**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**FACULDADE DE MEDICINA**

**PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS**

# PLASMA SEMINAL COMO TERAPIA LOCAL EM REPRODUÇÃO ASSISTIDA

RAFAEL MOTA PINHEIRO

Orientador: Prof. Dr. Edison Capp

Porto Alegre, julho de 2007.

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RAFAEL MOTA PINHEIRO

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## LISTA DE ABREVIATURAS

ACP – Análise de componente principal

CAA – célula apresentadora de antígeno

DC – Células dendríticas

ET – Transferência de embrião

FIV – Fertilização *in vitro*

FSH – Hormônio folículo-estimulante

G-CSF - Fator estimulador de colônias de granulócitos

GnRH – Hormônio liberador de gonadotropina

hCG+7 – 7 dias após a administração de 10.000 UI de gonadotropina coriônica humana

HO-1 – Heme-oxigenase-1

ICSI – Injeção intracitoplasmática de espermatozóide

IGF – Fator de crescimento insulina-símile

IGFBP – Proteínas ligantes de IGF

IL – Interleucina

ITF – *Intestinal trefoil factor*

IVI – Instituto Valenciano de Infertilidad (Valência, Espanha)

LIF – *Leukemia Inhibitory Factor*

LH+2 – 2 dias após o pico urinário de hormônio luteinizante

LH+7 – 7 dias após o pico urinário de hormônio luteinizante

OMS – Organização Mundial da Saúde

PBMC – Células mononucleares periféricas

PGE2 – Prostaglandina do tipo E2

Th0 – Linfócitos T-*helper* tipo 0

Th1 – Linfócitos T *helper* tipo 1

Th2 – Linfócitos T-*helper* tipo 2

Th3 – Linfócitos T-*helper* tipo 3

Treg – Linfócitos T regulatórios

TNF – Fator de necrose tumoral

TGF – Fator de crescimento transformador

VEGF – Fator de crescimento endotelial vascular

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**SUMÁRIO**

**RESUMO**

O plasma seminal é uma fração do ejaculado masculino, produzido por contribuição de várias glândulas acessórias masculinas. Deveria ser reconhecido, como ocorre em outras espécies, como agente necessário ao “condicionamento” do endométrio, favorecendo o processo de implantação do embrião. A composição e conseqüente atividade imunossupressora do plasma seminal parece estar relacionados com o favorecimento da viabilidade da gravidez. Nessa tese, em estudo em que foram comparadas amostras de plasma seminal de pacientes considerados férteis frente a amostras de homens considerados inférteis (com oligozoospermia ou astenozoospermia) não foi encontrada diferença estatística entre níveis de diversas citocinas envolvidas no processo implantação. Amostras de plasma seminal dos dois grupos estudados demonstraram ter o mesmo potencial em diminuir a secreção de fator de necrose tumoral (TNF)-alfa e estimular a secreção de interleucina (IL)-6 e IL-8 por células mononucleares periféricas (PBMC), comprovando a atividade imunomoduladora do plasma seminal. A habilidade do sistema imune materno em manter tolerância frente ao feto pode ser reflexo do ambiente criado nos tecidos envolvidos e não somente de citocinas liberadas exclusivamente por células do sistema imune. Em outro estudo aqui apresentado, demonstrou-se que o perfil de expressão gênica de células endometriais epiteliais cultivadas *in vitro* pode ser modulado pela ação de plasma seminal em cultura. Análises de ontologia gênica apontam a regulação negativa de genes envolvidos com a resposta imune e estimulação de agentes envolvidos com a quimiotaxia, podendo estar relacionados com a expansão de linfócitos T reguladores observados durante gestações bem sucedidas. Regulação positiva de 133 genes e resolução negativa de 12 genes endometriais epiteliais foram descritas pela primeira vez nesse trabalho de tese e podem contribuir para a compreensão do processo envolvido na simbiose materno-fetal. Em ensaio clínico apresentado nessa tese envolvendo pacientes submetidas à Fertilização *in vitro* (FIV) ou Injeção intracitoplasmática de espermatozóides (ICSI) voluntárias a participar do estudo piloto duplo-cego, observou-se que pacientes que recebiam plasma seminal intracervical e intravaginal no dia da coleta dos oócitos exibiam maior taxa de gravidez do que a do grupo controle (salina). Ainda permanece pouco esclarecido o exato mecanismo pelo qual o plasma seminal é capaz de levar a aumento relativo de 45% na taxa de gravidez em mulheres que receberam plasma seminal, por vias intracervical e intravaginal, no dia da coleta dos oócitos.

**ABSTRACT**

Seminal plasma (SP) is a fraction of the masculine ejaculate, produced by contribution of some masculine accessory glands. Seminal plasma must be recognized, as it occurs in other species, as a necessary agent for “conditioning” of the endometrium, favoring the embryo implantation process. The SP imunossupressor activity seems to be responsible for the viability of the pregnancy. In this thesis, we compared SP from fertile patients with samples from infertile (oligozoospermia or astenozoospermia) patients. There were no significant differences between levels of several cytokines in the different groups. SP samples of both groups had similar effects in inhibiting TNF-alpha secretion and stimulating the secretion of IL-6 and IL-8 from peripheral mononuclear cells (PBMC), indicating that SP displays imunomoduladory activity. The ability of the maternal immune system in keeping tolerance to the embryo, can be related to the environment created in surrounding tissues and not only to cytokines produced by immune cells. We also show the profile genomic change of epithelial endometrial cells cultivated *in vitro*, under SP stimulation. Gene ontology analysis pointed genes related to negative regulation of genes enrolled in the immune response. SP stimulatory effect, on gene expression in endometrial epithelial cells, notably of chemokines, could be related with the expansion of T reg cells observed during successful gestations. Up-regulation of 133 genes and down-regulation of 12 epithelial endometrial genes might contribute to a better understanding of the process involved in the maternal-fetal symbiosis. In a clinical trial involving patients submitted to the FIV/ICSI and volunteers to participate of a double-blind pilot study. We observed that patients receiving SP by intracervical and intravaginal routes at the time of oocyte retrieval, showed greater pregnancy rates when compared to the control group (saline). The molecular mechanisms underlying the conditioning effect of SP, which could explain the 45% relative increase in pregnancy rate in patients receiving local seminal plasma treatment, remain to be established.

# INTRODUÇÃO

## INFERTILIDADE

Estudos em países em desenvolvimento estimaram que, no ano de 2002, mais de 186 milhões de mulheres em idade reprodutiva (15 a 49 anos), que já foram casadas pelo menos uma vez, eram inférteis devido a infertilidades primária ou secundária (Rutstein, 2004). Em alguns países em desenvolvimento estudados, o número de mulheres inférteis representaria mais de um quarto das mulheres em idade reprodutiva (Rutstein, 2004).

A definição epidemiológica de infertilidade recomendada pela Organização Mundial da Saúde (OMS) se refere à incapacidade da mulher em conceber um filho após exposição ao risco de gravidez por mais de dois anos (Oms, 1975). O termo infertilidade primária é definido como a incapacidade da mulher em dar à luz a um filho, devido à inaptidão de conceber ou de suportar a gravidez até o nascimento de uma criança viva. Infertilidade secundária é considerada como a incapacidade de gerar um recém-nascido vivo após ter concebido um filho anteriormente (Oms, 2001).

A inabilidade de gerar um filho pode se transformar em drama para vários casais, trazendo sentimentos de perda e fracasso. Casais sem filhos podem ser privados de importantes participações em família, tais como aniversários, batizados, confirmações, bar mitzvahs e casamento de seus descendentes gerando sentimentos de exclusão. Entre os fatores associados à infertilidade, dados da Organização Mundial da Saúde destacam o número maior de divórcios e separação de casais inférteis; menor número de uniões em mulheres com esterilidade primária e menos uniões monogâmicas entre pacientes inférteis (Figura 1) (Oms, 2001).

**Divorce and separation by childlessness and sterility status**

Percentage of women who were divorced or separated at the time of the survey, by whether they were childless, primarily sterile, or secondarily sterile, Demographic and Health Surveys 1994-2000

| | Childless (no living children) | | | Primarily sterile (ever-married women) | | | Secondarily sterile[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| Country | No | Yes | Diff. | No | Yes | Diff. | No | Yes | Diff. |
| **Latin America/Caribbean** | | | | | | | | | |
| Brazil | 10.7 | 15.8 | 5.1 | 10.2 | 17.4 | 7.2 | 7.8 | 15.3 | 7.5 |

**Remarriage by childlessness and sterility status**

Percentage of women who have had more than one union, by whether they are childless, primarily sterile, or secondarily sterile, Demographic and Health Surveys, 1994-2000

| | Childless (no living children) | | | Primarily sterile (ever-married women) | | | Secondarily sterile | | |
|---|---|---|---|---|---|---|---|---|---|
| Country | No | Yes | Diff. | No | Yes | Diff. | No | Yes | Diff. |
| **Latin America/Caribbean** | | | | | | | | | |
| Brazil | 15.2 | 16.8 | 1.6 | 15.6 | 11.2 | -4.4 | 12.8 | 16.3 | 3.5 |

**Monogamous first union by childlessness and sterility status**

Percentage of women who are in a monogamous first union, by whether they are childless, primarily sterile, or secondarily sterile, Demographic and Health Surveys 1994-2000

| | Childless (no living children) | | | Primarily sterile (sexually experienced) | | | Secondarily sterile | | |
|---|---|---|---|---|---|---|---|---|---|
| Country | No | Yes | Diff. | No | Yes | Diff. | No | Yes | Diff. |
| **Latin America/Caribbean** | | | | | | | | | |
| Brazil | 70.9 | 69.3 | -1.6 | 71.3 | 67.0 | -4.3 | 73.7 | 66.6 | -7.1 |

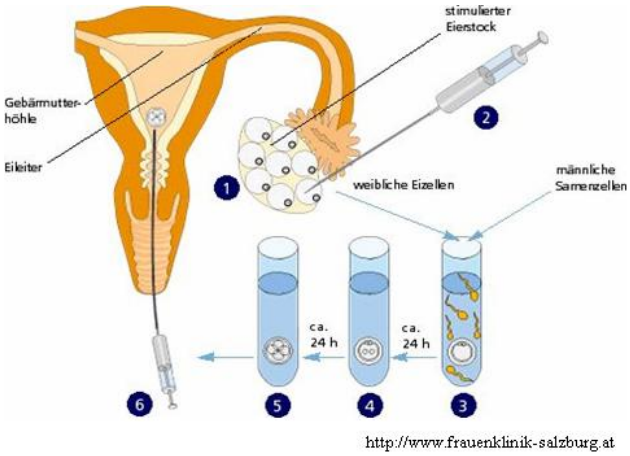
**Figura 1: Conseqüências da infertilidade** segundo relatório "*Infecundity, Infertility and Childlessness in Developing Countries*" (Rutstein, 2004) que inclui entrevistas de 12.612 mulheres no Brasil em 1996. Painel superior: Porcentagem de mulheres que eram divorciadas ou separadas no momento da coleta dos dados divididas em 3 grupos, sendo: (*childless*) as mulheres sem filhos vivos no momento da entrevista, (*primarily sterile*) as mulheres incapazes de dar à luz uma criança viva por não poder gerar ou conceber a gestação até o nascimento e (*secondarily sterile*) as mulheres que tenham tido pelo menos um filho vivo e agora são incapazes de gerar outro, mostrando maior número de divórcio ou separação nos três grupos. Painel central: Porcentagem de mulheres que tinham tido mais do que uma união estável. Ente os três grupos, as mulheres com esterilidade primária tiveram um menor número de uma segunda união. Painel inferior: Porcentagem de mulheres em uma primeira união monogâmica.

## REPRODUÇÃO ASSISTIDA

No mundo, estima-se que existam 35 a 70 milhões de casais inférteis que optaram por técnicas de reprodução assistida para superar a infertilidade (Schultz e Williams, 2002). Mais de um milhão de crianças nasceram após o emprego de técnicas de fertilização *in vitro* (FIV), desde a publicação do trabalho de Steptoe e Edwards (1978) e a tendência é de crescimento. Em vários países, a FIV é responsável por 2 a 4% das crianças nascidas anualmente (Nyboe Andersen e Erb, 2006).

As técnicas de FIV e Injeção Intracitoplasmática de espermatozóides (ICSI) são as duas tecnologias de reprodução assistida mais utilizadas na atualidade (Figura 2).

A




B

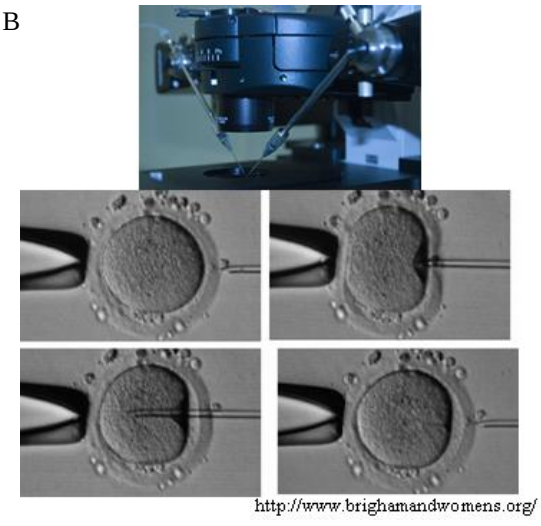



**Figura 2:** Painel (A) Procedimentos para a Fertilização *in vitro* (FIV) (1) oócitos; (2) punção e coleta oócitos; (3) FIV; (4) e (5) cultivo; (6) transferência do embrião. Painel (B) A técnica de Injecão intracitoplasmática de espermatozóides (ICSI) é utilizada em determinadas situações em substituição à FIV.

Apesar de inúmeros avanços na área da reprodução assistida, mais de 70% das transferências de embrião falham em implantar. Andersen e colaboradores (2007) demonstraram, na Europa, taxas de gravidez de 29,6% por transferência de embrião (ET) com o uso de FIV e 28,7% após ICSI (Injeção Intracitoplasmática de Espermatozóide) (Andersen, Goossens *et al.*, 2007). A causa exata da baixa taxa de implantação é desconhecida (Cooke, 2000).

Gravidezes múltiplas são muito mais comuns após FIV (15 a 30%) do que após concepção natural (1,05 a 1,35%) (Guttmacher, 1953; Bollen, Camus *et al.*, 1991). Uns dos fatores que mais influencia a taxa de múltipla gravidez é o número de embriões transferidos. E há preocupação com o fato de um alto índice de múltiplos nascimentos estar diretamente relacionado a maiores riscos de prematuridade, baixo peso ao nascer e morte perinatal em crianças nascidas após FIV.

## IMPLANTAÇÃO DO BLASTOCISTO

A receptividade uterina é definida como um estado em que o endométrio se encontra receptivo à implantação do blastocisto (Psychoyos, 1986). O processo de implantação bem sucedido não depende somente da qualidade do embrião, mas é resultado de uma rede complexa de interação molecular entre o endométrio receptivo e o blastocisto maduro (Simon, Moreno *et al.*, 1998; Norwitz, Schust *et al.*, 2001; Ledee-Bataille, Lapree-Delage *et al.*, 2002) (Figura 3).

A B

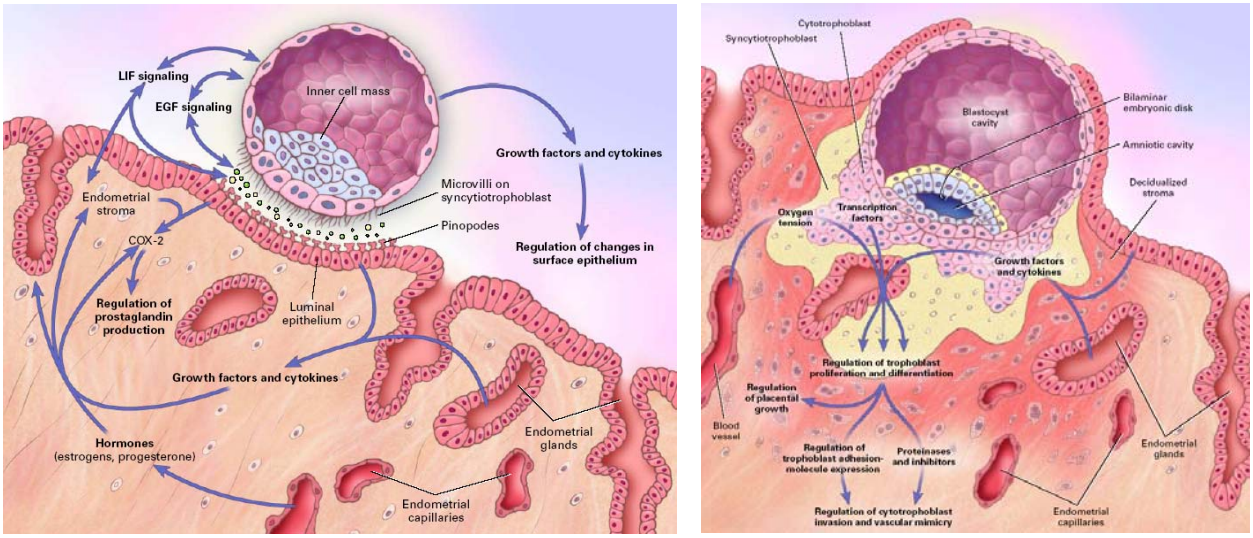



**Figura 3:** Aposição e adesão e implantação do blastocisto. Painel (A) aposição e adesão do blastocisto (aproximadamente 6 a 7 dias após a concepção) em endométrio receptivo; Painel (B) implantação do blastocisto (aproximadamente 9 a 10 dias após a concepção) (Norwitz, Schust *et al.*, 2001)

No começo da década de 90 do século passado, pesquisadores envolvidos no desenvolvimento e uso de técnicas de reprodução assistida identificaram o intervalo entre os dias 20 a 24 como o período ótimo para a implantação do blastocisto, em um ciclo menstrual regular de 28 dias (Bergh e Navot, 1992). Esta fase tem sido denominada de "janela de implantação". Os recentes avanços de técnicas de biologia molecular têm trazido pistas que ajudam a esclarecer o delicado e complexo mecanismo de implantação do blastocisto (Horcajadas, Pellicer *et al.*, 2006).

