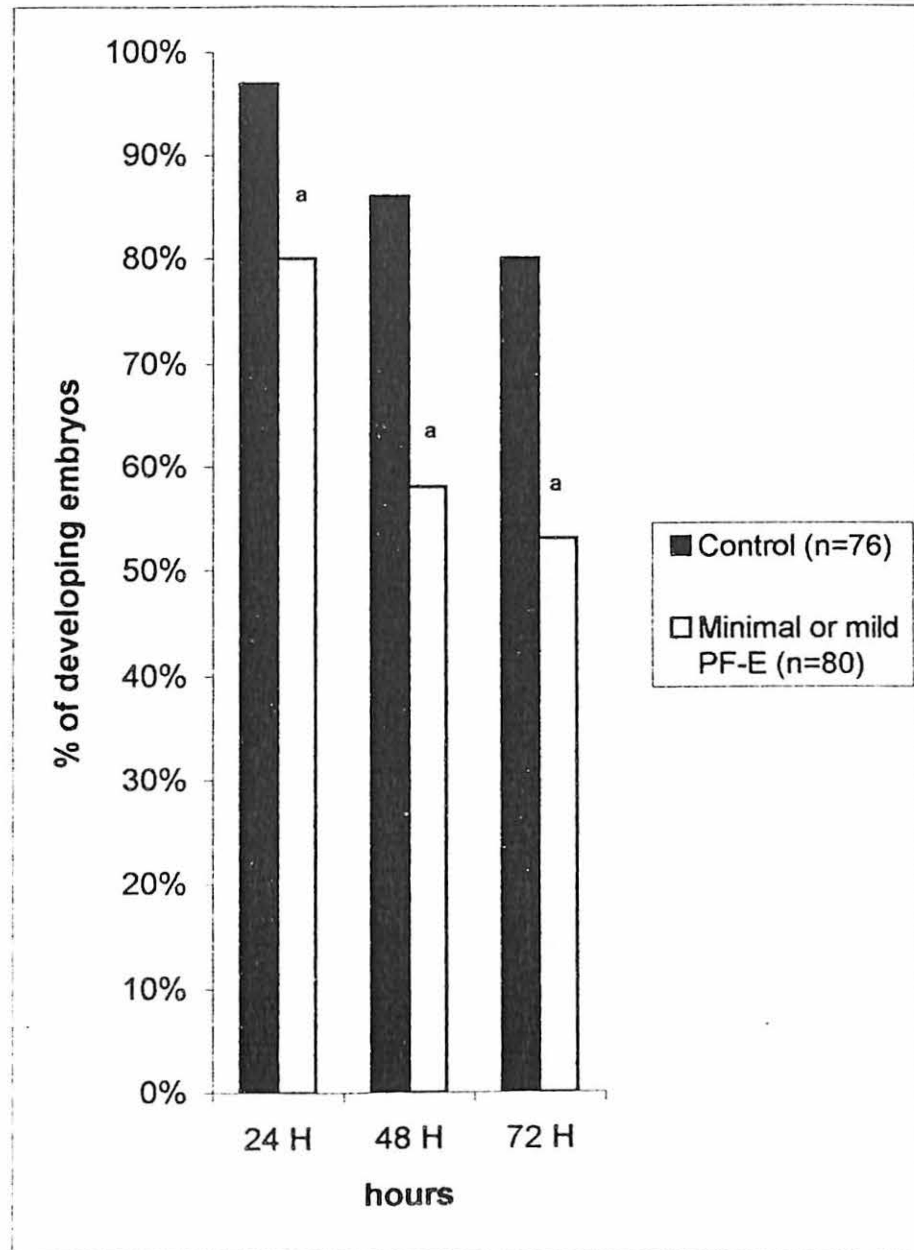


Figure 4.1: Effects of minimal or mild PF-E on 2-cell mouse embryo development. [a] $p<0.001$ versus control. () refers to number of embryos used in the experiment.

