# The Long Road to Culture of Embryos in a Safe Culture Medium: Synthetic Protein-Free Culture Medium (1985-2005)

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# Egg/Sperm/Embryo Handling & Culture Media

- Have you ever wondered:
  - what the handling & culture media **contains?**
  - Are its ingredients **safe?**
    - For your patients?
    - For the babies?
    - To yourself and other healthcare workers?
  - Is it defined and remains unaltered with every new batch?

# Disadvantages of the Present-Day Embryo Culture Media

- Contains donor human/animal (in the past) serum proteins
- Or serum replacement products derived from human donors
- Not defined; batch to batch variation is common affecting quality of embryos generated;
- **may be hazardous** Risk of disease transmission by protein-bound agents (viruses/prions)

# Sterilization not 100% efficacious

- **Proteins cannot be sterilized. Stringent purification and sterilization measures cannot eradicate with absolute certainty all unknown/known pathogens** (Truyen et al., 1995).

**Extreme heat treatment may NOT DESTROY PRIONS**

- **Proteins in culture medium - risk of disease transmission. e.g. HIV, Hepatitis, BSE/CJD, etc**

Kemmann, Hum Reprod 13: 1777, 1998

# Donor Proteins in Culture Medium

- Proteins are non-uniform/ non-consistent because each batch is from different groups of donors
- **Regulatory profile**: PFM anticipated to comply with regulations

# Disease Transmission in IVF

- **At least three documented cases but many more; unreported …. Check out Medico-Legal Journals**
- **Transmission of hepatitis - Pooled sera - IVF >200 patients affected** (van Os et al. 1991. Am J. Obstet. Gynecol. 165:152-159)
- **CJD? - A donor of serum proteins for a media manufacturing company in USA died of CJD**
- **Extend of damage not known**
- **CJD take as long as 20 years to manifest.** (Kemmann, 1998, Human Reprod. 13:1777)
- **Medico-legal cases – In medico legal journals numerous cases under pending judicial investigation**

# European Union Tissue & Cell Directive 2004

## EU Directive 2004/23/EC:

Urge member states to move away from use of non-uniform biological supplements (that creates batch variations) in healthcare products

Biologicals create possible contamination risk situations by pathogens, prions, RNA, DNA, other hitherto unknown agents, etc

- Members states must comply by April 2007

A major challenge to researchers & service providers alike.

Time-restricted urgency to seek for alternatives to the use of non-uniform biological supplements in health care products.

# Advantageous to use safe and defined synthetic Protein-Free media

- **MAKES SENSE TO ELIMINATE**
  - Use of potentially hazardous donor serum or serum replacement substances derived from human donors
- **COMPLETELY SYNTHETIC MEDIA**
  - Will remain consistent and therefore there will be no batch to batch variation – there will be no variation in the quality of embryos generated

# The Scenario in the 1980s

# Scenario in the 1980s

- Pregnancy rate was a dismal **9 -15%**
- That means **85-91%** of the patients will not become pregnant after ART treatment
- Naturally most Governments refused support for ART because
  - it was expensive /not cost effective
  - not efficacious and
  - because there were urgent matters such as sanitation and basic healthcare facilities that needed attention compared to infertility.

# Scenario in the 1980s

- **Reasons for the poor ART pregnancy rates** were multi-factorial
- But some of the main reasons were:
  - **Use of sub-optimal** tissue/mouse embryo culture media (not entirely meant for human application)
  - **Use of patient's own serum** in culture media that affected quality of embryos generated
  - **Lack of R&D** to develop embryo culture media specific for human embryos
  - Media was home-made and QC/QA was non-compliant/sub-optimal
- Clearly these sub-optimal conditions have to be overcome if we are going to equal or better the natural fecundity rate of 20%

# Scenario in the 1980s

- We embarked on an extensive and meticulous R&D venture beginning 1985 to
  - Develop Embryo Culture Media (ECM) that is specific for:
    - (i) mammalian embryos
    - (ii) human embryos and thence develop
  - **A synthetic formulation** that is:
    - Completely defined
    - No batch to batch variation
    - Very efficacious
    - Completely safe for human application
    - That complies with healthcare regulations