Alterações hormonais durante o ciclo menstrual normal em mulheres normovulatórias são capazes de controlar a expressão de centenas de genes na transição do endométrio desde a fase proliferativa até as fases secretórias precoce, intermediária e tardia (Figura 4) (Talbi, Hamilton *et al.*, 2006).

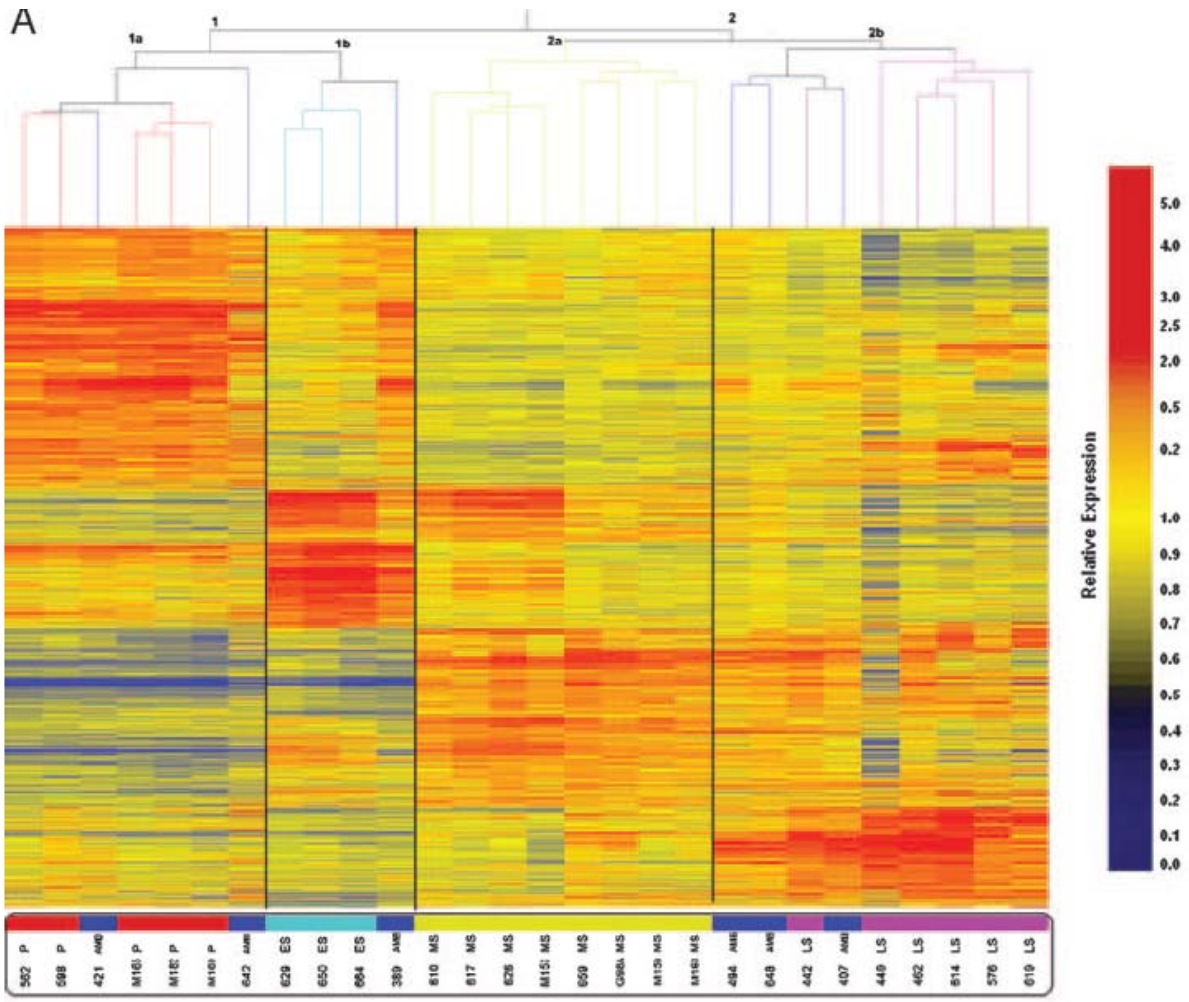



**Figura 4:** Expressão gênica durante as diferentes fases do ciclo menstrual natural - Representação da expressão relativa de genes em biópsias endometriais, em diferentes fases do ciclo menstrual natural (Talbi *et al.*, 2006). Cada linha horizontal representa um gene e cada coluna representa uma única amostra. As amostras foram agrupadas de acordo com a fase do ciclo, sendo P=proliferativa (amostras em vermelho), ES=secretória precoce (em azul claro), MS=secretória intermediária (em verde oliva), LS=secretória tardia (em roxo) e AMB=biópsia ambígua (em azul escuro). A expressão relativa dos genes é demonstrada em uma escala de cores que varia de azul (gene com expressão relativa baixa) a vermelho (gene com expressão relativa alta).

Durante o período correspondente à janela de implantação, um processo dinâmico é observado, com alterações histológicas no endométrio. Ao avaliar a expressão de moléculas em biópsias endometriais coletadas de mulheres no estado pré-receptivo e compará-la com aquela observada em biópsias obtidas de mulheres no período em que o endométrio está receptivo à implantação do blastocisto, várias centenas de genes mostraram-se significativamente alterados (Carson, Lagow *et al.*, 2002; Kao, Tulac *et al.*, 2002; Horcajadas, Riesewijk, Dominguez *et al.*, 2004; Horcajadas, Riesewijk, Martin *et al.*, 2004; Mirkin, Nikas *et al.*, 2004; Horcajadas, Sharkey *et al.*, 2006). A lista de genes candidatos a pertencerem à janela de implantação pode incluir mais de 400 genes, e ainda não existe consenso (Horcajadas, Pellicer *et al.*, 2006).

Os genes com maior regulação positiva em amostras de endométrio receptivo são aqueles envolvidos em transporte de colesterol (apoliproteína E), na síntese e ação de prostaglandinas (PG) (fosfolipase A2 e receptor para PGE2), em síntese de proteoglicanos (glucoroniltransferase), proteínas secretórias (glicodelina, mamaglobina, dickkopf-1), fator de crescimento transformador (TGF)-beta e proteínas ligantes do fator de crescimento insulina-símile (IGF) - IFGBP. Também há alteração em marcadores de transdução de sinal, componentes da matrix extracelular (osteopontina, laminina) e síntese de neurotransmissores (monoamino oxidase), assim como em vários moduladores da resposta imune, genes envolvidos em processos de detoxificação (metalotioneínas) e genes envolvidos no transporte de íons (Carson, Lagow *et al.*, 2002; Kao, Tulac *et al.*, 2002; Horcajadas, Riesewijk, Dominguez *et al.*, 2004; Talbi, Hamilton *et al.*, 2006).

A regulação negativa de alguns genes também parece ser importante na formação do endométrio receptivo. Vários fatores de transcrição, envolvidos na via do

TGF-beta, moduladores da resposta imune e outros genes envolvidos em funções celulares diversas têm a expressão diminuída. O ITF (*intestinal trefoil factor*), matrilisina e alguns membros da via de sinalização de receptores acoplados à proteína G e da via *Wnt* também estão significativamente reprimidos durante a janela de implantação (Kao, Tulac *et al.*, 2002).

## RECEPTIVIDADE ENDOMETRIAL EM CICLOS DE FIV/ICSI

A estimulação ovariana está intimamente relacionada com o desenvolvimento de tecnologias de reprodução assistida (Macklon, Stouffer *et al.*, 2006). O objetivo do protocolo de estimulação é induzir o desenvolvimento de múltiplos folículos dominantes para o amadurecimento de vários oócitos e, com isso, melhorar a probabilidade de concepção, seja *in vivo* (estimulação ovariana com inseminação intra-uterina) ou *in vitro* (estimulação ovariana com coleta cirúrgica dos oócitos e FIV ou ICSI) (Figura 5).

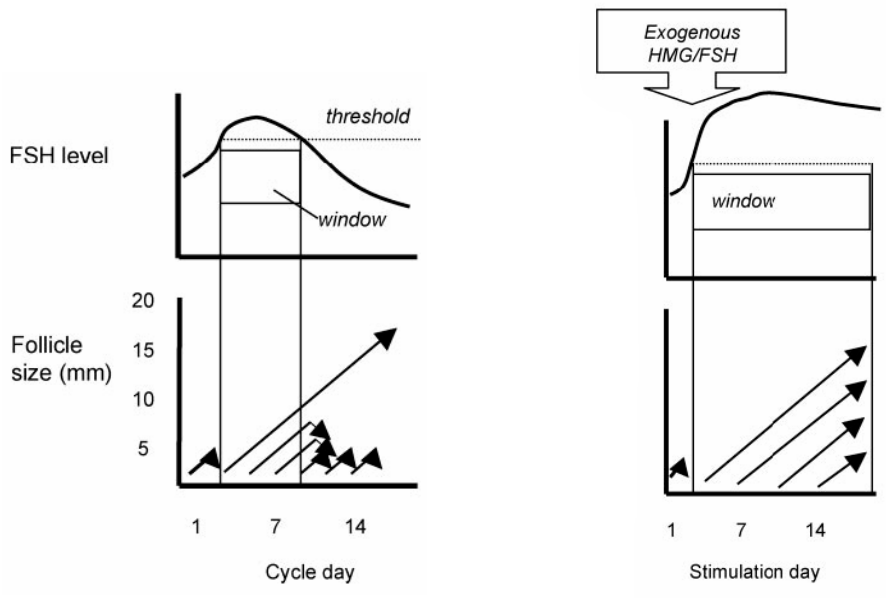



**Figura 5:** Níveis de FSH em ciclos naturais e em ciclos de estimulação ovariana. Limiar de FSH sanguíneo e desenvolvimento folicular em ciclos naturais (à esquerda) e manutenção de altos níveis de FSH (à direita) administrado exogenamente, permitindo desenvolvimento de múltiplos folículos (Macklon, Stouffer *et al.*, 2006)

Somente em 1987, pesquisadores sugeriram o efeito negativo da estimulação ovariana no desenvolvimento do endométrio receptivo (Martel, Frydman *et al.*, 1987). Ao avaliarem, em biópsias de 13 pacientes, a estrutura da superfície do epitélio uterino em ciclos de estimulação ovariana à procura de marcadores da janela de implantação, como protusão apical típica desse período do ciclo (pinopódios), aqueles pesquisadores demonstraram que somente 2 pacientes apresentavam esse último achado histológico.

A exposição do endométrio a níveis suprafisiológicos de estradiol, em reposta ao protocolo de estimulação ovariana, é considerado o principal responsável pelo efeito prejudicial à receptividade endometrial (Macklon e Fauser, 2000). Os níveis de estrogênio elevados podem aumentar a sensibilidade à ação da progesterona e levar ao adiantamento do estágio de desenvolvimento do endométrio, deslocando a janela de implantação (Jacobs, Balasch *et al.*, 1987).

Em 2005, pesquisadores da Fundação IVI (Instituto Valenciano de Infertilidad, Valência/Espanha) publicaram dados de expressão gênica de biópsias endometriais de pacientes do programa de doação de oócitos do IVI-Valência, coletadas durante a janela de implantação. As amostras foram obtidas da mesma paciente, em dois momentos. A primeira amostra foi coletada durante o ciclo natural (em LH+7 = 7 dias após o pico urinário de hormônio luteinizante), e a segunda, no dia hCG+7 (7 dias após a administração de 10.000 UI de gonadotropina coriônica humana) do ciclo estimulado. Ao comparar a expressão gênica de biópsias endometriais coletadas em hCG+7 ou em LH+7 do ciclo natural, a expressão de 558 genes estava alterada, e, por análise de componente principal, o perfil de expressão de hCG+7 foi comparável ao perfil de expressão de biópsias de endométrio não-receptivo (LH+2) (Horcajadas, Riesewijk *et al.*, 2005) (Figura 6).

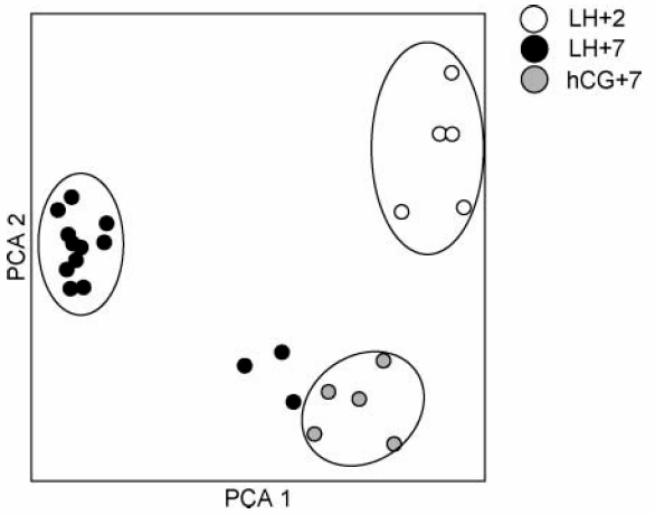



**Figura 6:** Análise de Componente Principal (PCA) de biópsias endometriais. PCA de 24 biópsias endometriais e 500 genes aleatoriamente selecionados usando algoritmo K-means. LH+2=biópsia de endométrio pré-receptivo; LH+7=endométrio receptivo em ciclo natural; hCG+7=dia de transferência do embrião em mulheres submetidas à estimulação ovariana para FIV/ICSI (Horcajadas, Riesewijk *et al.*, 2005).

Os autores espanhóis especulam que o perfil de expressão gênica encontrado em amostras de endométrio coletadas no dia que seria o da transferência do embrião (hCG+7), em pacientes submetidas ao protocolo de estimulação ovariana controlada (protocolo longo, com uso de agonista de hormônio liberador de gonadotropina - GnRH, sem suplementação de progesterona), não é favorável à implantação do blastocisto. Este fato seria o principal responsável pelas baixas taxas de gravidez observadas em ciclos de estimulação ovariana com FIV ou ICSI (Horcajadas, Riesewijk *et al.*, 2005).

## INTERVENÇÕES CLÍNICAS PARA AUMENTAR TAXA DE GRAVIDEZ EM FIV/ICSI

O processo de implantação do blastocisto requer sincronia perfeita no diálogo entre o embrião e o endométrio (Dominguez, Remohi *et al.*, 2002). As taxas de implantação ainda são muito baixas, mesmo quando embriões de alta qualidade (por critérios morfológicos e cromossômicos) são transferidos (Andersen, Goossens *et al.*, 2007). Taxas de insucesso ao redor de 75% após FIV são fonte de frustração, tanto para os pacientes quando para os médicos envolvidos. A transferência de vários embriões,

com o intuito de elevar a taxa de sucesso, ainda é o maior problema causado pela FIV (Fauser, Devroey *et al.*, 2005).

O contexto altamente competitivo em que a FIV normalmente está inserido tem levado, com alguma freqüência, a serem adotadas precocemente novas alternativas para melhorar taxas de sucesso, mesmo antes de se ter comprovado sua segurança e a eficácia (Boomsma e Macklon, 2006).

Tratamentos medicamentosos, adjuvantes ao protocolo de estimulação ovariana usado em ciclos de FIV/ICSI, são frequentemente utilizados de forma empírica, com o intuito de aumentar a taxa de implantação do embrião (Boomsma e Macklon, 2006). No presente momento, ainda não existem evidências que suportem o uso de ácido acetilsalicílico (Gelbaya, Kyrgiou *et al.*, 2007; Khairy, Banerjee *et al.*, 2007), doador de óxido nítrico (Lee, Wu *et al.*, 2004), ácido ascórbico (Griesinger, Franke *et al.*, 2002), glicocorticóide (Boomsma, Keay *et al.*, 2007) ou metformina (Costello, Chapman *et al.*, 2006) para aumentar a taxa de gravidez após FIV.

