CHAPTER 14

Actions of Seminal Plasma Cytokines in Priming Female Reproductive Tract Receptivity for Embryo Implantation

Sarah A. Robertson, John J. Bromfield, Danielle J. Glynn, David J. Sharkey and Melinda J. Jasper

Abstract

Embryo implantation is critically dependent on a supportive uterine environment. Uterine receptivity is the culmination of a cellular and molecular transformation mediated locally by paracrine signals under the governance of ovarian steroid hormones, with cells and cytokines of the immune system playing integral roles in this process. Semen is now recognised as contributing to endometrial preparation for embryo implantation, through the agency of specific factors in the seminal plasma fraction of the ejaculate. Transforming growth factor-β (TGFβ) and other immunoactive moieties derived from male accessory glands interact with epithelial cells in female reproductive tissues to induce pro-inflammatory cytokine expression and initiate an inflammatory cascade. The consequences are recruitment and activation of macrophages, granulocytes and dendritic cells which have immune-regulatory and tissue remodelling roles. The cytokines elicited by seminal activation also exert embryotrophic effects and contribute to optimal preimplantation embryo development. This review summarises our current understanding of the molecular and cellular basis of interactions between seminal plasma and the female reproductive tract, and explores the potential mechanisms through which seminal plasma influences the establishment of pregnancy.

Introduction

Exposure to semen elicits striking changes in cytokine expression and in resident leukocyte populations in female reproductive tract tissues. A dramatic infiltration of activated inflammatory cells including macrophages, dendritic cells and granulocytes is evident after seminal contact in the cervix and uterus of all species so far studied. The molecular and cellular basis of this post-mating inflammatory response has been explored most thoroughly in mice.[1,2,3] The response is initiated when seminal plasma moieties interact with estrogen-primed uterine epithelial cells to induce a surge in synthesis of pro-inflammatory cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6 and an array of chemokines including monocyte chemotactic protein (MCP)-1, KC, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES.[2,4] The response is transient, with resolution of inflammation by the time of embryo implantation on day 4 of pregnancy in the mouse. Similar effects are seen in pigs,[5] where instillation of seminal plasma into the uterine lumen at estrus induces expression of GM-CSF, IL-6 and MCP-1, which recruit macrophages and dendritic cells into the endometrial stromal tissue.[6]

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Cellular changes comparable to those seen in the mouse and pig appear to take place in the human cervix. Intercourse is known to elicit neutrophil recruitment into the superficial epithelium of the cervical tissues,[7] but changes in the deeper cervical stromal tissues have been more difficult to study. In a recent study examining the local effects of natural insemination in peri-ovulatory women, we have shown that intercourse induces an inflammatory reaction across the full thickness of the cervical epithelium and subjacent stromal tissues with a striking infiltration of macrophages, dendritic cells, and lymphocytes in both compartments.[8] Leukocyte influx requires contact between seminal fluid and the female tract tissues since no inflammatory response was seen following condom-protected intercourse. Regulation of the cervical leukocytic infiltrate occurs by activation of pro-inflammatory cytokines GM-CSF, IL-6 and IL-8.[9] In vitro studies suggest that the effects of seminal plasma may extend to the uterus in women.[10,11]

In this chapter we will review recent advances in our knowledge of the molecular regulation of this response, including the identity and interaction between active constituents in seminal plasma, and examine the potential physiological consequences in terms of female reproductive function and pregnancy success. The review will largely focus on events in mice, but where relevant information is available, human and other species will be discussed.