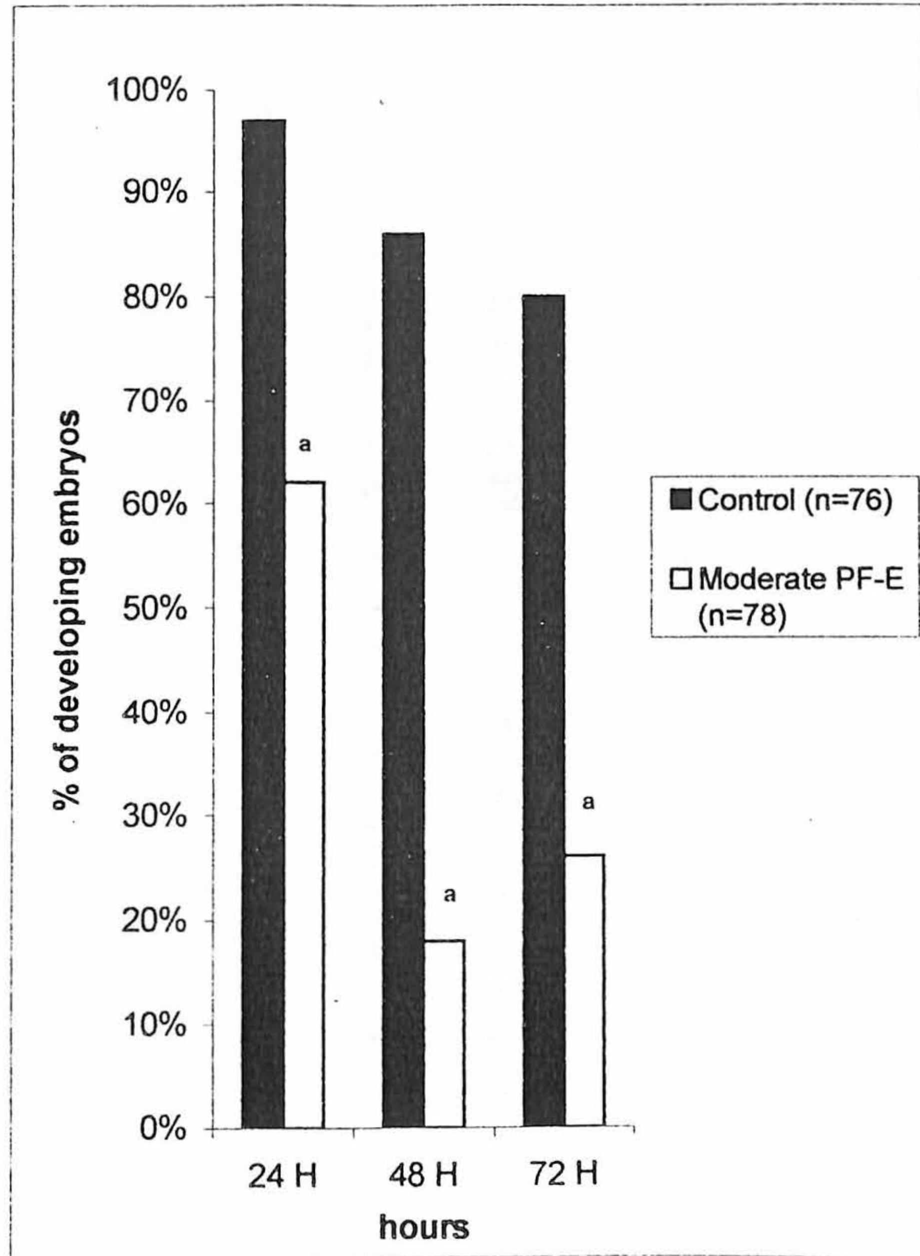


Figure 4.2: Effects of moderate PF-E on 2-cell mouse embryo development. [a]$p<0.001$ versus control. () refers to number of embryos used in the experiment.