Em 2003, polêmico trabalho foi publicado por grupo israelense (Barash, Dekel *et al.*, 2003), onde os autores demonstram taxa de gravidez clínica de 66,7% em pacientes que foram submetidas a procedimento de coleta de biópsias endometriais nos dias 8, 12, 21 e 26 do ciclo anterior ao da estimulação ovariana e da transferência do embrião, em comparação com taxa de 30,3% no grupo controle ($P$=0,00009) (Barash, Dekel *et al.*, 2003). Em janeiro de 2007, outro grupo israelense demonstrou taxa de gravidez de 30% em pacientes com história de mais de quatro transferências de embrião que não resultaram em gravidez e foram submetidas à coleta de biópsias endometriais (usando Pipelle® Cornier, França) no ciclo anterior (Raziel, Schachter *et al.*, 2007). Outras formas de trauma ao endométrio também demonstraram efeitos positivos sobre a receptividade endometrial (Humphrey, 1969; Finn e Martin, 1972).

A relação entre a lesão endometrial com a melhora no processo de implantação de embriões fertilizados *in vitro* pode envolver dois mecanismos. O primeiro tem como embasamento resultados de estudos em animais, realizados no início do século passado, em que a indução de lesão local no endométrio induziu decidualização e melhorou a receptividade uterina (Loeb, 1907). O segundo mecanismo que poderia justificar o aumento da taxa de gravidez após lesão endometrial está relacionada ao fato de que seguem-se a essa lesão eventos característicos do processo de cicatrização, como liberação de citocinas e fatores de crescimento. Tais eventos podem exercer efeitos favoráveis à receptividade uterina e, assim, contribuir com a melhora do processo de implantação do blastocisto e aumento das taxas de gravidez encontradas após lesão endometrial (Sharkey, 1998; Simon, Moreno *et al.*, 1998; Li, Chen *et al.*, 2007).

## PLASMA SEMINAL E TRATO REPRODUTOR FEMININO

O plasma seminal é uma fração do ejaculado masculino produzido por contribuição de várias glândulas acessórias masculinas (próstata, vesícula seminal, epidídimos e glândula bulbo-uretral). O plasma seminal deveria ser reconhecido como um agente necessário ao “condicionamento” do endométrio, por contribuir ativamente para o favorecimento do processo de implantação do embrião (Robertson, 2005; Robertson, O'leary *et al.*, 2006; Robertson, 2007).

A função do plasma seminal em favorecer a receptividade endometrial em mamíferos pode ser comparada às funções das glândulas acessórias masculina em insetos (Gillott, 2003). O fluido seminal de *Drosophila melanogaster* mostrou-se responsável por induzir alterações (possivelmente musculares) necessárias no trato reprodutivo da fêmea para favorecer o correto armazenamento dos espermatozóides, que serão utilizados posteriormente na fertilização (Adams e Wolfner, 2007).

O plasma seminal é capaz de modular a expressão gênica de interleucina (IL)1-beta, IL-6 e *Leukemia Inhibitory Factor* (LIF) em células endometriais epiteliais humanas (Gutsche, Von Wolff *et al.*, 2003) e regula positivamente a secreção de IL-8, IL-6, GM-CSF e MCP-1 de células ectocervicais humanas cultivadas *in vitro* (Sharkey, Macpherson et al., 2007).

Alterações induzidas por plasma seminal em tecidos reprodutivos femininos acabam refletindo sobre promoção da sobrevivência dos gametas masculinos, fertilização do oócito, desenvolvimento do embrião e processo de implantação do blastocisto (Figura 9) (Robertson, 2005).

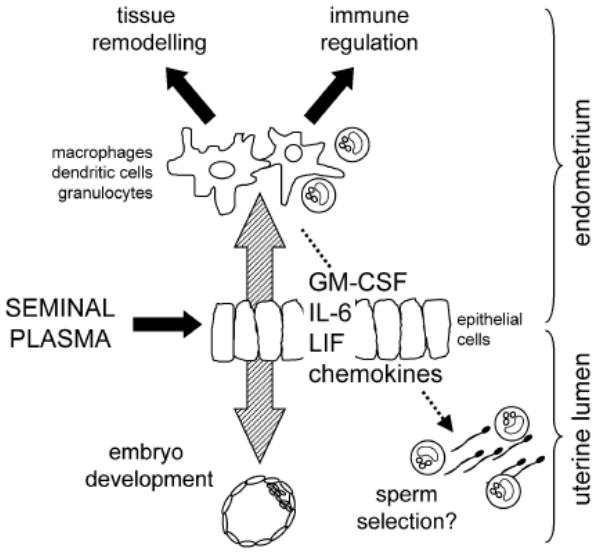



**Figura 9:** Sinalização induzida por plasma seminal no trato reprodutivo feminino, facilitando a implantação do embrião. Citocinas e quemocinas produzidas em resposta a constituintes do plasma seminal levam a recrutamento e ativação de células inflamatórias, controlando a resposta imune na interface materno-fetal (Robertson, 2005).

Evidências clínicas das alterações induzidas por plasma seminal favoráveis ao processo de implantação foram demonstradas em 1986, quando pesquisadores depositaram sêmen na vagina de pacientes submetidas à FIV no dia da fertilização do oócito. Observaram, então, taxa de implantação de 53%, em comparação com taxa de implantação de 23% no grupo controle (Bellinge, Copeland *et al.*, 1986).

Pesquisadores australianos e espanhóis realizaram ensaio clínico em 2000, envolvendo grupos de pacientes incentivados a manter ou abster-se de relações sexuais durante os dias que antecediam a transferência do embrião fertilizado *in vitro* (Tremellen, Valbuena et al., 2000). O estudo não apontou diferenças significativas na taxa de gravidez nos dois grupos. Porém, o grupo incentivado a manter relações sexuais apresentou razão de chances de manter a gravidez até a 8ª semana maior do que o grupo em que não houve contato com o plasma seminal (RC [IC95%] = 1,48 [1,01-2,19]) (Tremellen, Valbuena *et al.*, 2000).

Estudos recentes sustentam a idéia de que o útero e as trompas de Falópio agem como uma bomba peristáltica, aumentando o transporte de espermatozóides para o oviduto ipsilateral ao ovário que apresenta o folículo dominante (Zervomanolakis, Ott *et al.*, 2007). Pesquisadores comprovaram a possibilidade de componentes do plasma seminal entrarem em contato com o epitélio uterino, mostrando que a aplicação de partículas marcadas depositadas na vagina, por meio de peristaltismo, alcançavam partes altas do sistema reprodutor feminino (Wildt, Kissler *et al.*, 1998; Kunz, Beil *et al.*, 2006; Zervomanolakis, Ott *et al.*, 2007).

## CONTROLE IMUNOLÓGICO DO ÚTERO

O trato reprodutor feminino superior (ovidutos, ovários, útero e endocérvice) apresenta similaridades funcionais e estruturais comuns ao sistema imune de mucosas do tipo I, em que a superfície da mucosa é coberta por uma simples camada epitelial (Iwasaki, 2007). O amplo sistema de drenagem linfática, somado à presença de células linfo-hematopoiéticas reguladoras no útero, forma uma importante rede no desencadeamento de resposta imune adaptativa local (Robertson, 2000).

Os diferentes subgrupos de linfócitos T são responsáveis por variações no tipo

da resposta imune, podendo induzir respostas celulares mediadas por anticorpos ou, ainda, proporcionar situação de anergia ou supressão ativa (Piccinni, Scaletti *et al.*, 2000; Robertson, 2000). A capacidade de "direcionamento" da resposta imune é reconhecida como a principal responsável pela proteção imunológica em mucosas e pelo desenvolvimento de tolerância imunológica (Trowsdale e Betz, 2006).

Os linfócitos do tipo Th1 (T *helper*-1) participam de repostas imune celulares, tais como hipersensibilidade tardia e ativação de macrófagos, enquanto os linfócitos Th2 estimulam a resposta humoral por meio de liberação de citocinas que induzem linfócitos B a proliferar, diferenciar e secretar anticorpos reativos ao antígeno exposto. Os linfócitos Th3 e T regulatórios (Treg) exercem potente atividade supressora e são mediadores importantes no desenvolvimento de tolerância ao inibir as respostas do tipo Th1 (Mosmann e Sad, 1996; Zenclussen, 2006).

A proporção relativa de fenótipos alternativos de linfócitos T envolvidos em determinado tipo de resposta imune é resultado da ativação de linfócitos T *naïve* em linfócitos Th1, Th2, Th3 ou Treg, em resposta aos sinais dados por células apresentadoras de antígenos (CAA, macrófagos e células dendríticas) (figura 7) (Robertson, 2000).

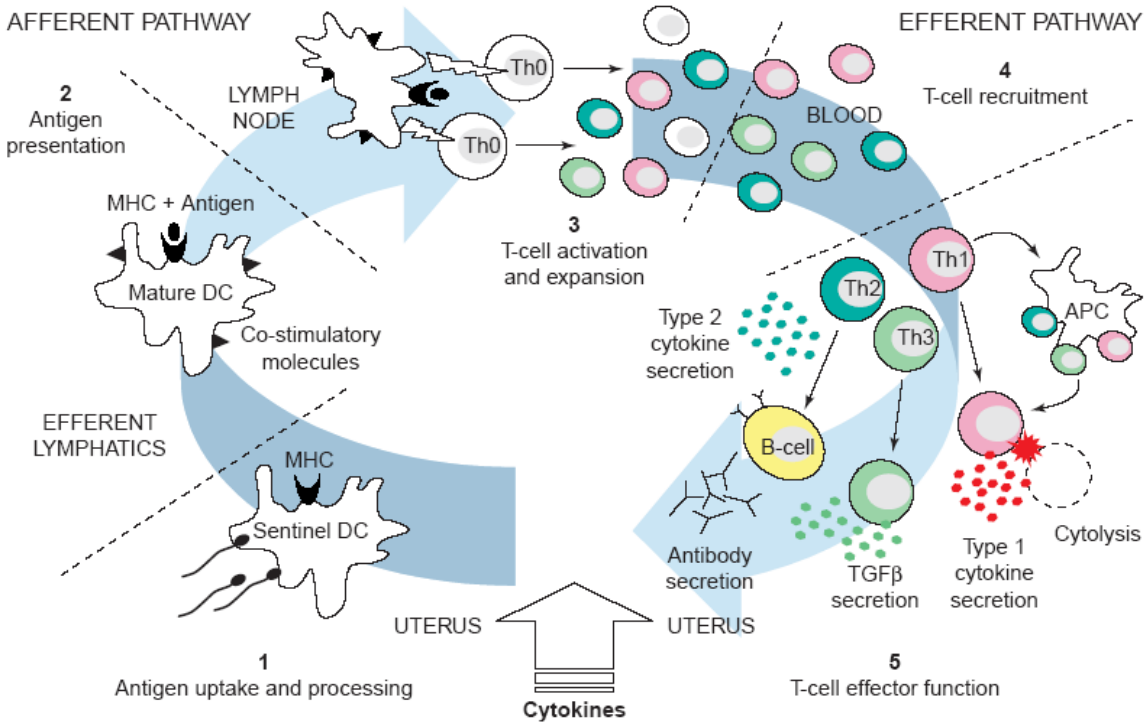



**Figura 7:** Vias aferentes e eferentes da resposta imune. (1) O antígeno é captado por CAA (célula apresentadora de antígeno). A apresentação do antígeno é feita no linfonodo (2) e é seguida por indução da ativação, proliferação e diferenciação de população específica de células efetoras (3), o que desencadeia recrutamento de células T sangüíneas (4) que irão se diferenciar e exercer suas funções efetoras, incluindo a síntese de anticorpos ou citocinas (5). Th0, Th1, Th2, Th3=T-*helper* tipo O, tipo 1, tipo 2 e tipo 3, respectivamente. IL=interleucina, DC=células dendrítica e TGF=fator de crescimento transformador (Robertson, 2000).

Durante a gestação, o sistema imune materno normalmente tolera a presença de alo-antígenos paternos. A idéia de que o sistema imune da mulher tolera o feto semi-alogênico foi apresentada em 1953 pelo brasileiro Sir Peter Medawar (Billingham, Brent *et al.*, 1953). Até hoje, a idéia ainda é considerada a melhor teoria que explica o paradigma da reprodução de mamíferos (Zenclussen, 2006).

Muitos pesquisadores preferem hoje em dia definir a simbiose materno-fetal como um estado de tolerância, em vez de considerar a gravidez como um estado de imunosupressão. Evidências de que fatores locais devam exercer papel importante na esquiva do ataque imune se tornarão mais claras recentemente (Zenclussen, 2006).

O balanço de citocinas Th1/Th2 no endométrio receptivo foi, por muito tempo, apresentado como o principal fator determinante da sobrevivência do feto no útero

materno. O paradigma de que “a alo-gravidez é um fenômeno Th2” hoje é considerado obsoleto (Chaouat, Ledee-Bataille *et al.*, 2004). Estudos demonstraram que o predomínio de linfócitos Th2 na interface materno-fetal não é essencial para o desenvolvimento normal da gravidez e que o controle de linfócitos Th1 pode ser dependente de linfócitos T reguladores (Treg) (Fallon, Jolin *et al.*, 2002; Kingsley, Karim *et al.*, 2002; Chaouat, Ledee-Bataille *et al.*, 2004; Zenclussen, 2006).

A expansão de linfócitos Treg durante a gravidez parece ser importante na supressão de uma resposta agressiva dirigida ao feto, visto que a ausência desse controle leva à falha de gravidez relacionada a ataque imunológico, com rejeição do feto (Aluvihare, Kallikourdis *et al.*, 2004). A habilidade de o sistema imune materno rejeitar enxertos alogênicos, enquanto mantêm tolerância frente ao feto, pode ser reflexo não somente de citocinas liberadas exclusivamente por células do sistema imune, como ser influenciado pelo ambiente criado nos tecidos envolvidos (Chaouat, Ledee-Bataille *et al.*, 2004).

Zenclussen (2006) levanta um cenário hipotético (figura 8), em que a população de linfócitos Treg se expandiria no tecido, tendo migrado por estímulo contínuo de antígenos em condições não-inflamatórias. Ao interagir com outras estruturas do tecido, aquela população criaria um microambiente privilegiado, caracterizado por alta expressão de TGF-beta, Heme-oxigenase-1 (HO-1) e LIF (*Leukemia Inhibitory Factor*).

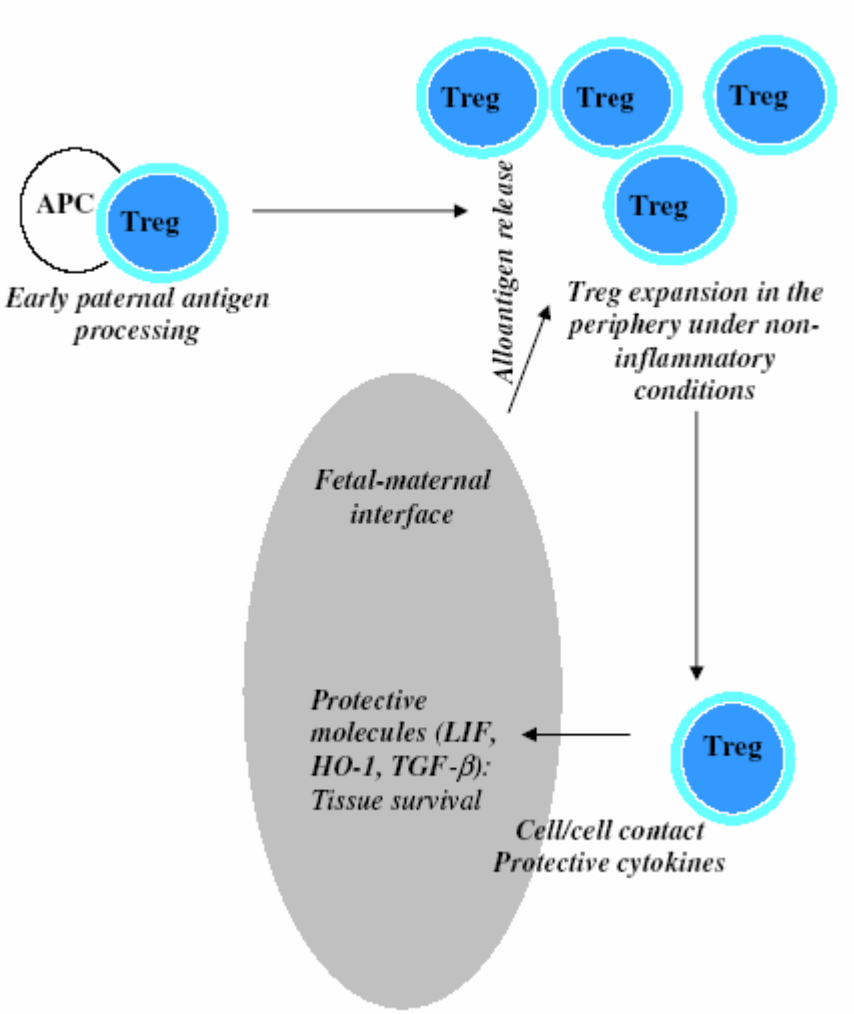



**Figura 8:** Linfócitos Treg em cenário hipotético na interface materno-fetal. Linfócitos Treg são criados após entrar em contato com antígenos paternos, provavelmente no trato genital. Essa subpopulação antígeno-específica se expandiria (provavelmente em linfonodos), em condições não-inflamatórias. Os Treg iriam, então, migrar para a interface materno-fetal, onde estaria envolvidos em um sítio imunológico privilegiado, caracterizado por alta concentração de moléculas protetoras (por exemplo, TGF-beta, LIF e HO-1). A falha em quaisquer dos níveis apresentados resultaria em rejeição fetal (Zenclussen, 2006)

## OBJETIVO

Avaliar o papel modulador do plasma seminal no processo de reprodução.