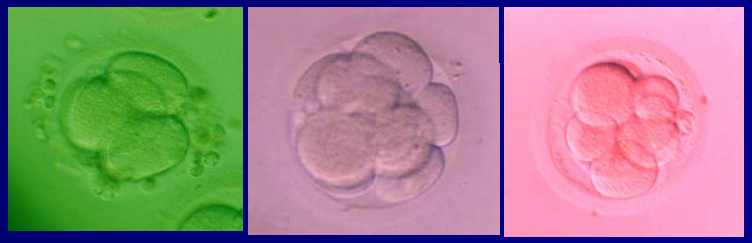
# PFM-11 Synthetic Protein-Free Media Products
# Outcome of 15-20 Years of Research

This research effort resulted in a synthetic ECM that is:

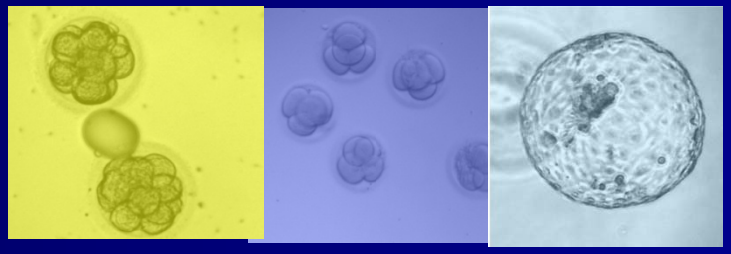
- Specific for human embryos
- Completely defined
- No batch to batch variation
- Very efficacious; better or similar current protein-containing ECM
- Appears very safe for human application
- That apparently complies with healthcare regulations

The subsequent slides will show how the PFM-11 media was formulated

# Development of Synthetic Protein-Free Culture Medium:



Above: Human embryos generated in protein-free medium.
Below: Human embryos generated in protein-containing medium



# Formulation of the Synthetic Protein-Free Medium (PFM-11)

- Two main functions of proteins in culture:

- 1. Physiological attributes
- 2. Physical attributes

# Formulation of the Synthetic Protein-Free Medium (PFM-11)

**Physical attributes in culture:**

- **1. Viscosity to culture medium**
- **2. Surfactant property**
  - These properties allow the handling of embryos with ease and prevent embryos sticking to sides of dishes and in pipettes

# Formulation of the Synthetic Protein-Free Medium (PFM-11)

## Physiological Roles of Proteins

- antioxidant & chelators of metal ions
- prevent membrane peroxidation/stabilize membranes
- source of energy
- capillary membrane permeability
- help solubilize lipids
- osmoregulation
- pH buffer
- transport of $CO_2$/hormones/vitamins/metals
  - serving as reservoirs for storage/release of
  - these components

# Role of proteins in Embryo culture medium: the prevailing wisdom

Is the presence of protein in CM a prerequisite for

- sperm capacitation?
- interaction of male and female gametes?
- fertilization and development of embryos?

Can absence of protein in CM cause ZP hardening?

- impair normal spermatozoa penetration?
- impair fertilization & development of embryos?
- impair normal zona hatching?

# Formulation of the Synthetic Protein-Free Medium (PFM-11)

**It is therefore clear that:**

- **No single substance can substitute serum proteins in culture media.**
- **However many components in appropriate combination/concentration may act synergistically to replace serum proteins**

# Plan of work

**Phase 1:** A systematic investigation to determine optimum concentration of various components normally found in the female reproductive tract and other components was undertaken using mouse zygote assay

[optimal conc. of individual components n=50 determined]

**Phase 2:** Based on these findings a number media were formulated and evaluated with mouse zygotes

**Phase 3:** The best of these media was then evaluated during routine human IVF procedures (medium contained HSA; Approved Trial)

**Phase 4:** Subsequently the protein in the medium was removed to create protein-free media evaluated with mouse embryos

# Plan of work

**Phase 5:** Protein-free medium evaluated with human 1PN and 3PN embryos (Approved Trial)

**Phase 6:** Effect of protein-free medium on survival of the human spermatozoa for 48hrs (Approved trial)

**Phase 7:** The capability of the protein-free medium to support the development of viable human embryos was determined during routine IVF with & without cumulus co-culture (Approved Trial)

**Clin trial:** Human embryos generated in the protein-free medium was performed (Approved Trial)

# Plan of work

## Phase 1, Experiments 1-50

Determination of the Optimal concentration of about 50 components, namely:

- 1. A. Acids
- 2. Anti-Oxidants (known & novel)
- 3. Osmolytes
- 4. Vitamins
- 5. Alternate Energy sources
- 6. Other components

Duration: >3 years

- Basal salt solution +BSA –
- Tested on SO mice zygotes. End point: :blastocyst formation

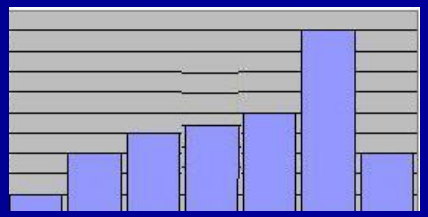
# Results
## Phase 1: Experiments 1- 50

Determination of tolerance and optimal concentration of Individual components

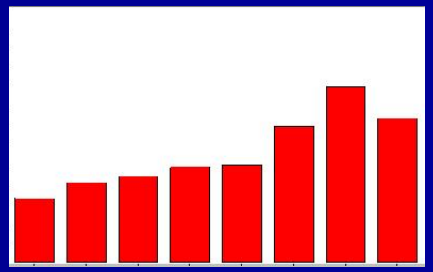
Beyond scope/ time does not permit the presentation of results of experiments

The **optimal concentration** identified and used for formulating new media

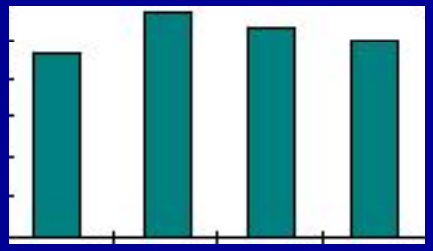
Component 1



Component 26



Component 50



**% Blastocysts**

# Plan of work
## Phase 2

- Based on results of investigations in Phase 1 (Experiments 1-50), many media were formulated.

- Test Media:
  - **TEST-1 TEST-2 TEST-3**
- Evaluated with mouse zygotes and compared with
- Control medium:
  - CM1 (Commercial medium)

# Results

## Phase 2: Experiment 51

Table 1: Development of 1-cell mouse embryos in test and control media

| Media | TEST-1 | TEST-2 | TEST-3 | CM1 | |
|---|---|---|---|---|---|
| %Blast | 97.3 | 94.7 | 98.2 | 84.2 | S |
| %Hatch | 78.8 | 57.0 | 72.6 | 62.2 | S 1 |

Blast=blastocyst formation; Hatch=blastocyst hatching

# Plan of work
# Phase 3

- The best media identified in Phase 2 (TEST Media-1&3) was then evaluated during routine human IVF procedures
- (Test Medium 6 contained HSA instead of BSA)
- Random selection all age groups
- Eggs fertilized/cultured in both test and control media. The best embryos selected irrespective media employed (Approved Trial)

# Results
# Phase 3: Experiment 54

**Table 2: Comparison between TEST-6 and control media on the development of human day 2 embryos**

| Media | No. hEggs | Fertil Rate % | Arrest @ 1-cell % | Blastomere number Mean | Grade Mean |
|---|---|---|---|---|---|
| **TEST-6** | **301** | **69.4** | **4.3S** | **2.9** | **3.3** |
| **CM1** | **370** | **71.6** | **7.3** | **3.1** | **3.0** |
| **CM2** | **374** | **70.1** | **8.8** | **3.2** | **3.0** |

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]
CM=Commercial medium
**All media, test and controls, contained HSA**

# Results
## Phase 4: Experiment 55

**Table 3: Development of late 2-cell mouse embryos when cultured in media supplemented with and without protein**

| Description | No. of 2-cell Mouse embryos | % Blastulation & Hatching/Hatched |
|---|---|---|
| TEST-6 Med (+ protein) | 66 | 95.5 |
| TEST-6 Med (- protein) | 69 | 100.0<br>p=0.2274 |

# Plan of work
## Phase 5

Protein-free medium evaluated with Human Embryos (Approved Trial)

- Preliminary Human Test Material 1PN & 3PN
- Protein-free medium was evaluated with human 1PN and 3PN (abnormal) embryos