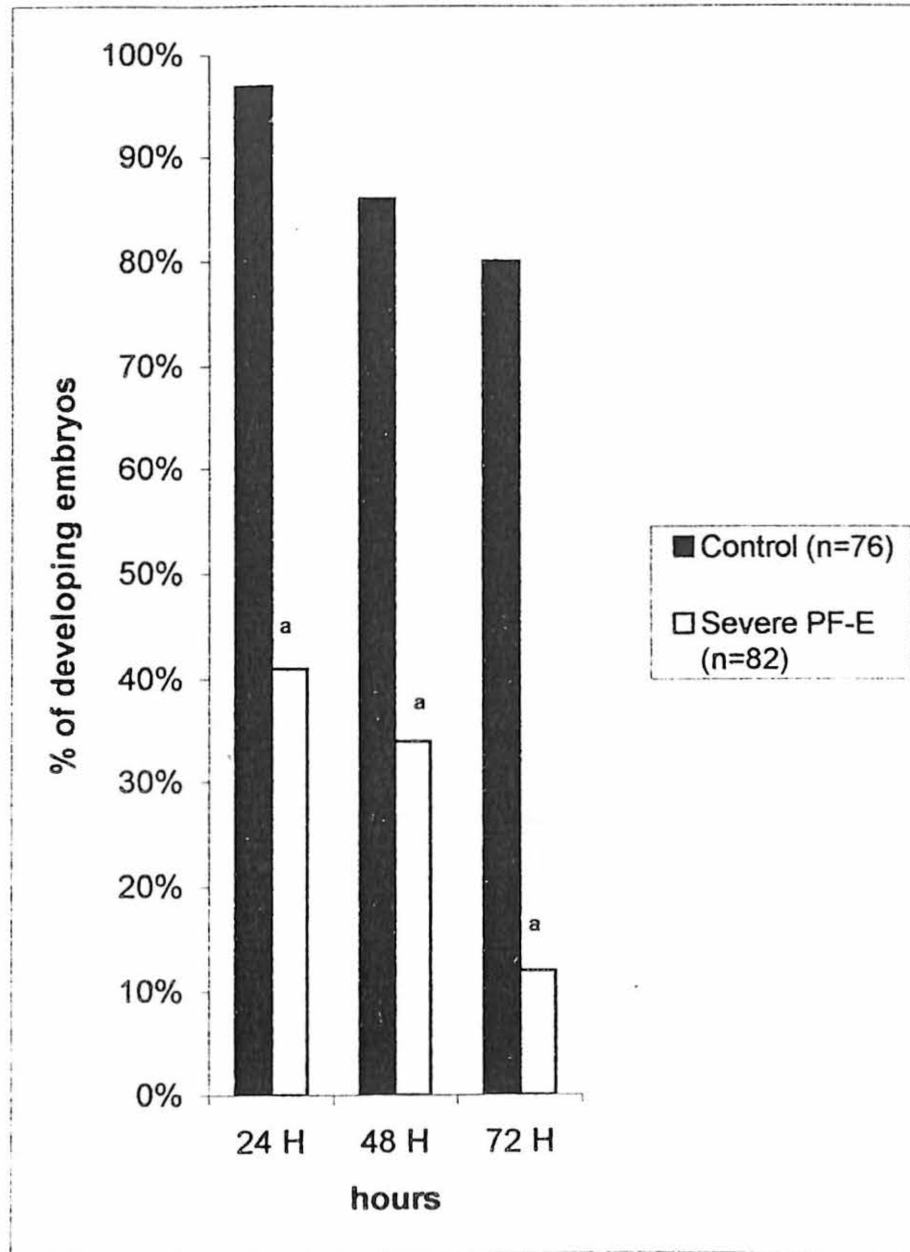


Figure 4.3: Effects of severe PF-E on 2-cell mouse embryo development. [a]$p<0.001$ versus control. () refers to number of embryos used in the experiment.