## OBJETIVOS ESPECÍFICOS

1. Avaliar o efeito imunomodulador do plasma seminal em células mononucleares periféricas (PBMC).

2. Avaliar o efeito modulador do plasma seminal sobre a expressão gênica de células endometriais epiteliais cultivadas *in vitro*.

3. Avaliar a relevância clínica da aplicação tópica de plasma seminal em pacientes submetidas a ciclos de fertilização *in vitro* (FIV) ou ICSI (injeção intracitoplasmática de espermatozóides), por meio da avaliação da taxa de gravidez avaliada por ultra-som na 7ª semana de gestação.

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**ARTIGOS**

**1.** *Seminal plasma – Immunomodulatory potential in men with normal and abnormal sperm count.*

**2.** *Genomic profile change of human endometrial epithelial cells induced by seminal plasma.* (Situação em 25.07.2007: a ser submetido)

**3.** *Intravaginal and intracervical application of seminal plasma in IVF / ICSI -treatment cycles – a double blind placebo-controlled randomised pilot study*. (Situação em 25.07.2007: submetido à *Fertility and Sterility*)

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# Seminal plasma—Immunomodulatory potential in men with normal and abnormal sperm count

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**Abstract**

*Objectives:* Seminal plasma elicits recruitment of immune cells into the cervix. It increases in mice in vivo and in humans in vitro the endometrial epithelial expression of those cytokines and growth factors, which play an essential role in implantation. To analyse if the stimulatory effect of seminal plasma correlates to the quality of the sperm count, the immunomodulatory potential of seminal plasma of fertile and infertile men was studied.

*Study design:* Seminal plasma from 34 volunteers with normal sperm count und from 28 men with oligozoospermia or asthenozoospermia was studied. Firstly, the concentrations of IL-6, IL-8, VEGF, TNFα, IL-1β, TGFβ1 und G-CSF were analysed by ELISA. Secondly, the immunomodulatory potential was studied by bioassays. Bioassays were set-up by isolation of peripheral mononuclear blood cells (PMBC), sensitized by stimulation with LPS. The assays were incubated with seminal plasma of both patient groups and secretion of IL-6, IL-8 and TNFα was analysed by ELISA.

*Results:* IL-6, IL-8, VEGF, TNFα, IL-1β, TGFβ1 and G-CSF were detected in seminal plasma. The bioassays revealed a significant increase of IL-6 and IL-8 and a decrease of TNFα by incubation with seminal plasma. The concentrations of all factors and the stimulatory and inhibitory potential of seminal plasma from men with oligozoospermia, asthenozoospermia and normozoospermia were not significantly different in ELISA- and bioassays.

*Conclusion:* The experiments revealed a similar immunomodulatory potential of seminal plasma from men with normal and abnormal sperm counts, suggesting that male infertility is probably not caused by differences in the activity of seminal plasma.


*Keywords:* Endometrium; Seminal plasma; Infertility; Sperm count

## 1. Introduction

Seminal plasma has conventionally only been considered as a transport and survival medium for mammalian sperm. However, there is increasing evidence that the introduction of seminal plasma into the female tract is related to the pregnancy outcome in humans, rodents and several other mammalian species. This effect is apparently linked to high concentrations of signalling agents such as prostaglandins, cytokines and growth factors [1], secreted by seminal vesicle and prostate glands. These molecules have several functions such as the interaction with epithelial cells in the cervix and inside the uterine cavity [2]. They induce a post-mating inflammatory response in mice [2] and stimulate the infiltration of subepithelial stromal tissues by macrophages, dendritic cells and granulocytes [3].

In humans, semen elicits recruitment of macrophages and lymphocytes into the epithelial layers and deeper stromal tissues of the cervix [4]. The regulation of the cervical leukocyte infiltration occurs by activation of pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) and others [5]. Seminal plasma stimulates the expression of those T-helper-1 (TH-1) cytokines in endometrial epithelial cells [6] which are thought to be relevant in human

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implantation such as leukaemia inhibitory factor (LIF) [7,8] and IL-6 [9], whereas T-helper-2 (TH-2) cytokines, which apparently do not support implantation [10], such as tumor necrosis factor alpha (TNFα), are not stimulated [6]. It was therefore speculated that seminal plasma ascends through the cervix by subendometrial and myometrial peristaltic waves [11,12], where it modulates endometrial receptivity. Other functions of seminal plasma such as activation of female immune response by draining of seminal antigens through lymph nodes [13] clearly demonstrate that seminal plasma must be recognised as a means for communication between male and female reproductive tissue. If, however, seminal plasma plays a role in reproductive function, an impaired biological activity of seminal plasma might contribute to infertility.

Several studies have addressed the concentration of cytokines and growth factors in human seminal plasma in fertile and infertile men [6,14–16]. These studies revealed conflicting results with increased, decreased or similar concentrations of cytokines in sperm of infertile men [15–18]. However, the complex pattern of cytokine concentration in seminal plasma and the partially conflicting results of previous studies demonstrate that the immunological potential of seminal plasma to mediate immunological signalling can hardly be estimated just by analysis of cytokine concentrations in seminal plasma.

We therefore analysed the concentration of a broad spectrum of cytokines in seminal plasma in men with normal and abnormal sperm count and we also set-up for the first time a highly reproducible bioassay involving peripheral mononuclear blood cells (PMBC), to gain additional information on the biological activity of seminal plasma in men with normal and abnormal sperm count to further assess the contribution of seminal plasma to male infertility and reproductive physiology.

## 2. Materials and methods

### 2.1. Patients

A total of 62 male patients attending our infertility clinic were included in the study. Thirty-four patients were classified as nomozoospermic and 28 men as non-normozoospermic. Sperm quality was determined according to the World Health Organization criteria [19]. Ejaculates were obtained at home or in the hospital after at least 5 days of sexual abstinence and were examined within 1 h after masturbation. Institutional Review Board approval was obtained from University of Heidelberg. Males with azoospermia were excluded as patients with obstructive azoospermia might have different composition of seminal plasma. Males with leukocytospermia were excluded as leucocytes increase the concentration of inflammatory cytokines. Oligozoospermia ($\leq$10 mio/ml) or asthenozoospermia (progressive motility $\leq$20%) were defined in this study as infertile whereas normozoospermia ($\geq$50 mio/ml) and progressive motility $\geq$25% were defined as fertile. Definition of oligozoospermia, asthenozoospermia and normozoospermia was defined on the basis of two sperm counts $\geq$12 weeks apart. Medium age of the fertile men was 36.8 years and of the infertile men was 37.3 years.

To set-up the bioassay-system, seminal plasma was collected from five healthy nomozoospermic controls, not attending the infertility clinic. Samples were mixed and stored at −80 °C to validate the culture system with the aliquots of the same mixed sample.

### 2.2. Bioassay

Cell culture of PBMCs was performed as described in detail elsewhere [9]. PMBCs were chosen for the following reasons: firstly, PBMCs have been proven to be a reproducible model to analyse paracrine interactions of endometrial cells [9], secondly, PBMCs produce several cytokines and can be found at high concentrations in the mid- and late secretory phases of the endometrium [21,22] and thirdly, the availability of these cells allowed us to set-up reproducible bioassays.

PBMCs were isolated from buffy coat from healthy blood donors by Ficoll-Hypaque density gradient centrifugation (Vacutainer CPT Cell Preparation Tube; Becton Dickinson, Franklin Lakes, USA). To perform all experiments with PBMCs from one individual donor, PBMCs were cryopreserved in RPMI-1640 medium without phenol red, inactivated fetal calf serum (FCS) (40%) and dimethyl sulfoxide (DMSO) (15%) (all from Sigma, Deisenhofen, Germany) in liquid nitrogen. Cells were seeded at a density of 250,000 per well into 0.4 μm culture plate inserts (Millipore, Bedford, USA), which were placed in 24-well tissue culture plates (Falcon, Oxnard, USA).

Cells were cultured for 1 day and then stimulated with lipopolysaccharides (LPS) for 24 h to initiate differentiation of monocytes to macrophages and to sensitize the cells to stimulation by seminal plasma. Characterization of cells after 3 days of culture had been performed previously by flow cytometric analysis [9] and had revealed an average of 20% monocytes/macrophages, 10% CD4-positive T lymphocytes, 25% CD8-positive T lymphocytes, 5% CD19-positive B lymphocytes and 5% CD16 +CD56-positive natural killer cells.

### 2.3. Validation of bioassay

PMBCs were stimulated with 0, 100 or 1000 ng/ml of LPS for 24 h. Cells were then stimulated with 10% seminal plasma for 24 h and concentrations of IL-6, IL-8 and TNFα were determined in culture supernatants by ELISA. These experiments were performed to define the conditions at which the cells were most susceptible to stimulation by seminal plasma. Samples of seminal plasma were not mixed. Afterwards PMBCs were stimulated with an optimized

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concentration of LPS (100 ng/ml) and were then incubated after 2 days of culture with 0% or 10% seminal plasma from each patient. Concentrations of IL-6, IL-8 and TNFα were analysed in culture supernatant after 24 h and increase or decrease of cytokine concentrations were determined. These experiments were performed to define the sensitivity of the bioassays and the functional viability of the cells.

Then PMBCs were cultured in different culture plates after stimulation with LPS (100 ng/ml) and 10% seminal plasma and concentrations of IL-6, IL-8 and TNFα were measured in different ELISA plates. These experiments were performed to define the fluctuation margin of IL-6, IL-8 and TNFα secretion in different bioassays (Interassay variation of the bioassays).

### 2.4. ELISA

Concentrations of IL-6, IL-8, vascular endothelial growth factor (VEGF), TNFα, IL-1β, transforming growth factor beta1 (TGFβ1) und granulocyte-colony stimulating factor (G-CSF) were determined in duplets using commercially available ELISA-Kits (R&D-Systems, Minneapolis, MN, USA). The ELISAs were performed according to the instructions of the manufacturer. In those ELISAs, which were used to determine IL-6, IL-8 and TNFα concentrations in the supernatants of the bioassays, we performed additional experiments to analyse the intra- and interassay variation in order to exclude a high degree of variations caused by the cell culture medium. The intra- and interassay variations were 0.3% and 13.3% (IL-6), 3.8% and 18.0% (IL-8) and 1.1% and 11.9% (TNFα).

Wilcoxon ranked sign test was used for statistical evaluation. Significance was assumed at the $p < 0.01$ and the $p < 0.05$ level.

## 3. Results

### 3.1. Validation of bioassay

Concentrations of IL-6, IL-8 and TNFα increased with increasing concentrations of LPS: after pre-treatment with 100 ng/ml LPS cytokine concentrations increased 4.7-fold (IL6), 2.2-fold (IL-8) and 7.8-fold (TNFα) in comparison to unstimulated controls. After stimulation with 1000 ng/ml LPS cytokine concentrations increased 4.4-fold (IL-6), 2.4-fold (IL-8) and 9.8-fold (TNFα) in comparison to unstimulated controls.

As cytokine concentrations increased significantly ($p < 0.05$) at 100 ng/ml and reached a plateau at 1000 ng/ml LPS, we decided to pre-treat the PMBCs with 100 ng/ml LPS for 24 h.

PMBCs were pre-treated with 100 ng/ml LPS for 24 h and were then incubated after 3 days of culture with 0% and 10% seminal plasma. Concentrations in culture supernatants increased significantly ($p < 0.01$) by 94% (IL-6) and 136% (IL-8) or decreased significantly ($p < 0.01$) by 58% (TNFα) (Fig. 1). The analysis of cytokine secretion by PMBCs after stimulation with seminal plasma demonstrated the sensitivity of the bioassay to react to different seminal plasma constituents and the differences in the seminal plasma mediated secretion pattern of TH-1 (IL-6) and TH-2 (TNFα) cytokines.

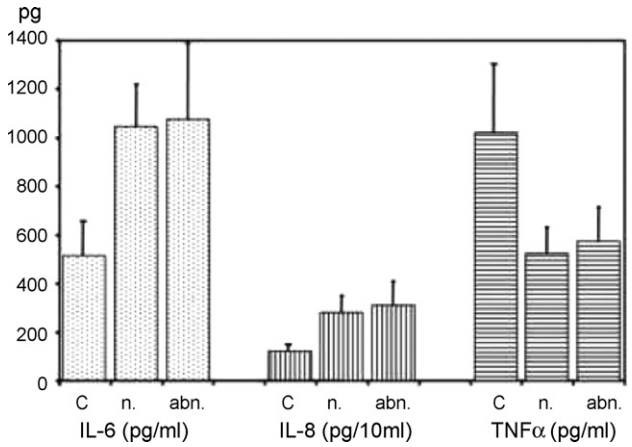


Fig. 1. Secretion of IL-6, IL-8 and TNFα by sensitized peripheral mononuclear blood cells (PMBC), stimulated with 10% seminal plasma from men with normal sperm count (n. = normozoospermia (≥50 mio/ml) and progressive motility ≥25%) or from men with abnormal sperm count (abn. = Oligozoospermia (≤10 mio/ml)) or asthenozoospermia (progressive motility ≤20%) (c = control: cytokine concentration by PMBCs without seminal plasma stimulation).

Interassay variation of the bioassay was analysed by stimulation of different culture plates of LPS pre-treated PMBCs with seminal plasma and analysis of culture supernatants in different ELISA plates. The interassay variations were 22% (IL-6), 17% (IL-8) and 36% (TNFα).

### 3.2. Cytokine and growth factor concentration in seminal plasma

ELISA analysis revealed a wide range of cytokine and growth factor concentrations (Table 1). Concentrations within the range of normal serum levels were found for IL-6, TNFα, IL-1β and G-CSF. Concentrations of TGFβ1 (10×), IL-8 (50×) and VEGF (100×) were much higher in comparison to serum. The concentrations of cytokines and growth factors were similar in nomozoospermic and non-normozoospermic men without significant differences.

### 3.3. Immunomodulatory potential of seminal plasma

PMBCs were pre-treated with 100 ng/ml LPS for 24 h and were then incubated after 2 days of culture with 10% seminal plasma from men with normal and abnormal sperm count. Analysis of cytokine concentrations did not reveal significant differences of IL-6, IL-8 and TNFα (Fig. 1). These results demonstrate, that the analysed complex immunological potential of seminal plasma to induce

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Table 1
Concentration of cytokines and growth factors (pg/ml) in seminal plasma in men with normal and abnormal sperm count

| | IL6 | IL8 | VEGF | TNFα | IL1β | TGFβ1 | G-CSF |
|---|---|---|---|---|---|---|---|
| Normal sperm count[a] ($n = 34$) | | | | | | | |
| Median | 18 | 1725 | 489,450 | 2.9 | 2.2 | 404 | 66 |
| Mean | 35 | 2005 | 523,935 | 5.5 | 2.8 | 554 | 82 |
| STD | 54 | 1465 | 248,376 | 5.8 | 2.4 | 446 | 55 |
| Abnormal sperm count[b] ($n = 28$) | | | | | | | |
| Median | 12 | 1460 | 450,500 | 1.0 | 1.2 | 450 | 44 |
| Mean | 25 | 2058 | 474,634 | 5.6 | 1.5 | 534 | 50 |
| STD | 33 | 1648 | 21,682 | 8.1 | 1.7 | 356 | 24 |

[a] Normozoospermia (≥50 mio/ml) and progressive motility ≥25%.
[b] Oligozoospermia (≤10 mio/ml) or asthenozoospermia (progressive motility ≤20%).

cytokine secretion by PMBCs in vitro is similar in nomozoospermic and non-normozoospermic men.

### 3.4. Comment

Seminal plasma has been recognised as a means for communication between male and female reproductive tissue. Recent studies in rodents and humans have shown that the introduction of semen into the female tract mediates molecular and cellular changes that facilitate conception and pregnancy [1].

In the endometrium of mammals [2], pigs [23] and possibly in humans [6], seminal plasma interacts with uterine epithelial cells and induces a post-mating inflammatory cascade [2].

Whereas the post-mating endometrial inflammatory response is still speculative in humans, is has clearly been shown that semen elicits recruitment of macrophages and lymphocytes into the epithelial layers and deeper stromal tissues of the human cervix [4]. This effect apparently contributes to the clearance of superfluous sperm and microorganisms introduced into the vagina. The regulation of the cervical leukocyte infiltration occurs by activation of the pro-inflammatory cytokines such as IL-6, IL-8 and others [5].