# Results

## Phase 5: Experiment 57

Table 4: Evaluation of protein-free medium on 1PN and 3PN human embryos

| Stage of Development | No. of embryos | % cleavage |
|---|---|---|
| Day 1 (1-Cell Stage) | n=24 | - |
| Day 2 (2- to 6-Cell) | n=24 | 100 |
| Day 3 (8-Cell) | n=23 | 95.8 |
| Day 4 (12- to 16-cell) | n=21 | 87.5 |
| Day 5 (compacted morula) | n=7 | 29.2 |
| Day 6/7 (early blastocyst) | n=3 | 12.5 (poor) |
| Day 7 (expanded blastocyst) | n=0 | 0 |

Finding comparable to previous reports on media containing proteins

# Plan of work
# Phase 6

**Effect of protein-free medium on survival of the human spermatozoa for 48hrs**

- To determine response of human spermatozoa to PF medium
- Control media : Commercial medium CM1

# Results
## Phase 6: Experiment 58

**Table 5: Human sperm survival characteristics in control medium CM1 and the protein-free media**

| Medium | 0hr mil/mL Mean | No. | 24hr (% Survival at 37°C) Mean | 48hr Mean |
|---|---|---|---|---|
| PF Medium | 6.6 | 22 | 79.0 | 41.7 |
| Control CM1 | 5.1 | 22 | 50.1 | 26.2 |
| Significance | | | p=0.0113 | p=0.2141 |

(Individual semen specimen was portioned equally for test and control groups; only normal specimen used)

# Plan of work
# Phase 7

- Phase 7.1

- The capability of the protein-free medium to support the development of human embryos was determined during routine ART procedures
- Random selection all age groups
- Eggs fertilized/cultured in both test and control media. The best embryos selected irrespective media employed (Approved Trial)

# Phase 7.2: Quality of day 2 human sibling embryos generated by conventional IVF in protein-free medium

| Medium | Fertil % | Arrested @ 1-cell stage % | Blast number Mean (1SD) | Grade Mean (1SD) | % ≥ 4 cells | % ≥3 Grade |
|---|---|---|---|---|---|---|
| Protein-Free Med ( - protein) | 85.3 (116/136) | 2.2 | 3.4 (1.0) | 3.1 (0.9) | 58.4 | 74.3 |
| Medi-Cult (+ protein) | 79.2 (118/149) | 8.1 | 3.4 (1.0) | 2.7 (0.8) | 56.2 | 58.1 |
| Significance | p=0.235 | p=0.052 | p=0.865 | p=0.001 | p=0.847 | p=0.017 |

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]
Control embryos generated by ICSI or IVF
Blast = blastomere

# Results
## Phase 7.1: Experiment 59

**Table 6: Quality of day 2 human sibling ICSI embryos when cultured in PF and control media**

| Medium | Fertilization Rate % | Blastomere number Mean (SE) | Grade Mean (SE) | No. of cleaved embryos |
|---|---|---|---|---|
| PF Medium | 87.1 (27/31) | 2.7 (0.225) | 3.6 (0.163) | 27 |
| Control CM1 | 74.5 (35/47) | 3.2 (0.215) | 3.2 (0.169) | 34* |
| Signif. | p=0.7785 | p=0.3581 | p=0.2141 | |

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]

- * All zygotes cleaved in PF medium but 1 arrested in Control medium

# Plan of work
# Phase 7

Phase 7.3

- Cumulus co-culture was employed to determine whether the PF was deficient
- If deficient, to further improve the quality of the PF medium to generate quality embryos

# Results
## Phase 7.3: Experiment 60

**Table 7: Quality of day 2 human sib. embryos cultured in Protein-free medium with cumulus co-culture and control media**

| Medium | Fertilization Rate % | % | Arrested @ 1-cell stage Mean | Blastomere no. Mean | Grade |
|---|---|---|---|---|---|
| **PF Med+CC** | **69.9 (51/73)** | | **0** | **3.5** | **3.3** |
| **Cont. CM1** | **55.1 (54/98)** | | **11.1*** | **2.8** | **2.7** |
| Signif. | p=0.0715 | | p=0.0290 | p=0.0358 | p=0.0164 |

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor] *6 arrested at zygote stage

# Plan of work
## Phase 7

### Phase 7.3

- Cumulus co-culture (i) tedious and time consuming, (ii) potential for contamination, and is (iii) not chemically defined
- 16 further modifications were tested to formulate another protein-free medium to eliminate the need for cumulus co-culture