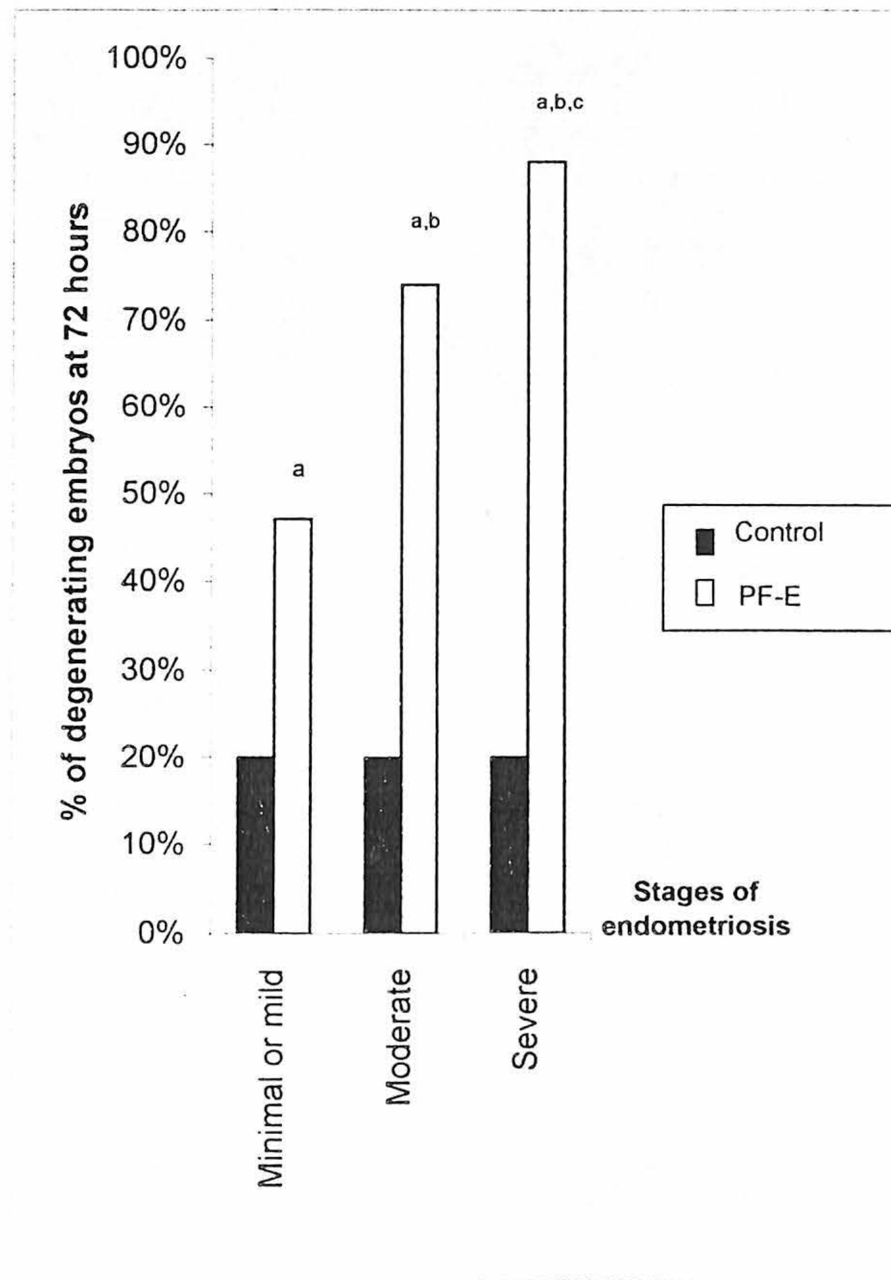
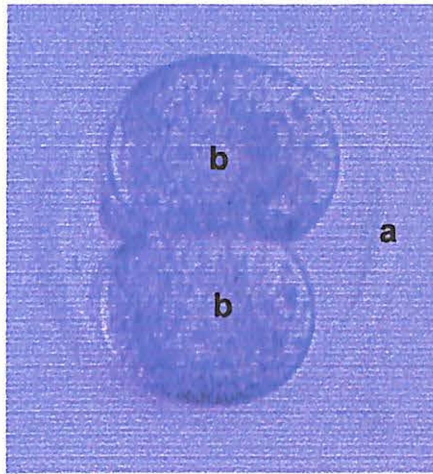
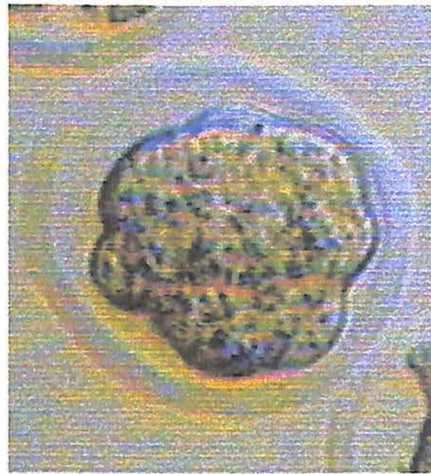


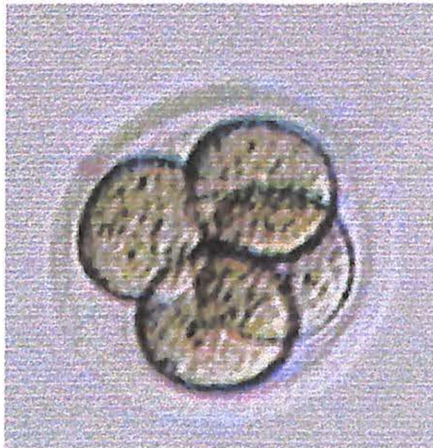
Fig 4.4: Percentage of degenerating embryos at 72 hours in different stages of PFE. a $p<0.001$ vs. control. b $p<0.001$ vs. minimal or mild PF-E. c $p<0.05$ vs. moderate PF-E.