Seminal plasma has not only local effects, it also modifies organs far distal to the reproductive tract. In mice the female response to seminal antigens is characterized by hypertrophy of lymph nodes draining the uterus and by lymphocyte activation [13,24]. It has been speculated that seminal plasma induces a male antigen-specific tolerance. Female mice exposed to seminal plasma show hyporesponsiveness to cell mediated Type-1 immunity to male MHC antigens [25]. The modulation of the maternal immune system might be necessary to accept paternal antigens which are found both in the conceptus and in semen [26].

The relevance of seminal plasma for reproduction in humans has been supported by several studies, which have demonstrated improved live birth rates in couples undergoing IVF-treatment when women engage in intercourse around the time of embryo transfer [27,28].

The broad spectrum of possible functions of seminal plasma on human reproduction has led to several studies analysing the concentration of cytokines and growth factors in seminal plasma of fertile and infertile men. Huleihel et al., 1996 [15] and Dousset et al., 1997 [17] found similar concentrations of IL-6 in fertile and infertile men whereas Eggert-Kruse et al., 2001 [18] described higher concentrations of IL-6 in infertile men. Concentrations of IL-1β were similar in both patient groups according to Huleihel et al., 1996 [15] whereas Dousset et al., 1997 [17] described higher concentrations in infertile men. TNFα was studied by Huleihel et al., 1996 [15] who found similar concentrations in fertile and infertile men. Concentrations of IL-8 were higher in infertile men [18]. All papers described differences of cytokine concentrations in men with leukocytospermia and genital tract infections. Genital tract infections led to increased concentrations of IL-6, IL-8 and IL-1-receptor antagonist and decreased concentrations of IL-10 and TNF-I receptor. As these studies revealed a general adverse effect of genital tract infection on cytokine concentrations in seminal plasma, we excluded men with leukocytospermia.

As previous studies revealed contradictory results, possibly as seminal plasma contains many different interacting mediators which cannot only be analysed by ELISA, the analysis of the functional capacity of seminal plasma requires a bioassay. A bioassay subsumes the function of several mediators and therefore allows us to perform a functional study of the immunological potential of seminal plasma in nomozoospermic and non-normozoospermic men.

We used PMBCs in our bioassay, since the post-mating inflammatory cascade in the human cervix and in mice and pigs involves mononuclear cells such as macrophages and granulocytes and the availability of these cells allowed us to set-up a reproducible bioassay system.

Interestingly, seminal plasma induced an increase of IL-6 and IL-8 secretion by PMBCs whereas the secretion of TNFα was reduced. These results support previous findings of our group [6], which described an increase of mRNA-expression of IL6 in endometrial epithelial cells by stimulation with 10% seminal plasma whereas TNFα-expression was not increased. IL-6 belongs to the group of TH-1 cytokines, which have been postulated to support endometrial implantation [10,22,29] whereas TNFα belong to the group of TH-2 cytokines, which have been described to have an adverse effect on endometrial function and implantation at high concentrations. It can therefore be speculated that seminal plasma exerts different effects on immune cells and thereby induces sophisticated cellular immune responses.

Our combined study of cytokine concentration in seminal plasma using ELISAs and bioassays revealed no differences of cytokine and growth factor concentrations

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and demonstrated the same immunomodulatory potential of seminal plasma of nomozoospermic and non-normozoospermic men.

The authors are fully aware, that the bioassay with PMBCs, even after careful validation as performed in this study, does not exactly copy the physiological conditions and therefore can not definitely proof the identical immunological properties of seminal plasma from men with normal and abnormal sperm count. However, as the culture model is very sensitive and as the model uses cells which can also be found in the cervix and the endometrium, it can be assumed, that first significant differences in the immunological properties of seminal plasma would have been picked up and second that the results of this study can be carefully transferred to human physiology, especially as we combined the bioassay with ELISA-analysis.

In contrast to previous studies we also analysed the concentration of TGFβ1 in seminal plasma in both patient groups. TGFβ1 had been shown to be the principal trigger for the induction of uterine inflammatory responses following mating in mice [3]. However, even the concentration of this growth factor was similar in fertile and infertile men.

Our experiments therefore strongly support that the constituents and the immunological stimulatory potential of seminal plasma of ejaculates without leukocytospermia are similar in both, men with normal and abnormal sperm count. Even though seminal plasma has a relevant and still poorly understood function in the physiology of the reproductive system, this function appears not to be dependant on the number and the motility of the spermatozoa.

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**Title:**

Gene Expression Pattern Induced by Seminal Plasma in Human Endometrial Epithelial Cells in Culture.

**Running Title:**

Seminal plasma effects on endometrial epithelial cells.

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**Abstract**

Studies in rodents, livestock species and humans have showed that introduction of semen into the female reproductive tract elicits molecular and cellular changes with physiological consequences bearing on conception and pregnancy. The present study used a functional genomics approach to investigate the effect of seminal plasma (SP) on primary human endometrial epithelial cell (EEC) cultured *in vitro*. We used CodeLink™ Gene Expression Bioarray system to interrogate changes in gene expression occurring in EEC by SP. Using this strategy we identified 145 significantly expressed genes. One hundred and seven genes with known protein products were up-regulated with a treatment control ratio more than 2.0. Among the most up-regulated genes there were members of the metallothionein family and some enrolled on the MAPK signaling pathway. Among the most down-regulated genes there were some chemokines and interferon inducible genes. Gene ontology analysis revealed an emphasis on genes involved with response to stimulus (25%) and immune responses (12%). The data here published contributes to the idea that EEC are able to build up a response to seminal plasma, inducing molecular and cellular changes in the endometrium that can have a favorable effect on uterine receptivity.

***Keywords:*** Seminal plasma/endometrium/gene expression profile

## Introduction

Seminal plasma is produced by contributions from several male accessory glands, including seminal vesicle, prostate, epididymis and bulbourethral glands (Pilch e Mann, 2006). Several of its components have been known for more than 40 years, and conventionally seminal plasma has been viewed as a transport and survival medium for mammalian sperm. But its role is now recognized to extend beyond this to targeting female tissues (Robertson, 2005). Studies in rodents, livestock species and humans have showed that introduction of semen into the female reproductive tract elicits molecular and cellular changes with physiological consequences bearing on conception and pregnancy and must be considered a factor that can contribute in in conditioning the female tract for pregnancy (Robertson, 2007).

The secretory products of the seminal vesicles encompass ions, oestrogen, testosterone, low molecular weight substances, several prostaglandins, cytokines and growth factors (Aumuller e Riva, 1992; Maegawa, Kamada *et al.*, 2002). Discovery and identification of potent immunoregulatory components in semen during the 1970s led to recognition that semen is immunoactive (Lord, Sensabaugh *et al.*, 1977). Recently, some sets of proteins engaged in well characterized biological process, notably metabolism and immune response was identified in seminal plasma using proteomic methods (Pilch e Mann, 2006).

It is hypothesized that insemination constitutes a 'priming' event, acting to induce maternal immune tolerance to paternal transplantation antigens (Thaler, 1989; Kelly e Critchley, 1997; Robertson, Mau *et al.*, 1997). As the embryo and subsequent fetus have been seen as a semi-allograft and immunologically foreign to the maternal uterus, a special interaction must be established between the maternal immune system and embryo/fetal cells during implantation and pregnancy, allowing survival and normal development of the fetus (Trowsdale e Betz, 2006). At the site of blastocyst apposition, the trophectoderm contacts the endometrial epithelial cells (EECs). This interaction occurs during a defined period, called the 'implantation window', when the endometrium is receptive for embryo implantation. The direct interaction between the embryo and

the endometrial luminal surface of the uterus is an important step in the initial process of human embryo implantation (Tabibzadeh e Babaknia, 1995).

In this study, using a functional genomic approach, we have analyzed the effect of seminal plasma on human endometrial epithelial cells. Our aim was investigate the hypothesis that seminal plasma conditioning effect on female tract could occurs also in human being contributing to uterine receptivity.

**Material Methods**

*Seminal plasma*

Semen samples were obtained from donors attending the Instituto Valenciano de Infertilidad (Valencia, Spain). Analysis of semen parameters concentration, viability, progressive motility, semen volume and leukocyte counts were carried out according to the appropriate World Health Organization guidelines. Semen was produced by manual masturbation and within 30 minutes of ejaculation. Following liquification, it was centrifuged at 700 g for 10 minutes. A second round of centrifugation was performed at 10000 g for 10 minutes at room temperature. All procedures are in agreement with the Declaration of Helsinki.

*Endometrial biopsies and cell culture*

Human endometrial biopsies was obtained from three fertile healthy premenopausal women by using a Pipelle catheter (Genetics, Namont-Achel, Belgium) under sterile conditions from the uterine fundus at the Instituto Valenciano de Infertilidad (Valencia, Spain). This study was conducted in accordance with the guidelines of The Declaration of Helsinki. Endometrial samples were minced into segments smaller than 1 mm and subjected to mild collagenase digestion for 1 hour (collagenase IA 0,1%, Sigma). Stromal cells were separated by washing the tissue with DMEM three times and throwing away the supernatant after 5 minutes time left for sedimentation of glandular epithelial cells. At the bottom of the conical tube, the epithelial cells were collected, cultured and grown to confluence in a steroid-depleted medium composed of 75% Dulbecco's Modified Eagle Medium and 25% MCDB-105 (Sigma, Madrid, Spain) containing antibiotics. This medium was supplemented with 10% fetal bovine serum and insulin, 5 µg/mL (Sigma).

*RNA isolation and quality*

Total RNA was extracted from human endometrium epithelial cells using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. RNA quality was assessed by loading

300 ng of total RNA onto an RNA Labchip and analyzed on an A2100 bioanalyzer (Agilent Technologies, Germany).

*RNA processing and gene expression profiling*

RNA samples were prepared and processed for microarray hybridization by the CodeLink Expression Assay Reagent Kit (Amersham). In brief, cDNA was synthesized from total RNA and purified by a QIAquick purification kit (Qiagen). Following cRNA synthesis, the target product was recovered by an RNeasy kit (Qiagen) and quantified by means use of Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Fragmented target cRNA (10 µg) was added to the hybridization solution, and a 250-µL quantity of denatured target solution was injected into each hybridization chamber of a CodeLink Whole Human Genome microarray (Amersham Biosciences/GE Healthcare), which contained oligonucleotide probes for approximately 55,000 different genes targets. Endometrial epithelial cells (EEC) treat with 10% seminal plasma (n = 3) and its respective non-stimulated EEC (n = 3) samples were hybridized and scanned. The normalized intensity of all genes and sequences of the CodeLink whole human genome bioarray (Amersham Biosciences/GE Healthcare) was submitted to statistical analyses in order to find genes which were significantly altered by seminal plasma treatment.

*Quantitative real-time PCR*

To verify, in part, the differential expression of selected genes, we synthesized cDNA from total RNA. RT-PCR reactions were carried out using LightCycler System (Roche). Primer sequences, product sizes, gene target sites and Genbank accession numbers for target sequences are shown in Table I. Approximately 100ng of cDNA was used for these analyses. The reactions contained 0.5 µM of each primer, and the corresponding amount of the LightCycler–FastStart DNA Master Hybridization Probe reaction mix (Roche Diagnostics) in a final volume of 20 µl. Amplification was performed in a LightCycler 1.5 (Roche) starting with 10 min denaturation at 95$^{o}$C, followed by

45 cycles of 10 s denaturation at 95$^{o}$C, 15 s annealing and 5 s extension at 72$^{o}$C. Melting curve analysis was performed after 20 s denaturation at 95$^{o}$C and hybridization for 20 s at 40$^{o}$C by increasing the temperature from 40$^{o}$C to 85$^{o}$C with a slope of 0.1$^{o}$C/min.

*Microarray data analysis and statistics*

Normalized data from the CodeLink software package were uploaded to SAM 3.00 (Significance Analysis of Microarrays, Stanford University) for analysis. We assessed the differential expression of genes by averaging the normalized samples and running a pair-wise analysis. To find genes that were up- or down-regulated in endometrial epithelial cells treated with seminal plasma, the genes that passed the intensity filter, with a delta of 0.08 and a treatment control ratio of $\geq 2$ or $\leq 0.5$ were included in the analysis. Gene ontology reports (Biological Process, Cellular Component, and Molecular Function) was made using DAVID 2007 (Database for Annotation, Visualization, and Integrated Discovery, N.I.H.) (Dennis, Sherman *et al.*, 2003).

**Results**

The impact of seminal plasma (SP) on gene expression in the human endometrial epithelial cell was detected by differentially expressed mRNAs using CodeLink™ Whole Human Genome Bioarray (Amersham Biosciences/GE Healthcare) containing ~55,000 gene targets. Our results have demonstrated that seminal plasma significantly affects the expression of 145 genes. One hundred and thirty-three of them were up-regulated while only 12 were down-regulated (Table II).

To validate microarray data, 5 genes were selected for Q-PCR. Three of them that showed to be up-regulated in response to SP: MT1G, HMOX1, SPP1, and the other two genes that were down-regulated: CXCL10 and CXCL11. Gene expression data analyzed by Q-PCR showed very similar gene expression than in microarray experiments. Figure 1.

Six genes were expressed >10-fold compared with non-treated endometrial epithelial cell (EEC). The gene encoding metallothionein 1H was up-regulated to the greatest extent (133-fold) by SP treatment. Other genes encoding members of the metallothionein family 1 were also up-regulated in EEC after SP treatment (metallothionein 1G, 34-fold; 1F, 12-fold; 1E, 7-fold; and the -1B, -1L, -1X ~6-fold). Metallothionein family IV mRNA was also induced by SP (2.5-fold). The use of data filtering for significant changes pointed that the 8 up-regulated metallothionein could be functionally classified into a cluster (enrichment score 7.49). Overall, the 112 genes changed by SP treatment could be classified functionally into at least seven gene clusters (enrichment scores from 7.49 to 0.35).

Genes affected by SP belonged to a variety of cellular, molecular and biological ontologies. Expression of EEC genes associated with different biological processes, molecular functions and cellular components significantly altered by SP treatment is shown at Table III. Within the biological processes affected by SP, more than 10% of the genes was pointed to be involved in response to chemical or external stimulus (angiopoientin-like 4, bombesin-like receptor 3, CCL2, CXCL10, integrin alpha 2, interleulin 11, kallikrein b, osteopontin, CXCL11, stanniocalcin 1, v-fos homolog with $P<0.005$). Genes controlling biological processes as development (22), apoptosis (8),

inflammatory response (6), and response to wounding (8) were also pointed with $P<0.005$ by DAVID2007 (Dennis, Sherman *et al.*, 2003).

Seminal plasma induced EEC mRNA modulation could be related with several molecular functions terms (Table III). Cation, metal, insulin-growth factor binding, growth factor and cytokine activity related genes were pointed with $P<0.05$. All metallothionein encoded genes induced by SP is related to metal ion binding. Different genes were also related to ion binding notably, heme oxygenase 1, signal transducer and activator of transcription 1, early growth response 1, superoxide dismutase 1 and integrin alpha 2.

Nineteen percent of the EEC significantly altered genes were shown to be related with cellular components ontology, being 15% of the total significantly altered genes related with the extracellular region ($P<0.001$) and are listed at Table IV. Using the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway tool, seminal plasma was able to up-regulated EEC genes which have a correlation with the MAPK signaling pathway (Table IV). The Toll-like receptor signaling pathway could be also be affected by seminal plasma treatment as pointed by KEGG tool and listed at table IV.

**Discussion**

Seminal plasma is composed by a mixture of secretions from several male accessory glands, including prostate, seminal vesicles, epididymis, and Cowper's gland. Despite the primary site of semen deposition being the cervix in women, the female genital tract is capable of transporting inert radioactively labeled material deposited in the vaginal fornix to the tubes and ovaries (Venter e Iturralde, 1979). The effects of seminal plasma can though extend to the uterus (Chu, Nocera *et al.*, 1996; Wildt, Kissler *et al.*, 1998; Gutsche, Von Wolff *et al.*, 2003; Kunz, Beil *et al.*, 2006; O'leary, Jasper *et al.*, 2006; Robertson, 2007).

It has been previously reported that human endometrial epithelial cells, when stimulated by seminal plasma *in vitro*, have the interleukin (IL)-1 beta, IL-6, leukemia inhibitory factor (LIF) mRNA expression up-regulated (Gutsche, Von Wolff *et al.*, 2003). Human ectocervical cells release in culture IL-8, GM-CSF and monocyte chemoattractant protein-1 (MCP-1) after stimulation with seminal plasma (Sharkey, Macpherson *et al.*, 2007).

Seminal plasma has long been credited to have potent ‘immunosuppressive’ activity, with the capacity to induce tolerance or to skew the class of an immune response which subsequently assist embryo implantation and placental development (Allen e Roberts, 1986; Robertson e Sharkey, 2001; Ochsenkuhn, O'connor *et al.*, 2006).

The impact of seminal plasma (SP) on gene expression in the human endometrial epithelial cell was detected by differentially expressed mRNAs using CodeLink™ Whole Human Genome Bioarray (Amersham Biosciences/GE Healthcare) demonstrating that seminal plasma significantly affects the expression of 145 genes. One hundred and thirty-three of them were up-regulated while only 12 were down-regulated. The uterine epithelium is the major producer of hematopoietic cytokines through the peri-implantation period, suggesting that these play a major role in orchestrating immunity at the uteroembryonic interface (Robertson, 2000).