# Results

## Phase 8: Experiment 63

### Table 8:Quality of day 2 human sibling embryos generated by conventional IVF or ICSI in protein-free medium

| Medium | Fertil % | Arrested @ 1-cell stage % | Blast number Mean | Grade Mean | % ≥ 4 cells | % ≥3 Grade |
|---|---|---|---|---|---|---|
| PF medium ( - protein) | 80.4 (320/398) | 2.8 | 3.7 | 3.0 | 65.0 | 68.0 |
| Control CM2 (+ protein) | 73.1 (293/401) | 7.0 | 3.4 | 2.8 | 55.4 | 58.4 |
| Significance | p=0.0178 | p=0.0092 | p=0.001 | p=0.0007 | p=0.022 | p=0.021 |

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor] **Culture technique: ULTRA MICRO-DROPLET, Ali et al., 2000**

# First pregnancies/births from embryos generated in synthetic protein-free medium, 1997/2000

The births of babies generated from the fertilization of eggs collected and inseminated in synthetic PFM using spermatozoa also prepared in the same protein-free medium

(Ali J, Shahata MAM., Al-Natsha SD. Formulation of a protein-free medium for human assisted reproduction. Hum. Reprod. Jan 2000, 15: 145-156).

# Results

## Phase 8: Clinical trial

Table 9: Summary of Embryos Transferred, Implantation, Viability and Delivery Rates of Embryos Generated in Protein-Free Medium

| Description | Protein-free | Control Medium (CM2) |
|---|---|---|
| Total no. of Patients | 114 | 1515 |
| No. of Patients Clin Pregnant (all ages) | 55 (48.2%; S) | 469 (31.0%) |
| No. of Clin..Preg Patients lost to Follow-up: | 5 | 16 |
| No. of Patients That Aborted | 12 (24%; S) | 150 (n=453; 33.1%) |
| No. of Patients That Delivered | 38 | 303 |
| % pregnancies that went to term | 76% (38/50; S) | 66.9% (303/453) |
| Delivery Rate | 35% (38/109; S) | 20.2% (303/1499) |
| Total No. of Embryos Transferred | 358 | |
| Average No. of Embryos Transferred: | 3.1 | ~3.0 |
| Implantation Rate: | 25% (83/358) | |
| No. of Babies Delivered | 53 (2 tripl; 13 tw.; 21 singl.) | |
| Embryo Viability Rate: | 14.8% (53/ 358) | |

# Results
# Phase 8: Clinical trial

Table 6: Summary of clinical pregnancies from day 2 embryos generated in the protein-free medium (Age factor)

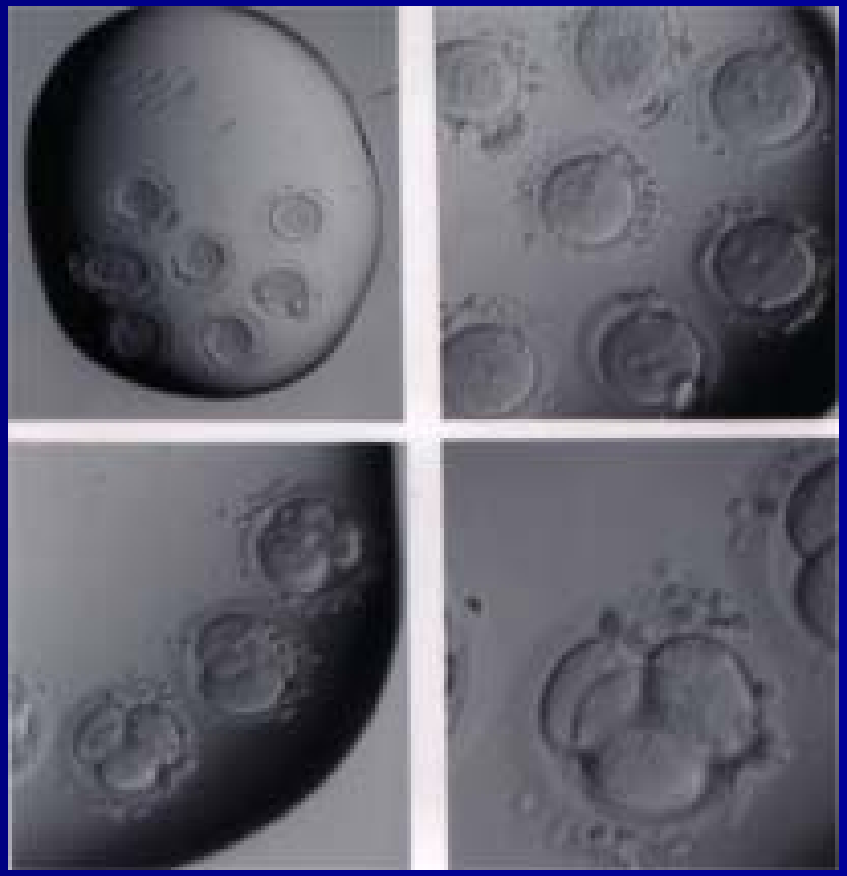
| | |
|---|---|
| CPR in women 39 years and below | 54.7% (52/95) |
| CPR in women 40yrs and above | 15.8% (3/19) |
| CPR Overall (all age groups) | 48.2% (55/114) |