2-cell embryo

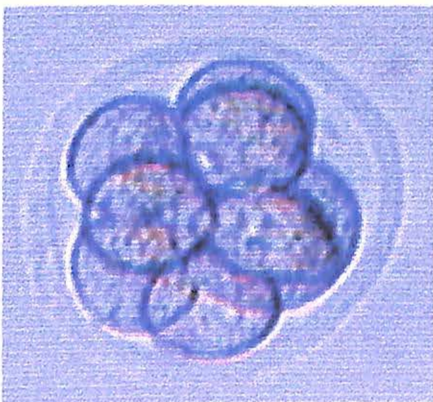


Morula

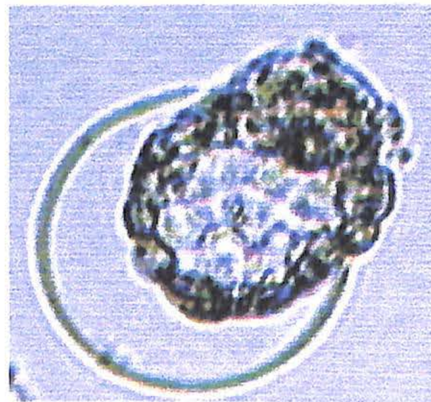


4-cell embryo

Blastocyst

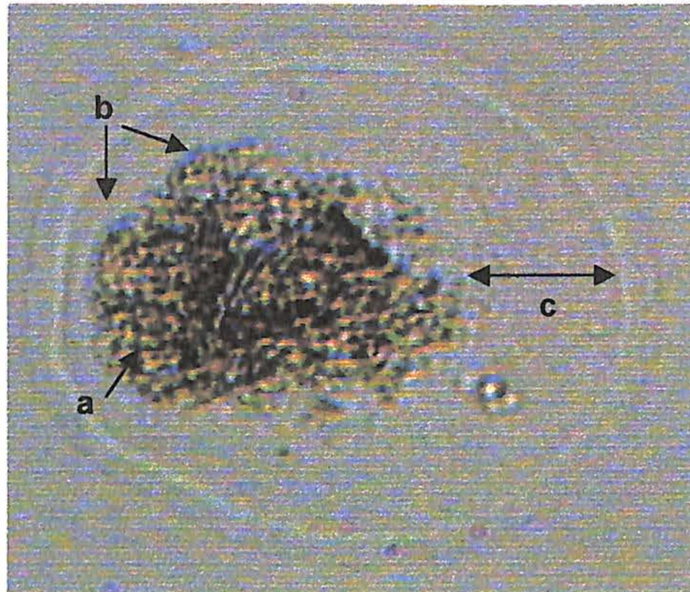


8-cell embryo



Hatching embryo

Figure 4.5: Photographs showing normal mouse embryos.
a: zona pellucida b: blastomere