The spatio-temporal patterns of TLRs gene expression in human endometrium may subserve fine-tuning of innate immunity in the endometrium. The negative effects of seminal plasma on the

Toll-like receptor signaling and INF-beta action remain to be fully elucidated. Detrimental properties of SP on the EEC TLR signaling pathway was pointed by KEGG pathways and related to the low expression of CXCL10 and CXCL11 and to the significantly down-regulation of the gene encoding STAT1 (signal transducer and activator of transcription 1, 91Kda).

Searching for the expression of interferon regulated genes altered in EEC by SP treatment, we found a down-regulation of interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon-induced protein 44-like (IFI44L) and interferon-inducible guanylate binding protein 1 (GBBP1).

Interleukin (IL)-6, CXCL8/IL-8, GM-CSF and CCL2/MCP-1 was demonstrated by previous studies, to be up-regulated by SP treatment (3 hours for mRNA expression and 24 hours for ELISA quantification of cell culture supernatant) (Gutsche, Von Wolff *et al.*, 2003; Sharkey, Macpherson *et al.*, 2007), surprisingly in our experiments, the 12 hours treatment of EEC with SP, led to a significantly down-regulation of CCL2.

Monocyte chemoattractant protein-1 (MCP-1) or CCL2, is a member of the beta-chemokine family, with a distinct reduction in staining intensity observed in periovultatory phase endometrium which remains low during the early and mid-secretory stage of the menstrual cycle (Jones, Kelly *et al.*, 1997). CCL2 is related to chemotactic activity for monocytes/macrophages, T lymphocytes, basophils and natural killer cells and can be released by endometrial epithelial and stromal cells in culture (Leonard e Yoshimura, 1990; Arici, Macdonald *et al.*, 1995). In our experiment using EEC, the expression of CCL2 was significantly down-regulated at the greatest extend by SP treatment (5-fold).

In a variety of contexts in which immune activities are changing, metallothionein (MT), a metal-binding protein, can be synthesized as a consequence of a variety of agents, including acute phase cytokines (Yin, Knecht *et al.*, 2005). Seminal plasma concentration of IL-1beta, IL-6 and TNF-alpha were 2.8 pg/mL, 35 pg/mL and 5.5 pg/mL, respectively in samples of normozoospermic

men (Von Wolff, Nowak *et al.*, 2007) and might contributed to the up-regulation to the greatest extent (MT1H, 133-fold) observed in EEC after seminal plasma treatment.

Metallothionein gene map close to the chemokines CCL17 and CX3CL1 and might also act as a chemotactic factor, speculating that MT serve as one of the many signals designed to draw immunocompetent cells to the required site able to modify immune activity *in vivo* (Yin, Knecht *et al.*, 2005). Others genes encoding members of the metallothionein family 1 were also up-regulated in EEC after SP treatment (metallothionein 1G, 1F, 1E, 1B, 1L and 1X).

The role of metallothioneins in endometrium remains unknown but genes for members of the metallothionein family are up-regulated 5.9-fold (MT1G), 3.8- (MT1F) and 3.5- (MT1B) during the implantation window (Kao, Tulac *et al.*, 2002). Our results demonstrated for the first time that seminal plasma was able to induce a significantly up-regulation of various metallothionein genes (MT1B, MT1E, MT1F, MT1G, MT1H, MT1L, MT1X, MTIV) in endometrial epithelial cells.

The epithelial lining capacity of the female reproductive tract to react to seminal plasma exposure after intercourse is consistent with the immunological competence required of the tissue and it has been speculated that seminal plasma elicits consequences in the female immune system which subsequently assist embryo implantation (Robertson e Sharkey, 2001; Robertson, 2007).

A T helper (Th)-2 immune response mounted at the maternal-fetal interface has been related to fetal survival in the maternal uterus (Lin, Mosmann *et al.*, 1993; Raghupathy, 1997), however, mice with quadruple-Th2 gene deletion do not show disturbed pregnancy (Fallon, Jolin *et al.*, 2002). Th1-type cytokines, which promote allograft rejection, may compromise pregnancy (Piccinni, 2006). Regulatory T cells (Treg) play important roles in immune system homeostasis, and may also be involved in immunotolerance by suppressing Th1 immune response (Cao, Wang *et al.*, 2007).

A hypotetical scenario is showed by Dr. Zenclussen (Zenclussen, 2006; Zenclussen, Gerlof *et al.*, 2006), where regulatory T cells can be generated in the periphery after encountering male antigens which would expand and migrate into the fetal-maternal interface and provoke a tolerant

microenvironment characterized by high levels of protective molecules (e.g. TGF-beta, LIF and HO-1). Recent data suggest heme oxygenase (HO)-1 as an important player in pregnancy tolerance with HO-1 augmentation related to rescue pups from maternal rejection (Zenclussen, Gerlof *et al.*, 2006; Zenclussen, Anegon *et al.*, 2006). Endometrial epithelial cells when stimulated with seminal plasma is able to up-regulate by 30 times the HO-1 mRNA expression. LIF mRNA was also demonstrated to be up-regulated by SP (Gutsche, Von Wolff *et al.*, 2003) and TGF-beta present in seminal plasma was identified as an important trigger of immune response (Letterio e Roberts, 1998; Robertson, Ingman *et al.*, 2002; Robertson, 2007).

Our results on endometrial epithelial cells come add evidences to support the concept of the conditioning effect of seminal fluid on female tract with the idea that the EECs are able to build up a response to seminal plasma and this could lead to important changes of the micro-environment existing adjacent and might be related with the increased pregnancy rate observed on patients receiving seminal plasma intravaginal and intracervical at the oocyte retrieval day of our double-blind placebo-controlled randomised pilot study (von Wolff *et al.*, unpublished data).

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**Figure 1.** Validation of microarray data by Q-PCR. Three up- and 2 down-regulated genes were selected for gene expression analysis by Q-PCR. Gene expression changes observed by Q-PCR (black bars) were very similar to those obtained by microarray analysis (grey bars) for each gene.

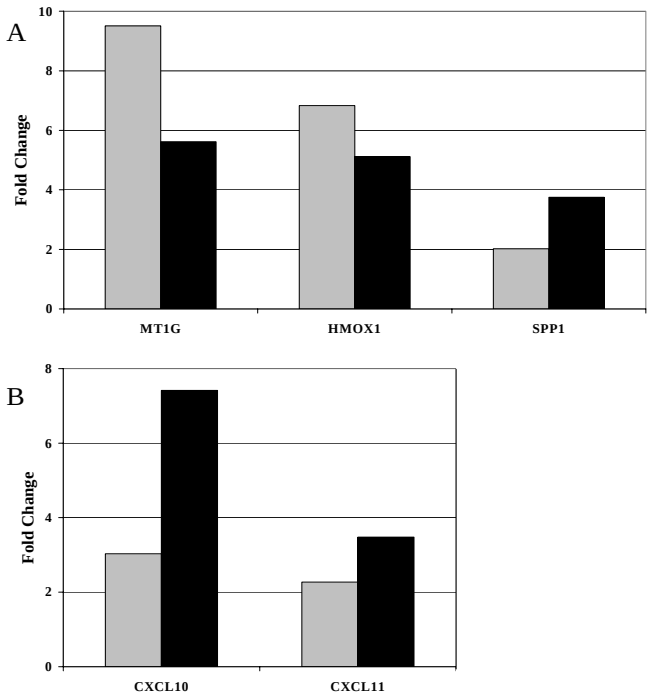

**Table I.** List of genes selected for validation by Q-PCR. Accession number (ACCN), gene name and oligonucleotide sequences are indicated.

| ACCN | Gene name | Primer | Sequence |
|---|---|---|---|
| NM_005950 | MT1G | Forward | TCCTGCAAGTGCAAAGAGTG |
| | | Reverse | GGAATGTAGCAAAGGGGTCA |
| NM_002133 | HMOX1 | Forward | TCCGATGGGTCCTTACACTC |
| | | Reverse | ATTGCCTGGATGTGCTTTTC |
| NM_000582 | SPP1 | Forward | TTGCAGTGATTTGCTTTTGC |
| | | Reverse | GCCACAGCATCTGGGTATTT |
| NM_001565 | CXCL10 | Forward | AAGGATGGACCACACAGAGG |
| | | Reverse | TGGAAGATGGGAAAGGTGAG |
| NM_005409 | CXCL11 | Forward | AGAGGACGCTGTCTTTGCAT |
| | | Reverse | AGATGCCCTTTTCCAGGACT |

**Table II:** The impact of seminal plasma (SP) on gene expression in the human endometrial epithelial cell detected by differentially expressed mRNAs using CodeLink™ Whole Human Genome

| GENBANK ACCESSION | GENE NAME | FOLD CHANGE |
|---|---|---|
| BQ188762 | CHEMOKINE (C-C MOTIF) LIGAND 2 | -5,55 |
| NM_001565 | CHEMOKINE (C-X-C MOTIF) LIGAND 10 | -3,03 |
| AK022231 | SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1, 91KDA | -2,70 |
| NM_001548 | INTERFERON-INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 1 | -2,50 |
| NM_013312 | HOOK HOMOLOG 2 | -2,44 |
| NM_006820 | INTERFERON-INDUCED PROTEIN 44-LIKE | -2,33 |
| NM_005409 | CHEMOKINE (C-X-C MOTIF) LIGAND 11 | -2,27 |
| NM_002053 | GUANYLATE BINDING PROTEIN 1, INTERFERON-INDUCIBLE, 67KDA | -2,17 |
| NM_014314 | DEAD (ASP-GLU-ALA-ASP) BOX POLYPEPTIDE 58 | -2,17 |
| NM_016323 | HECT DOMAIN AND RLD 5 | -2,00 |

| GENBANK ACCESSION | GENE NAME | FOLD CHANGE |
|---|---|---|
| NM_005951 | METALLOTHIONEIN 1H | 133,74 |
| NM_005950 | METALLOTHIONEIN 1G | 33,70 |
| NM_002133 | HEME OXYGENASE (DECYCLING) 1 | 30,68 |
| NM_005949 | METALLOTHIONEIN 1F (FUNCTIONAL) | 12,44 |
| NM_005252 | V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG | 11,50 |
| AV747163 | KIAA1432 | 11,23 |
| NM_017899 | TESCALCIN | 8,13 |
| BG505162 | METALLOTHIONEIN 1E (FUNCTIONAL) | 7,02 |
| NM_000596 | INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 1 | 6,81 |
| BG747999 | METALLOTHIONEIN 1L | 6,76 |
| NM_005947 | METALLOTHIONEIN 1B (FUNCTIONAL) | 6,49 |
| NM_005952 | METALLOTHIONEIN 1X | 6,45 |
| NM_000424 | KERATIN 4 | 6,14 |
| NM_004418 | DUAL SPECIFICITY PHOSPHATASE 2 | 5,41 |
| NM_000839 | GLUTAMATE RECEPTOR, METABOTROPIC 2 | 5,34 |
| NM_005684 | G PROTEIN-COUPLED RECEPTOR 52 | 5,23 |
| NM_012320 | LYSOPHOSPHOLIPASE 3 (LYSOSOMAL PHOSPHOLIPASE A2) | 5,12 |
| NM_139314 | ANGIOPOIETIN-LIKE 4 | 4,91 |
| NM_173354 | SNF1-LIKE KINASE | 4,88 |
| NM_030913 | SEMA DOMAIN, TRANSMEMBRANE DOMAIN (TM), AND CYTOPLASMIC DOMAIN, (SEMAPHORIN) 6C | 4,75 |
| NM_021194 | SOLUTE CARRIER FAMILY 30 (ZINC TRANSPORTER), MEMBER 1 | 4,64 |
| NM_000641 | INTERLEUKIN 11 | 4,60 |
| AA577781 | MICROTUBULE ASSOCIATED SERINE/THREONINE KINASE FAMILY MEMBER 4 | 4,16 |
| NM_007036 | ENDOTHELIAL CELL-SPECIFIC MOLECULE 1 | 4,13 |
| BQ020357 | HWKM1940 | 4,12 |
| NM_022131 | CALSYNTENIN 2 | 4,11 |
| NM_205855 | HWKM1940 | 4,09 |
| NM_001638 | APOLIPOPROTEIN F | 3,97 |
| NM_014191 | SODIUM CHANNEL, VOLTAGE GATED, TYPE VIII, ALPHA | 3,83 |
| NM_000582 | SECRETED PHOSPHOPROTEIN 1 (OSTEOPONTIN, BONE SIALOPROTEIN I, EARLY T-LYMPHOCYTE ACTIVATION 1) | 3,75 |
| AA781411 | FIBROBLAST GROWTH FACTOR 6 | 3,63 |
| NM_203413 | CHROMOSOME 17 OPEN READING FRAME 81 | 3,59 |
| NM_001727 | BOMBESIN-LIKE RECEPTOR 3 | 3,45 |
| NM_005261 | GTP BINDING PROTEIN OVEREXPRESSED IN SKELETAL MUSCLE | 3,37 |
| AA463818 | INTERFERON INDUCED TRANSMEMBRANE PROTEIN 5 | 3,19 |
| AK024261 | DISRUPTED IN RENAL CARCINOMA 3 | 3,14 |
| NM_005382 | NEUROFILAMENT 3 (150KDA MEDIUM) | 3,13 |
| CD244731 | SIMILAR TO HYPOTHETICAL PROTEIN SB153 ISOFORM 1 | 3,13 |
| NM_001945 | HEPARIN-BINDING EGF-LIKE GROWTH FACTOR | 3,10 |
| NM_015675 | GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, BETA | 3,04 |
| NM_000442 | PLATELET/ENDOTHELIAL CELL ADHESION MOLECULE (CD31 ANTIGEN) | 3,03 |
| T78439 | TIMP METALLOPEPTIDASE INHIBITOR 3 (SORSBY FUNDUS DYSTROPHY, PSEUDOINFLAMMATORY) | 3,00 |
| NM_199243 | G PROTEIN-COUPLED RECEPTOR 150 | 2,93 |
| AB067496 | KIAA1909 PROTEIN | 2,91 |
| NM_000167 | GLYCEROL KINASE | 2,90 |