Semen Exposure and Pregnancy Outcome

A case for semen exposure contributing to optimal pregnancy outcome can be made based on data from several mammalian species. While the practise of artificial insemination shows that seminal plasma is not mandatory for initiation of pregnancy, there is evidence that the success and quality of the pregnancy, particularly as measured by growth trajectory of the fetus, are compromised if females are not exposed to seminal plasma. Experiments in which the seminal vesicle, prostate or coagulating glands are surgically removed from mice, rats and hamsters prior to mating each show that seminal vesicle fluid is the most vital nonsperm component of the ejaculate.[12-15] In mice, embryo transfer protocols generally employ recipients exposed to seminal plasma by mating to vasectomised males, but fetal loss and abnormality is considerably greater when pseudopregnancy is achieved without exposure to male fluids.[16] When recipient females are mated with seminal vesicle deficient males, transferred embryos yield fetuses with altered growth trajectories and this is associated with changes in placental development.[17] In rats, implantation rates and fetal growth are similarly impaired unless females are inseminated prior to embryo transfer.[18] In pigs, artificial insemination with diluted semen reduces litter sizes but mating with a vasectomised male or administration of heat-killed semen restores litter size and improves farrowing rate.[19,20]

Clinical studies in humans have shown that live birth rates in couples undergoing IVF treatments are significantly improved when women are exposed to semen at the time of embryo transfer.[21,22] Furthermore, treatment of women suffering from recurrent spontaneous abortion with seminal plasma pessaries has been reported to improve pregnancy success.[23] In preeclampsia, there is a cumulative benefit of chronic exposure to semen, with limited sexual experience or use of barrier methods of contraception being linked with increased risk,[24,25] and evidence from women where a change in male partner has occurred suggesting that the effect is partner-specific.[26] Markedly increased rates of preeclampsia are also evident in pregnancies initiated by donor oocytes or semen,[27] when prior exposure to sperm or conceptus antigens has not occurred.

Active Factors in Semen

Experiments in mice from which accessory glands were surgically removed showed that the active inflammation-inducing moieties in semen are derived from the seminal vesicle, the principal constituent of seminal plasma in the mouse.[2] Using protein chromatographic techniques and neutralising antibodies, TGFβ was identified as the key component for induction of uterine epithelial GM-CSF synthesis following mating in mice.[28] The TGFβ content of seminal

vesicle fluid is 70 ng/ml;[29] approximately five-fold the content of serum, and similar to that of colostrum which is the most potent biological source of TGFβ known. Seminal vesicle TGFβ synthesis is testosterone dependent, with a severe reduction evident after castration, and partial recovery after administration of exogenous steroid hormone.[29]

TGFβ is also identified as the principle active moiety in human semen in experiments using primary and transformed human cervical keratinocyte cultures.[10,11,30] This cytokine was initially identified in the plasma fraction of human semen when it was recognised to confer inhibitory bioactivity in prostatic carcinoma cell lines.[31] Unlike TGFβ in serum, which is present exclusively in the latent form complexed with β2-microglobulin, approximately 25% of TGFβ in human and rodent seminal plasma exists in the mature, active form. Subsequently it was shown using isoform-specific immunoassays that TGFβ in human semen is principally of the TGFβ1 isoform, with a lower content (5-10%) of TGFβ2.[32,33] The content of TGFβ3 approximates that of TGFβ1 yielding a final concentration of approximately 300 ng/ml total TGFβ (Sharkey and Robertson, unpublished data). Responsiveness of both murine uterine epithelial cells and human cervical epithelial cells to TGFβ is maximal at ovulation.[10] Whether this reflects a differential expression in TGFβ receptors or other components of the docking or signal–transducing repertoire of molecules remains to be elucidated.