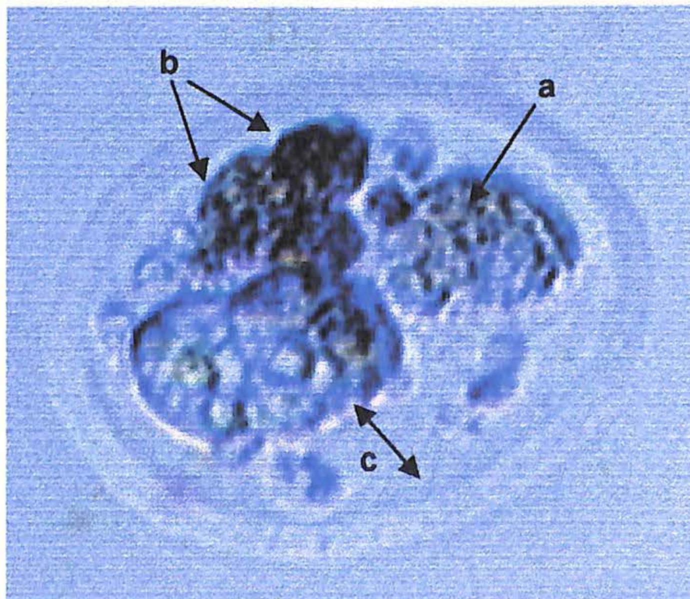
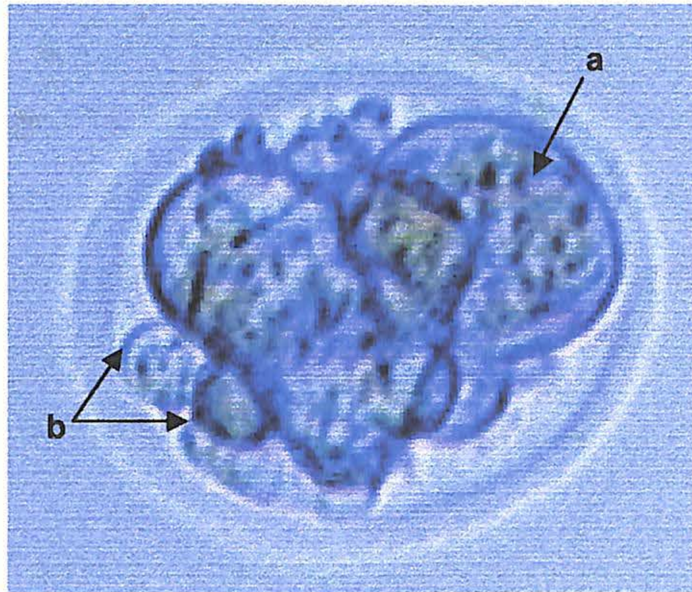


Figure 4.6: Photographs showing degenerated mouse embryos.
a: dark granular cytoplasm
b: fragmentations
c: cell mass retracted from the zona pellucida



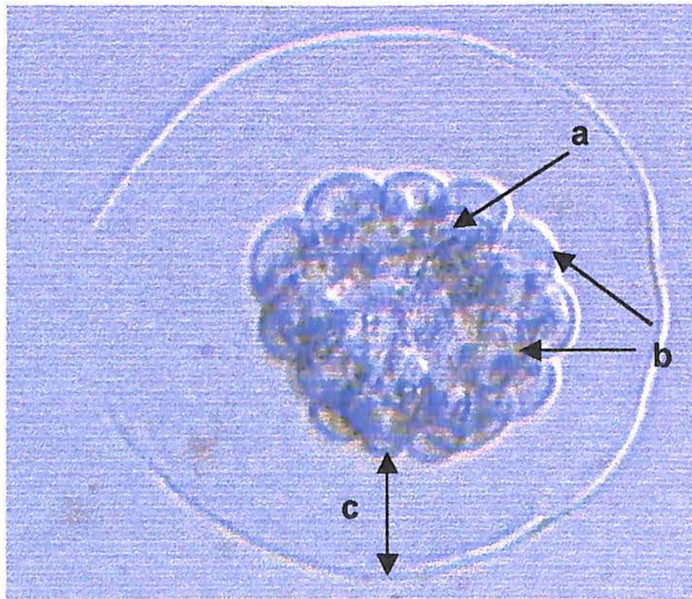


Figure 4.7: Photographs showing degenerated mouse embryos.
a: dark granular cytoplasm
b: fragmentations
c: cell mass retracted from the zona pellucida

4.4 Discussion

Although it is generally agreed that endometriosis is associated with infertility, causality is not well defined. The potential influence of peritoneal fluid or its cellular components as mediators of infertility has only been examined in the last decade. Experimental data seem to indicate that there is a direct embryotoxic effect of peritoneal fluid as one of the underlying causes of infertility. The presence study examines the possibility of embryotoxicity as a mechanism of infertility in women with endometriosis.

Heat-inactivated of PF-E is found to be toxic to early mouse embryos as compared to the control. Embryotoxicity appears to increase with increased severity of endometriosis. By 72 hours, the highest percentage of degenerated embryos is noted in severe PF-E, followed by moderate and minimal or mild PF-E (88%, 74% and 47% respectively). The percentage of degenerated embryos in the control group is 20%. Embryotoxicity is therefore most mark in severe endometriosis, indicating the presence of perhaps more toxic factors with increased severity of endometriosis.

The presence finding supports the hypothesis that embryotoxicity may be a mechanism of infertility in endometriosis. The embryotoxicity as observed is congruent with the findings of several studies (Morcos *et al.*, 1985; Tan *et al.*, 1989; Illera *et al.*, 2000; Gomez-Torres *et al.*, 2002). The relationship with severity of endometriosis however appears to contradict the finding of Dodds *et al.*, 1992 and Awadalla *et al.*, 1997.