| | | |
|---|---|---|
| NM_181710 | ZINC AND RING FINGER 4 | 2,90 |
| NM_178820 | F-BOX PROTEIN 27 | 2,88 |
| NM_021972 | SPHINGOSINE KINASE 1 | 2,80 |
| NM_004951 | EPSTEIN-BARR VIRUS INDUCED GENE 2 (LYMPHOCYTE-SPECIFIC G PROTEIN-COUPLED RECEPTOR) | 2,73 |
| AK093618 | HYPOTHETICAL PROTEIN LOC284551 | 2,71 |
| NM_004732 | POTASSIUM VOLTAGE-GATED CHANNEL, SHAKER-RELATED SUBFAMILY, BETA MEMBER 3 | 2,67 |
| AW193472 | SIMILAR TO IMPORTIN ALPHA-2 SUBUNIT (KARYOPHERIN ALPHA-2 SUBUNIT) (SRP1-ALPHA) (RAG COHORT PROTEIN 1) | 2,65 |
| NM_002575 | SERPIN PEPTIDASE INHIBITOR, CLADE B (OVALBUMIN), MEMBER 2 | 2,58 |
| AI123967 | CHROMOSOME 11 OPEN READING FRAME 49 | 2,56 |
| NM_015185 | CDC42 GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 9 | 2,52 |
| NM_004417 | DUAL SPECIFICITY PHOSPHATASE 1 | 2,51 |
| NM_002061 | GLUTAMATE-CYSTEINE LIGASE, MODIFIER SUBUNIT | 2,51 |
| NM_152759 | LEUCINE RICH REPEAT CONTAINING 43 | 2,51 |
| NM_032935 | METALLOTHIONEIN IV | 2,51 |
| NM_004816 | CHROMOSOME 9 OPEN READING FRAME 61 | 2,48 |
| NM_004811 | LEUPAXIN | 2,48 |
| BF510814 | SH3 AND MULTIPLE ANKYRIN REPEAT DOMAINS 3 | 2,48 |
| NM_014291 | GLYCINE C-ACETYLTRANSFERASE (2-AMINO-3-KETOBUTYRATE COENZYME A LIGASE) | 2,47 |
| NM_019025 | SPERMINE OXIDASE | 2,47 |
| NM_018602 | DNAJ (HSP40) HOMOLOG, SUBFAMILY A, MEMBER 4 | 2,46 |
| AW979075 | NUCLEAR RECEPTOR COACTIVATOR 2 | 2,44 |
| NM_005635 | SYNOVIAL SARCOMA, X BREAKPOINT 1 | 2,39 |
| NM_173086 | KERATIN 6E | 2,37 |
| AK027128 | ZINC FINGER PROTEIN 277 | 2,37 |
| NM_005345 | HEAT SHOCK 70KDA PROTEIN 1A | 2,33 |
| NM_014851 | KELCH-LIKE 21 (DROSOPHILA) | 2,33 |
| NM_020645 | NUCLEAR RECEPTOR INTERACTING PROTEIN 3 | 2,32 |
| BF573354 | CHROMOSOME 14 OPEN READING FRAME 34 | 2,31 |
| NM_174926 | HYPOTHETICAL PROTEIN MGC17839 | 2,28 |
| NM_033226 | ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 12 | 2,27 |
| BU739449 | TRANSMEMBRANE, PROSTATE ANDROGEN INDUCED RNA | 2,27 |
| NM_021010 | DEFENSIN, ALPHA 5, PANETH CELL-SPECIFIC | 2,15 |
| BF515657 | CHROMOSOME 8 OPEN READING FRAME 36 | 2,12 |
| NM_182491 | ZINC FINGER, AN1-TYPE DOMAIN 2A | 2,12 |
| NM_006145 | DNAJ (HSP40) HOMOLOG, SUBFAMILY B, MEMBER 1 | 2,11 |
| W90798 | HYPOTHETICAL PROTEIN MGC14376 | 2,11 |
| NM_031474 | NUCLEAR RECEPTOR INTERACTING PROTEIN 2 | 2,11 |
| NM_004496 | FORKHEAD BOX A1 | 2,08 |
| NM_145657 | GS HOMEOBOX 1 | 2,08 |
| NM_201632 | TRANSCRIPTION FACTOR 7 (T-CELL SPECIFIC, HMG-BOX) | 2,08 |
| NM_000892 | KALLIKREIN B, PLASMA (FLETCHER FACTOR) 1 | 2,07 |
| NM_014861 | KIAA0703 GENE PRODUCT | 2,07 |
| AV726947 | FAMILY WITH SEQUENCE SIMILARITY 66, MEMBER C | 2,06 |
| NM_004083 | DNA-DAMAGE-INDUCIBLE TRANSCRIPT 3 | 2,05 |
| R19491 | FAMILY WITH SEQUENCE SIMILARITY 80, MEMBER B | 2,05 |
| BM988108 | INTEGRIN, ALPHA 2 (CD49B, ALPHA 2 SUBUNIT OF VLA-2 RECEPTOR) | 2,05 |
| NM_032145 | F-BOX PROTEIN 30 | 2,04 |
| BM555828 | HYPOTHETICAL PROTEIN LOC284591 | 2,04 |
| U46768 | STANNIOCALCIN 1 | 2,04 |
| NM_003582 | DUAL-SPECIFICITY TYROSINE-(Y)-PHOSPHORYLATION REGULATED KINASE 3 | 2,03 |
| BI910683 | SUPEROXIDE DISMUTASE 1, SOLUBLE (AMYOTROPHIC LATERAL SCLEROSIS 1 (ADULT)) | 2,03 |
| NM_000422 | KERATIN 17 | 2,02 |
| NM_001964 | EARLY GROWTH RESPONSE 1 | 2,01 |
| NM_005524 | HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA) | 2,01 |
| NM_003364 | URIDINE PHOSPHORYLASE 1 | 2,01 |
| AI418777 | ANTIGEN P97 IDENTIFIED BY MONOCLONAL ANTIBODIES 133.2 AND 96.5 | 2,00 |
| NM_053044 | HTRA SERINE PEPTIDASE 3 | 2,00 |

**Table III:** Gene ontology classification of significantly altered EEC gene expression induced by SP.

| Biological processes | Cellular components | Molecular functions |
|---|---|---|
| Response to stimulus | Extracellular space | Cation binding |
| Development | Extracellular region | Insulin-like growth factor binding |
| Regulation of apoptosis | | Receptor binding |
| Response to nutrient levels | | Protein binding |
| Inflammatory response | | Growth factor activity |
| Response to wounding | | Chemokine activity |
| Organ development | | |
| Immune response | | |
| Regulation of biological process | | |
| Regulation of enzyme activity | | |

**Table IV:** SP inducing EEC to up-regulate some genes related to extracellular region, MAPK and toll-like receptor signaling pathway (KEGG Pathways)

| Genbank accession | Gene Name | SP fold induction |
|---|---|---|
| **Extracellular region** | | |
| NM_000596 | INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 1 | 6,81 |
| NM_139314 | ANGIOPOIETIN-LIKE 4 | 4,91 |
| NM_000641 | INTERLEUKIN 11 | 4,60 |
| NM_007036 | ENDOTHELIAL CELL-SPECIFIC MOLECULE 1 | 4,13 |
| NM_001638 | APOLIPOPROTEIN F | 3,97 |
| NM_000582 | SECRETED PHOSPHOPROTEIN 1 (OSTEOPONTIN) | 3,75 |
| AA781411 | FIBROBLAST GROWTH FACTOR 6 | 3,63 |
| NM_001945 | HEPARIN-BINDING EGF-LIKE GROWTH FACTOR | 3,10 |
| T78439 | TIMP METALLOPEPTIDASE INHIBITOR 3 | 3,00 |
| NM_021010 | DEFENSIN, ALPHA 5, PANETH CELL-SPECIFIC | 2,15 |
| NM_000892 | KALLIKREIN B, PLASMA (FLETCHER FACTOR) 1 | 2,07 |
| U46768 | STANNIOCALCIN 1 | 2,04 |
| AI418777 | ANTIGEN P97 | 2,00 |
| NM_053044 | HTRA SERINE PEPTIDASE 3 | 2,00 |
| NM_005409 | CHEMOKINE (C-X-C MOTIF) LIGAND 11 | 0,44 |
| NM_001565 | CHEMOKINE (C-X-C MOTIF) LIGAND 10 | 0,33 |
| BQ188762 | CHEMOKINE (C-C MOTIF) LIGAND 2 | 0,18 |
| **MAPK signaling pathway** | | |
| NM_005252 | V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG | 11,50 |
| NM_004418 | DUAL SPECIFICITY PHOSPHATASE 2 | 5,41 |
| AA781411 | FIBROBLAST GROWTH FACTOR 6 | 3,63 |
| NM_015675 | GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, BETA | 3,04 |
| NM_004417 | DUAL SPECIFICITY PHOSPHATASE 1 | 2,51 |
| NM_005345 | HEAT SHOCK 70KDA PROTEIN 1A | 2,33 |
| NM_004083 | DNA-DAMAGE-INDUCIBLE TRANSCRIPT 3 | 2,05 |
| **Toll-like receptor signaling pathway** | | |
| NM_005252 | V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG | 11,50 |
| NM_005409 | CHEMOKINE (C-X-C MOTIF) LIGAND 11 | 0,44 |
| AK022231 | SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1, 91KDA | 0,37 |
| NM_001565 | CHEMOKINE (C-X-C MOTIF) LIGAND 10 | 0,33 |

**Running title:**

**Intracervical application of seminal plasma**

**Title page**

# Intravaginal and intracervical application of seminal plasma in IVF/ICSI-treatment cycles – a double blind placebo-controlled randomised pilot study

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**Conflict of interest**

There is no conflict of interest

**Capsule**

Intravaginal application of seminal plasma at the time of follicle aspiration in 137 IVF/ICSI treatment cycles increased pregnancy rate by 11,6%. Around 450 patients will be needed to reach significance.

**Abstract**

**Objective:** To evaluate if intravaginal application seminal plasma at the time of follicle aspiration in IVF/ICSI treatment cycles has the potential to increase pregnancy rate. To calculate the number of patients needed to achieve significance in a multicentre trial.

**Design:** Double blind placebo-controlled randomised pilot study.

**Setting:** University department of gynaecological endocrinology and reproductive medicine.

**Patients:** 168 patients undergoing IVF/ICSI treatment**.**

**Interventions:** Cryopreserved seminal plasma from the patient's partner or sodium chloride (placebo) was injected into the cervix and the posterior fornix of the vagina just after follicle aspiration.

**Main outcome measure:** Clinical pregnancy rate.

**Results:** 168 patients agreed to participate in the study. Participation was limited to one treatment cycle. 31 patients (18%) were excluded from the study, mainly due to cancelled embryo transfers. 70 patients received placebo, 67 seminal plasma. Clinical pregnancy rates were 25.7% (18/70) in the placebo group. Clinical pregnancy rate in the seminal plasma group reached 37.3% (25/67), corresponding to a relative increase of 45%.

**Conclusions:** Even though significance was not reached in this pilot study, the data suggest that seminal plasma has the potential to improve pregnancy rate. It is estimated that around 450 patients need to be recruited to reach significance in a multicentre study.

**Key words: endometrium/seminal plasma/in vitro fertilisation/implantation**

## Introduction

Implantation failure is one of the remaining major obstacles in assisted reproductive techniques. One of the reasons for low implantation rates might be the dysregulation of endometrial function in IVF (in-vitro fertilisation) and ICSI (intracytoplasmatic sperm injection) cycles, caused by non-physiological stimulation with gonadotrophins (1). To overcome this problem, reproductive biologists and endocrinologists have focussed on the regulation and dysregulation of endometrial function to develop therapeutic strategies for the treatment of dysregulated endometrium to increase pregnancy rates.

The high demand for an effective therapy that will increase implantation rates has led to the introduction of several systemic therapies which, however, are not based on clear pathophysiological concepts. Therapies such as application of immunoglobulins, aspirin, heparin (2) and the subcutaneous application of cytokines like Leukaemia inhibitory factor (LIF) have therefore not proven to be efficient.

The failure of systemic therapies, the application of therapies without clear pathophysiological concepts and our increasing knowledge about the complex regulation of endometrial function has prompted us to analyse systematically the concept of a therapy involving local physiological stimulation of the genital tract using seminal plasma (SP).

This concept is supported by studies in mice in which seminal plasma proved to be essential in implantation (3). In humans, seminal plasma, which contains a high concentration of biologically active mediators (4), stimulates in vitro those factors such as LIF and Interleukin-6 (IL-6) (5) which have been described to play an important role in human implantation. Furthermore, seminal plasma has been shown in our laboratory to stimulate or inhibit in Ishikawa cells many of those factors that are downregulated respectively upregulated in patients undergoing stimulation with gonadotrophins for IVF or ICSI (6).

Some clinical evidence is given by Tremellen et al., 2000 (7), who studied the pregnancy rate in patients abstaining from or engaging in sexual intercourse around the time of embryo transfer. They described a similar pregnancy rate but a significant higher number of viable embryos at 6-8 weeks of pregnancy in patients having intercourse. However, even though this study possibly supports the concept of sexual intercourse to improve the likelihood of successful embryo implantation and development, it remains unclear which of the multiple factors involving intercourse might be responsible for the result of the study.

As cell culture experiments have shown seminal plasma to modulate endometrial function, we hypothesized that the local application of SP into the vagina and the cervix can prime the endometrium at the time of follicle aspiration and thereby could increase pregnancy rates.

## Materials and Methods

### Subjects

All couples undergoing IVF or ICSI treatment at the Department of Gynaecology and Reproductive Medicine, Heidelberg, between February 2004 and January 2007 were considered for enrolment in the trial. 269 women 18 – 42 years of age in a stable sexual relationship were eligible for enrolment and were asked to participate in the study. 101 patients refused to participate. Participation was limited to one treatment cycle per couple. Patients were instructed not to engage in intercourse between 5 days before and 5 days after follicle aspiration. The exclusion criteria included the presence of hepatitis B, C or human immunodeficiency virus, leukocytospermia or other signs of infection and men with < 500µl of seminal plasma. The study was approved by the local ethical committee and the patients' approval was given by written consent.

Clinical IVF / ICSI protocol

In all patients the long stimulation protocol was used. Pituitary desensitization was performed by nasal application of 0.4mg nafarelinacetate, starting in the luteal phase of the previous cycle. After downregulation of the pituitary gland, patients received in addition either 150 to 300 IU of recombinant follicle stimulating hormone (FSH) (Gonal F, Serono, Munich, Germany) or 2-4 ampoules of highly purified human menopausal gonadotrophin (HMG) (Menogon HP, Ferring, Hamburg, Germany) per day. Dosage was adjusted according to estradiol concentrations and vaginal ultrasound scan. Once an adequate ovarian response had been confirmed, FSH and HMG were discontinued and 10000 IU of human chorionic gonadotrophin (hCG) (Predalon, Organon, Oberschleissheim, Germany) were administered. Transvaginal oocyte retrieval was scheduled 34-36 hours after hCG administration and was performed under general anaesthesia. Fertilization was achieved by standard IVF or ICSI. 1-3 oocytes at the pronuclear stage were selected and cultured for another 1- 2 days and were then transferred back into the uterus. Selection of fertilized oocytes after the pronuclear stage was not performed due to the German embryo protection law. Intravaginal progesterone (600 mg/day) was used as luteal support.

Randomisation

Randomisation was performed by randomisation lists (block randomisation every 20 patients) one day before the oocyte aspiration. As the pregnancy rate of IVF/ICSI-treatments is highly dependant on the age of the patients and on the number of previous treatment cycles, the randomisation process was stratified according to the expected pregnancy rate. Stratum I (expected low pregnancy rate) was defined: ≥36 years of age or <36 years of age and transfer of ≥6 embryos in ≥3 embryo transfers without a pregnancy in previously performed IVF/ICSI-cycles. All other patients were allocated to Stratum II (expected high pregnancy rate).

Preparation and application of seminal plasma

Semen samples were obtained ≥ 1/2 week before follicle aspiration by masturbation from the patient's partners and were collected in sterile flasks. SP was extracted by centrifugation of ejaculates at 700 g for 10 min. A second round of centrifugation was performed at 10000 g for 10 min. to extract definitely all spermatozoa. 500-1500µl (depending on the amount of extracted SP) of supernatant was stored in 2ml syringes at -20°C. For placebo, 500-1500µl of sterile sodium chloride was also stored in 2ml syringes at -20°C. Blinding of the samples was achieved by using a placebo (sodium chloride) which could not be differentiated from SP due to the same optical appearance. SP and placebo samples were stored in separate freezers in exactly the same syringes.

On the day of oocyte collection blinded SP or placebo samples were taken out of the freezer by the technician around 30 – 60 min. before the aspiration of the follicles and were transferred to the operating theatre. Follicles were aspirated and bleeding was stopped. An insemination catheter (Laboratoire C.C.D., Paris, France) was introduced 2-3 cm into the cervical canal and 500µl of SP was injected into the cervix. The remaining fluid was injected into the posterior fornix of the vagina. Follicle aspiration and embryo transfer were performed by different gynaecologists.

Analysis

Couples were excluded from final analysis if they did not reach embryo transfer. The outcome measure was the clinical pregnancy rate 4-5 weeks after follicle aspiration. The SP and placebo groups were compared according to age, cause of infertility, proportion of patients undergoing IVF or ICSI, average number of transferred embryos, average number of blastomeres per transferred embryo and to the quality of the embryo at the time of transfer (according to 8, 9). Quality of embryos was defined as followed: Grade 1: Embryo with blastomeres of equal size, no cytoplasmic fragments; Grade 2: Embryo with blastomeres of

equal size, minor cytoplasmic fragments or blebs; Grade 3: Blastomeres with distinctly unequal size, few cytoplasmic fragments or none; Grade 4: Embryo with blastomeres of equal or unequal size, significant cytoplasmic fragmentation; Grade 5: Embryo with blastomeres of any size, severe or complete fragmentation.

Fisher's Exact test was used to compare the pregnancy rates in both groups. Significance level alpha was set to 5%. No sample size calculation was done due to the pilot character of this study. Result of this trial was intended to be used to calculate sample size needed to detect significant differences in pregnancy rates.

**Results**

168 patients were enrolled over the course of the study. 82 patients were allocated to stratum I and 86 patients to stratum II. A total of 31 patients were excluded since they failed to reach embryo transfer due to fertilization failure, cryopreservation of all embryos in case of imminent ovarian hyperstimulation syndrome (OHSS) or other reasons such as degradation of embryos or failure to aspirate oocytes (Figure 1). Characteristics of trial participants in the verum and placebo group were similar according to age, to the proportion of patients undergoing IVF or ICSI, to the average number of transferred embryos and to the cause of infertility. The cause of infertility was pathological sperm count (male factor), occlusion of one or two tubes or severe peritubal adhesions (tubal factor) or unknown (idiopathic) factors. Other factors were defined as severe endometriosis and polycystic ovarian syndrome. The number of blastomeres and the average quality of embryos was slightly but not significantly lower in the verum group. The overall expected pregnancy rate was around 27% according to the Department’s average pregnancy rate, according to the German average pregnancy rate

under the conditions of the German Embryo protection law (www.deutsches-IVF-register.de) and according to the average number of transferred embryos.

SP was analysed several times to exclude remaining contamination with spermatozoa. None of the samples contained any spermatozoa after centrifugation. As some of the SP was expected to reach the uterine cavity, we expected painful cramps in some of the patients. However, painful cramps or infections were not found neither in the verum nor in the placebo group.