Other inflammation-inducing moieties present in semen are likely to synergise with TGFβ in targeting female tract cells, and may act differentially between species and even between individuals in a population. ϖ Prostaglandin E (PGE) is abundant in human semen as the 19-hydroxy form, but is undetectable in rodent and porcine seminal plasma. In vitro experiments with cultured human cervical explants show that 19-hydroxy PGE promotes expression of chemotactic IL-8 and inhibits expression of the anti-inflammatory molecule secretory leukocyte protease inhibitor (SLPI).[34] Another abundant seminal plasma cytokine is IL-8, which synergises with TGFβ to induce IL-1β, IL-6 and LIF from endometrial epithelial cells.[11]

Bacterial lipopolysaccharide similarly acts to induce cytokine synthesis in murine and human uterine and cervical epithelial cells, presumably through binding to Toll-like receptors TLR2 and TLR4. Of emerging interest is the impact of different 'probiotic' versus pathogenic bacterial species in the male and female tract flora.[35] Through differential binding to TLRs and other pattern recognition receptors on the surface of reproductive tract epithelial cells, the relative abundance of different bacterial species would further influence the character of the cytokine response.[36] Finally, we find that the type-1 cytokine interferon (IFN)-γ acts as a potent inhibitor of TGFβ signalling both in human and mouse epithelial cells.[37] Together this provides an emerging picture of multiple active seminal constituents acting in concert to elicit expression of several cytokines in the female tract, and implies likely variation in the strength and pattern of response elicited by individual seminal fluids within a male population.

Consequences of the Post-Mating Inflammatory Response

The inflammatory response accompanying insemination impacts on several reproductive processes by virtue of the wide variety of potential actions of the leukocytes recruited into the endometrial and cervical tissues. Four categories of effector function are postulated; (1) clearance of superfluous sperm and microorganisms introduced into the uterus at mating; (2) activation of female immune responses specific to paternal transplantation proteins and other antigens present in semen; (3) tissue remodelling associated with preparation of endometrial receptivity; and (4) activation of expression of cytokines and growth factors implicated in preimplantation embryo development (Fig. 1).

The distribution of seminal material within the female tract after coitus would constrain the tissues infiltrated by inflammatory cells and thus the range of downstream effects in a species-specific manner. In rodents and pigs the ejaculate fills the uterine lumen and clearly can directly access the implantation site, but in humans an impact on the uterine environment is more difficult to envisage, with semen deposition occuring at the external os of the cervical canal. Of relevance to this is the observation in humans that seminal plasma constituents including TGFβ are bound to the postacrosomal region of the sperm head and thus presumably