Generally, the percentage of degenerated embryos was observed to increase after 24 hours of culture. By 72 hours, the majority of the degenerated embryos that do not reach the blastocyst stage or greater are noted to contain mainly fragments or cell mass retracted from the zona pellucida.

However, it is uncertain from the present study whether the embryos underwent the process of necrosis or apoptosis as a result of peritoneal fluid embryotoxicity. It could be a mix of both processes. Future studies could focus on the ultrastructural changes associated with embryotoxicity to differentiate the mechanism of cell degeneration. According to Betts and King (2000), most of the mechanism in cell death is not restricted to a single mode of death (apoptosis or necrosis); there is some overlap in the initial cellular responses and triggers of each pathway of cell death.

It is acknowledged that there are essential cellular components for cell survival such as the plasma membrane, mitochondria, lysosomes, the nucleus or other cellular organelles that have their own functions (Kanduc *et al.*, 2002). Plasma membrane, mitochondria and the nucleus and its DNA, have been predominant areas of study as 'vulnerable site' whose destruction or malfunction threatens the functioning of the cellular unit.

It can be hypothesized that the embryotoxic factor may disrupt the essential cellular components organization, especially the mitochondria, an essential component for intermediary metabolism and the most sensitive to changes in media composition. Mitochondria are important sites of aerobic respiration,

which produce over 85% of all ATP production (Benos & Balaban, 1983). They have also reported that the major source of ATP in the *in vitro* mammalian pre-implantation embryo is aerobic respiration, which is presumed similar to *in vivo.*

In addition, an intact plasma membrane is also important for embryo survival as it provides both a physical and a selective physiological barrier with the extracellular environment (Overstrom, 1996). This membrane is essential for maintenance of ionic gradient, intracellular pH, solute-specific exchange and co-transport mechanism (Overstrom, 1996). If membrane integrity is compromised probably by the embryotoxic factors, there will be a disruption in the ability to regulate cellular homeostasis such as pH and osmotic pressure that may result in developmental failure (Hansen, 2002).

Nucleus is important for DNA synthesis, which is required for protein synthesis. In conjunction with the activation of the embryonic genome, there are major changes occur in protein synthesis as the embryo growth. These changes are necessary for cell division, compaction, blastocyst formation and hatching.

The presence study confirm the embryotoxicity of PF-E, however the mechanism of actions still remains unclear. The possible mechanisms of embryotoxicity is outline in Figure 4.8. The effects of intermediary metabolism and interleukins will be examined to elucidate further the mechanism of embryotoxicity in endometriosis.