(CPR = Clinical Pregnancy Rate = +ve Sac & FHB)

The protein-free medium appears to be similar to or better than media containing proteins

# Efficacy of the Protein-Free medium Muneera's Babies

- 6 of 7 eggs fertilized by ICSI in PF medium
- 5 of 6 eggs fertilized by IVF in PF medium
- 3 day 2 ICSI (4/5-cell) embryos > ET
- Positive blood test, 3 sacs seen/ +ve FH
- 3 boys delivered, now 9 yrs old



# Independent evaluation of PFM

- The protein-free medium will be/is being independently evaluated in a number of labs worldwide
- Evaluated with excellent outcome for mouse embryos in the USA
- Multicentre IUI clinical trial on PFM currently ongoing in India with excellent outcome – 26% PR

# The PFM-11 Synthetic ECM is UNIQUE

- Unique because a completely different approach was taken in its formulation/development and contains components not found in standard ECM.
- Contains a macromolecule “MetCel” that is very safe. MetCel has been used as a
  - food thickener for over 30yrs,
  - in ocular solutions for 25 yrs or more with no adverse reports.
  - a negative control in cancer research as it is non-teratogenic/non-carcinogenic
  - is an antioxidant
- Contains a unique sugar component “MT”.
  - Saturated MT was/is used for clinical application with no adverse outcome.
  - An antixidant,
  - enhances embryo development by a presently unknown mechanism (antioxid?)

# Previous reports on protein-free embryo culture

- Mouse: Brinster, 1960s, Cholewa and Whitten, 1970, Mehta and Kiessling 1990, Spindle, 1995
- Rabbit: Li et al., 1996
- Primates: Schramm and Bavister, 1996
- Human: Ali, 1997-8,9, 2000, 02a,b, 03, 06 (Abst), Ali et al. 2000,01,04, 06
  - Mohr & Trounson, 1986; Serta Kiessling 1997 (Abst); Parinaud et al. 1999– BUT proteins used for sperm prep/fertilization

# Summary of Overall Results

1. Fertilization rate,
2. quality of **e**mbryos,
3. implantation rate &
4. zona hatching NOT IMPAIRED IN PF Medium

– clinical pregnancy rate

– and live birth rate in PF medium are similar to or better than those generated in media containing serum proteins

# Conclusion

- We now have an efficacious (Regulation-compliant?, GMP-compliant?) protein-free (PFM-11) medium for human ART
- Approval for certification compliance pending with FDA and EU
- The PFM-11 appears to be SAFE.
- Excellent clin. pregnancy rate of 55% in women <39
- Children born from embryos generated in the PFM are now about 9 to 13 years old and apparently normal and healthy
- The PFM-11 is the product of a long, meticulous and systematic investigation and is thus anticipated to meet the expectations of ART workers
- PFM Reproducible/No batch to batch variation
- Potential for transmission of protein-bound pathogens to IVF patients can now be eliminated
- No longer dependent on serum proteins

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- John Curtin School of Medical Research Australian National University
- Others: individuals/institutions
- Investigation took years of
- prime family time.
- My family for their patience
- THANK YOU