Stratification was performed to improve accuracy of the study by avoiding mixing of patients with high and low expected pregnancy rates. Around 10% lower pregnancy rate in stratum I, including patients ≥36 years or previous implantation failure, than in stratum II confirmed the adequacy of stratification. Adequacy of randomisation was confirmed by similar characteristics of trial participants in the two trial arms. Accuracy of the study was further increased by restricting the analysis to the clinical rather than the biochemical pregnancy rate.

Transfer of 125 embryos in the verum group resulted in a clinical pregnancy rate of 37.2% (25/67) in comparison to 25.7% (18/70) following the transfer of 131 embryos in the placebo group. The difference in pregnancy rate was 11.6%, corresponding to a relative increase of 45% (odds ratio 1.72, 95% CI 0.8-3.6). However, the difference in pregnancy rate was still not significant (p=0.19). To reach significance, assuming a difference in pregnancy rate of 13 %, it was calculated that 211 patients had to be included in each patient's group.

**Discussion**

A growing body of clinical evidence as well as basic research on human and non-human endometrium indicate that seminal plasma might play a role in human implantation.

Clinical evidence is given by Bellinge et al., 1986, (10) who deposited semen in the vagina of patients undergoing IVF treatment at the time of oocyte fertilisation and found an

implantation rate of 53%, compared with 23 % in the control group. Coulam et al., 1995, (11) performed a placebo controlled clinical trial, depositing vaginal capsules containing seminal plasma or placebo and described an implantation rate of 80% in patients in the seminal plasma group, compared to 67% in the placebo group. Tremellen et al., 2000, (7) randomised patients either to abstain from or engage in vaginal intercourse and found similar pregnancy rates but a significantly higher proportion of viable embryos at 6-8 weeks of pregnancy in the group engaging in vaginal intercourse. These clinical studies are based on the concept that not only spermatozoa but also seminal plasma are transported passively through the cervix and reach the uterine cavity where they it may interact with endometrial components. Leyendecker at al., 1996, (12) and Kunz et al., 1997, (13) have demonstrated that technetium-labelled albumin macrospheres, placed at the external os of the cervix, reached the tubes within one minute due to subendometrial and myometrial peristaltic waves. However, in patients undergoing in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI), seminal plasma and spermatozoa do not get into contact with the uterine cavity. The blastocyst is retransferred without any seminal plasma and spermatozoa and patients usually abstain from sexual intercourse before and after oocyte retrieval. We therefore suggest that the reduced implantation rates in IVF/ICSI could be increased by endometrial priming with seminal plasma.

This suggestion is supported by basic research on the endometrium of patients undergoing controlled ovarian hyperstimulation (COH). COH, as used in IVF/ICSI procedures, resulted in dysregulated expression of more than 300 genes (1): Microarray technique was applied to compare gene expression in COH cycles at LH (luteinizing hormone) + 7 with unstimulated cycles of the same patients at hCG (human chorionic gonadotrophin) + 7. Interestingly, we found that stimulation of Ishikawa cells, as a highly reproducible in vitro model of endometrial epithelial cell function, with seminal plasma up-regulated respectively down-regulated several of those factors which had been down-regulated respectively up-regulated

by COH (6), indicating the potential of seminal plasma to correct COH-induced endometrial dysregulation. Furthermore, in vitro stimulation of primary endometrial epithelial cells (5) with seminal plasma had shown to upregulate several endometrial factors such as LIF and IL-6, which are thought to play an important role in human implantation and the early stages of pregnancy (14-16).

These data are further supported by studies in mammals (17) and pigs (18), in which seminal plasma interacts with uterine epithelial cells and induces a post-mating inflammatory cascade. Transforming growth factor β (TGFβ), which is found at high concentration in seminal plasma (3, 5), triggers a surge in synthesis of cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-6. These pro-inflammatory cytokines trigger the recruitment and activation of inflammatory cells in endometrial stroma, including macrophages and granulocytes, activating maternal immune activity to accept the invading embryo.

However, as all these studies only support but do not prove the concept of seminal plasma having the potential to improve implantation, we have performed a double blind placebo-controlled randomised study either to further support or to discard the clinical relevance of this concept. Before we started the study, statistical calculations had shown that around 400 patients were required in each study arm to detect a 10% higher pregnancy rate in the verum group. As recruitment of patients proved more difficult than expected, we first performed a pilot study to exactly calculate sample size needed to detect significant differences in pregnancy rates.

The interim analysis revealed an 11.6 % higher pregnancy rate in the verum group. Systematic comparison of SP and placebo group characteristics further supported the trend towards an effect of seminal plasma as embryo quality was lower in the SP group, expecting an even higher difference in pregnancy rate.

Around 500µl of seminal plasma was injected into the cervical canal. Even though none of the patients complained about abdominal cramps, as typically found after instillation of prostaglandins into the uterine cavity, it can be expected that some of the SP ascended through the cervical canal into the uterine cavity.

Different speculative concepts of the effects of SP on the female reproductive tract can be discussed:

First, some of the SP might reache the uterine cavity via the cervical canal, where it might expose some direct stimulatory effect on the endometrium.

Second, the concept of vascular counter current transfer (19) between the vagina and the uterus provides a very interesting model to explain some stimulatory effect of SP on the endometrium without ascension through the cervical canal. This concept is based on the observation that application of progesterone in the vagina resulted in doubling of concentration in the uterine arterial blood compared with peripheral arterial blood (20). The progesterone concentration in endometrial cells was even 10-20 times greater after vaginal administration compared with parental administration in doses resulting in identical peripheral plasma values (21). Some constituents of SP might therefore reach the endometrium by this called “first pass effect” (22) where they stimulate endometrial function.

The third speculative mechanism by which SP might improve implantation is based on the concept that seminal plasma provides the female with paternally derived alloantigens, modulating the female immune system to better accept the embryo (23). This concept is supported by experiments in mice. Johansson et al., 2000, (24) demonstrated activation and expansion of female lymphocyte populations after mating, triggered by constituents of seminal plasma derived from seminal vesicle glands.

Tremellen et al., 2000 (7) found in contrast to our study only a 2.4% increase of pregnancy rate in patients engaging in intercourse around the time of embryo transfer. As our study only provides non-significant data, interpretation of the different results is difficult. Furthermore,

the concepts of both studies are quite different. First, we applied SP without any semen. Second, instillation of SP was performed at the time of follicle aspiration whereas Tremellen et al. recommended intercourse around the time of embryo transfer.

Even though both studies are difficult to compare, it can be speculated that the different results are due to the timing of SP exposure. Our in vitro experiments support the concept that supraphysiological concentrations of SP might possibly neutralize damaging effects of the ovarian hyperstimulation on the quality of the endometrium (6). If SP had such an neutralizing effect, it can be assumed that stimulation by SP at the time of embryo transfer is too late to improve endometrial function.

The concept of seminal plasma stimulating endometrial function raises the question whether such an effect depends on sperm quality. We therefore initially analysed the concentration of a broad spectrum of cytokines in the seminal plasma of men with normal and abnormal sperm count and – in addition - set up a highly reproducible bioassay involving peripheral mononuclear blood cells (PMBC) to gain information on the biological activity of seminal plasma (4). Both assays revealed neither differences in cytokine and growth factor concentrations nor demonstrated differences of the immunomodulatory potential of the seminal plasma of men with normal and abnormal sperm counts. We concluded that endometrial priming by seminal plasma would not be dependent on the number and the motility of the spermatozoa.

As mentioned above, seminal plasma interacts in mammals (17) and pigs (18) with uterine epithelial cells and induces a post-mating inflammatory cascade, triggered by several factors of SP. TGFβ has been found to be the principal trigger for the induction of uterine inflammatory responses in mice (3). Other inflammation inducing moieties present in SP are prostaglandin E and interleukin-8 (25). The great diversity of immunologically active constituents supports the concept that priming of the endometrium by seminal plasma might be rather dependent on a cocktail of several factors than on a single stimulatory agents.

In conclusion, our data support but do not prove the concept of seminal plasma having the potential to increase pregnancy rate. If the data can be confirmed by another large study, seminal plasma could be used as a model to develop a local therapy for endometrial stimulation in COH treatment cycles.

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## Table 1

## Characteristics of trial participants and outcome

| | **Seminal plasma (Verum), n=67** | **Sodium chloride (Placebo), n=70** |
|---|---|---|
| Mean Age (years) | 34,4 (23-42) | 34,1 (23-42) |
| Ätiology of infertility (% of total) | | |
| Male factor | 54 | 63 |
| Tubal factor | 24 | 16 |
| Idiopathic | 17 | 16 |
| Others | 5 | 5 |
| Patients with previous implantation failure** (% of total) | 19 | 16 |
| IVF (% of total) | 46 | 37 |
| ICSI (% of total) | 54 | 63 |
| Median no. of transferred embryos | 1,9 (1-3) | 1,9 (1-3) |
| Embryo score *** (% of total) | Total: n = 125 | Total: n = 131 |
| 1 | 33 | 28 |
| 2 | 34 | 50 |
| 3 | 3 | 5 |
| 4 | 27 | 16 |
| 5 | 3 | 1 |
| Medium embryo score | 2,3 | 1,9 |
| No. of blastomeres / transferred embryo | 3,6 | 4,0 |
| Clinical pregnancy rate Stratum I (%) | 31,0 | 19,4 |
| Clinical pregnancy rate Stratum II (%) | 42,1 | 30,8 |
| Total clinical pregnancy rate (%) | 37,3 (25/67) | 25,7 (18/70) |
| Abortion rate (%) | 4,4 | 4,3 |

* Stratum I: expected low pregnancy rate: ≥36 years of age or <36 years of age and transfer of ≥6 embryos in ≥3 embryo transfers without a pregnancy in previously performed IVF/ICSI-cycles. Stratum II: expected high pregnancy rate: all other patients.
** Previous implantation failure: Transfer of ≥6 embryos in ≥3 embryo transfers without a pregnancy.
*** Quality of embryos according to Veeck, 1991 (7): 1 = highest quality; 5 = lowest quality. See Chapter “Materials and Methods”, “Analysis”.

Figure 1

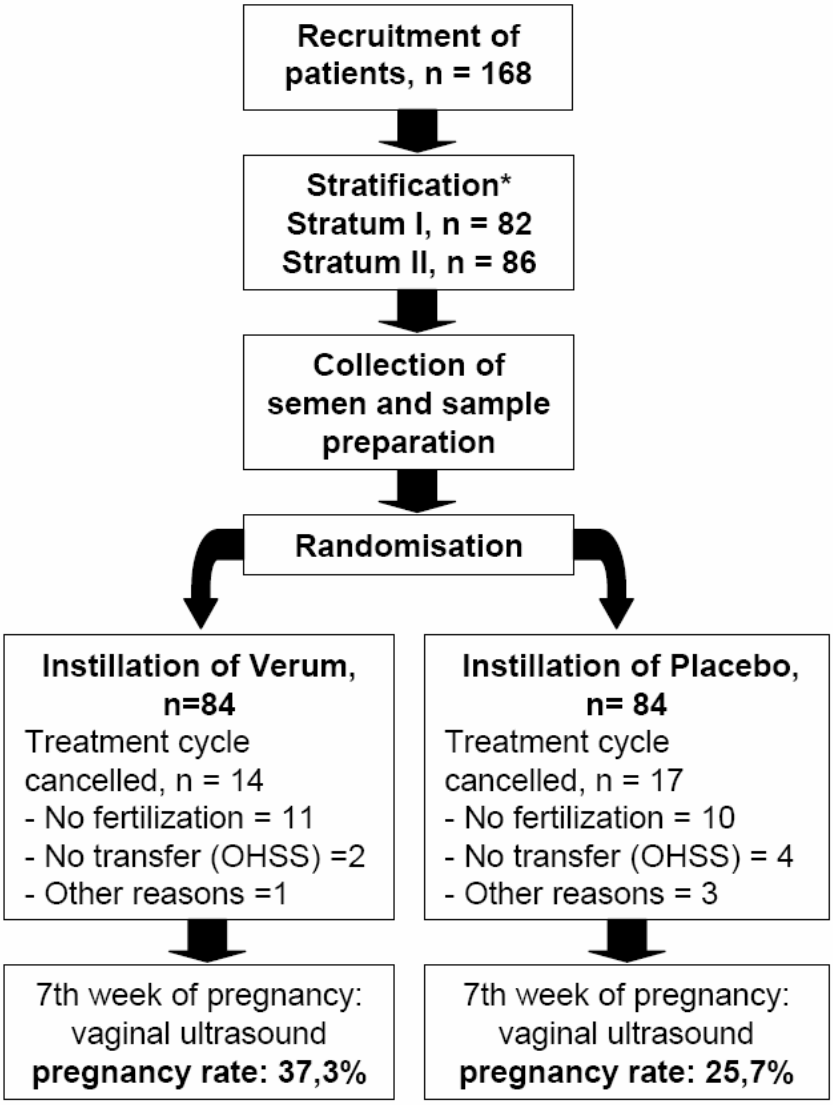


**Figure 1**

Assignment of study subjects

Characteristics of trial participants and outcome of treatment assignment

## CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A idealização da tese aqui apresentada teve início no ano de 2004. Naquela ocasião, dois grupos de pesquisa se destacavam no estudo das funções do papel do plasma seminal. O grupo de pesquisa em Saúde Reprodutiva da Universidade de Adelaide – Austrália, sob coordenação da Dra. Sarah Robertson, publicou vários trabalhos analisando o papel do plasma seminal em modelos animais.

O processo de implantação do blastocisto e o desenvolvimento da receptividade endometrial em humanos parecem ocorrer de forma diferente do observado em modelos animais. A extrapolação direta dos resultados observados em experimentação animal não se mostrou de grande utilidade para melhor entender o processo que ocorre em humanos, visto que existem genes cruciais no processo de implantação animal que não correspondem ao observado em humanos.

O segundo grupo que desenvolve pesquisa com plasma seminal utilizando células humanas é o do grupo do Departamento de Ginecologia, Endocrinologia e Distúrbios da Fertilidade da Universidade de Heidelberg – Alemanha. Em 2003, o grupo alemão demonstrou que o plasma seminal era capaz de modular citocinas em células epiteliais endometriais humanas. Em 2004 teve início o ensaio clínico utilizando plasma seminal como intervenção clínica para aumentar taxa de gravidez em pacientes submetidas a ciclos de IVF/ICSI.

Os resultados apresentados nessa tese vêm adicionar informações a respeito do efeito condicionante do plasma seminal no tecido endometrial. Pela primeira vez na literatura mundial foi realizada análise de microarranjos de DNA de células endometriais epiteliais cultivas *in vitro* estimuladas com uma solução de plasma seminal.

A análise de expressão gênica e das vias de sinalização de células endometriais epiteliais estimuladas pelo plasma seminal foi capaz de acrescentar dados à hipótese do efeito “condicionante” do plasma seminal no endométrio humano. A modulação de genes envolvidos na resposta imune local, favorecendo o desenvolvimento de um micro ambiente favorável à proliferação e migração de linfócitos T reguladores, pode estar relacionado com o aumento da taxa de gravidez observada no ensaio clínico piloto, por favorecer o desenvolvimento do mecanismo de tolerância.

O efeito imunossupressor de componentes do plasma seminal ainda merece maiores estudos. Ao comprovarmos o efeito imunomodulador do plasma seminal utilizando linfócitos, apontar genes possivelmente importantes para o desenvolvimento da tolerância local e o aumento da receptividade endometrial causada pelo plasma seminal, estamos abrindo uma nova linha de pesquisa. Não há no mercado internacional na atualidade, nenhuma intervenção clínica para aumentar taxa de gravidez em ciclos de FIV/ICSI.

Todo o trabalho apresentado nessa tese foi realizado em colaboração com instituições de ensino européias durante os anos de 2005 – 2007. Duas semanas após a defesa pública dessa tese, o Ministério da Ciência e Tecnologia, por intermédio da Financiadora de Estudos e Projetos – FINEP abriu uma chamada pública de propostas de apoio a projetos de pesquisa em genômica com objetivo de fortalecimento da infra-estrutura nacional de pesquisa na área. Parte dos resultados aqui apresentados foram utilizados na elaboração de uma proposta submetida à chamada pública (GENOPROT 07/2007) com o intuito de criação de um laboratório de análise de expressão gênica com fins de aplicação terapêutica na Universidade de Blumenau-SC (FURB).

O uso de tecnologias de genômica e proteômica pode contribuir na elucidação do complexo mecanismo de condicionamento do plasma seminal em tecidos

endometriais ao expandir o conhecimento sobre vias de sinalização envolvidas e na identificação de possíveis alvos moleculares para o desenvolvimento de fármacos. A seleção, caracterização e expressão de proteínas do plasma seminal que exercem os efeitos condicionantes observados, poderão contribuir no aprimoramento do uso de tecnologias de reprodução assistida.

Ao elevar taxa de gravidez com o uso de plasma seminal com terapia local em reprodução assistida estaremos contribuindo com a diminuição do número de gravidezes múltiplas (ao garantir melhor sucesso com a transferência de menos embriões), aumentando a satisfação de pacientes submetidas a FIV/ICSI e reduzindo custos gerais relacionado à diminuição do alto risco de prematuridade, baixo ou muito baixo peso ao nascer e morte perinatal relacionada à gravidezes múltiplas.