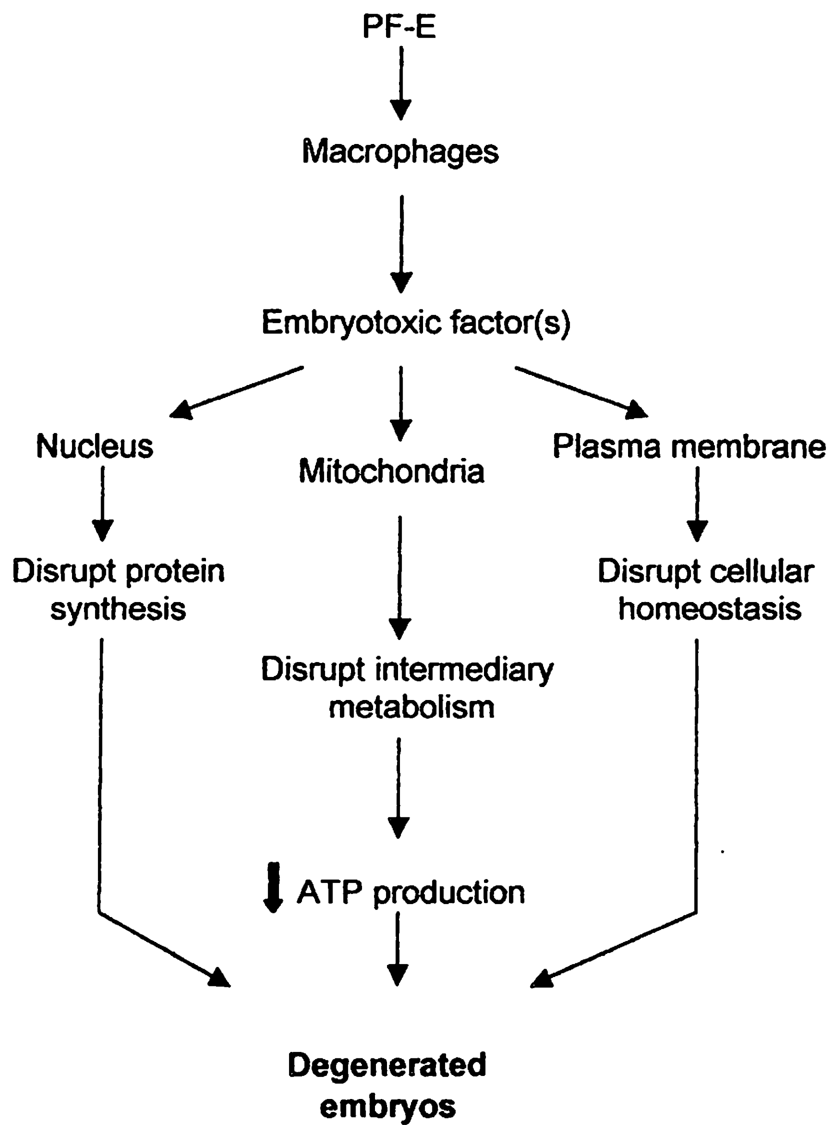


Figure 4.8: Possible mechanisms of embryotoxicity in endometriosis

5. PYRUVATE IN EMBRYO GROWTH AND POTENTIAL SUPPORT AGAINST THE EMBRYOTOXICITY OF PF-E

5.1 Introduction

Carbohydrates and amino acids are the most significant nutrients to the developing human pre-implantation embryo (Devreker & Englert, 2000). The patterns of uptake and utilization of energy substrates such as pyruvate, lactate, glucose and glutamine have been described in pre-implantation embryos of many mammalian species (Overstorm, 1996).

In order to have successful development of embryos in culture, it is important to consider their nutrient requirements. According to Conaghan *et al.*, (1993), the nutrient that supplied in embryo culture media mainly at concentrations that are well above those presence *in vivo*, however, excessive amounts of substrates may be detrimental to embryo health or produce abnormal patterns of development. Nutrient requirements for preimplantation embryo have been studied mainly in experimental animals (Devreker & Englert, 2000).

The development competence of embryos *in vitro* is significantly reduced compared to *in vivo*-produced embryos (Thompson, 2000), however, providing appropriate nutrients in the culture media can enhance the *in vitro* development. In recent years, the focus of studies on nutrient requirement for energy sources in early embryos has been on consumption and utilization of exogenous substrates. However, the contribution of exogenous nutrients to the energy needs is poorly understood. One of the nutrients that have been studied

extensively is pyruvate, an essential substrate for the early preimplantation mouse and human (Hardy *et al.*, 1989).

Pyruvate is a three-carbon (triose) ketoacid, produced at the end stages of glycolysis. It acts as an energy source by being oxidized in the mitochondrion (Butcher *et al.*, 1998). The ATP production from this substrate involved the acid citric cycle and oxidative phosphorylation (Thompson, 2000). Though pyruvate is an essential component of all the media used for human *in vitro* fertilization (Conaghan *et al.*, 1993), little is known regarding its role on early embryo growth and the mechanism by which it enters the embryo. Studies have shown that pyruvate uptake by human embryos has been correlated with embryo viability and the ability to develop to the blastocyst stage (Hardy *et al.*, 1989).

Several authors agreed that human embryo in the early stage (pre-compaction) has an initial preference for pyruvate over glucose as a nutrient (Hardy *et al.*, 1989; Conaghan *et al.*, 1993). As development proceeds, the embryonic metabolism shifts to utilize glucose as the primary source of ATP (Thompson, 2000; Gardner *et al.*, 2002). In the routine culture of preimplantation embryos, a single culture media is used (Sakkas *et al.*, 1993), however, due to a difference in nutrient requirements, it is plausible that optimal development of the mammalian embryo in culture requires the two or more media since culture conditions that support excellent development of the blastocyst, probably detrimental to the zygote (Gardner, 1998). Furthermore, a static environment does not allow for the metabolic and developmental changes as they have during normal development (Sakkas *et al.*, 1993).