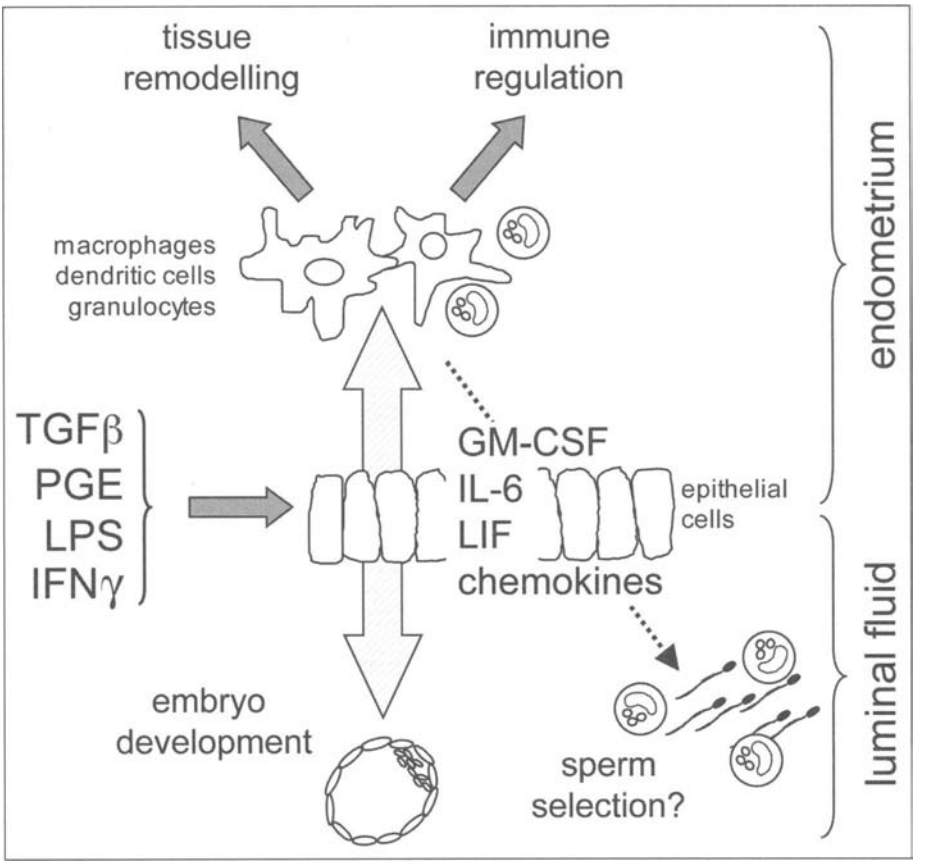

Figure 1. Schematic illustrating potential roles for the post-mating inflammatory cascade in promoting uterine receptivity and embryo implantation. Seminal plasma cytokines including TGFβ, 19-OH PGE and IFNγ, as well as bacterial LPS, synergise to activate expression of pro-inflammatory cytokines in uterine and cervical epithelial cells after mating. Cytokines including GM-CSF, IL-6, LIF and several chemokines such as MCP-1, KC and RANTES act to recruit and activate macrophages, dendritic cells and neutrophils into the endometrial stroma. Infiltrating macrophages and dendritic cells have roles in processing and presentation of seminal antigens to activate maternal immune tolerance. Macrophages further act to secrete growth factors, MMPs and other enzymes and angiogenic molecules to promote tissue remodelling and vasculogenesis. Neutrophils passing into the luminal space participate in clearance of debris and micro-organisms introduced at mating, but may also contribute to sperm selection through phagocytosis of redundant sperm. Cytokines secreted into the luminal fluid target the developing embryo.

are carried together with sperm into the higher tract.[38] Furthermore delivery of seminal plasma as well as sperm from the cervix into the uterus and Fallopian tubes is served by a process of rapid and sustained uterine peristaltic contractions which transport macromolecular material to the tube ipsilateral to the dominant follicle independently of any motile or chemotactic properties of sperm.[39]

Sperm Selection and Clearance of Seminal Debris

One obvious role for the abundant populations of neutrophils that emigrate between epithelial cells into the uterine lumen is phagocytosis and clearance of micro-organisms and seminal debris remaining in the tract after intromission. The higher reproductive tract is normally

sterile, with insemination providing the opportunity for access by commensal micro-organisms originating from male and female tissues including sexually transmitted pathogens. In mice, bacteria are prevalent in the uterus after insemination but sterility is recovered within 24 h, even in GM-CSF null mutant mice where impaired macrophage function predisposes to uterine infection in virgin animals.[40] The physiological significance of seminal plasma in uterine clearance is illustrated in livestock species where rapid resolution of the uterine inflammatory response is linked with pregnancy success.[41,42]

Phagocytic activity in the cervical or uterine lumen targets spermatozoa as well as bacteria. Sperm selection is an interesting potential function of female leukocytes recruited across the luminal surface of the tract; through differential resistance to phagocytosis individual sperm appear to be selected for fertilisation competence and on the basis of other morphological or antigenic parameters,[43,44] such as haplotype in the MHC-linked t-complex.[45] Whether female immune cells can actively select and inactivate sperm within a single ejaculate or distinguish between sperm of different mates in polyandrous species requires investigation. While antibody and complement-mediated opsonisation would provide a potential mechanism for selection, the molecular basis of discrimination and the identity of any target structures remain to be characterised.

Priming the Maternal Immune System to Paternal Antigens

Macrophages and dendritic cells comprise the major populations of cells recruited into the endometrial stromal tissue after exposure to semen. Both have professional antigen processing and presenting ability and are implicated in initiating active immune responses to paternal MHC and other antigens in semen. The consequences of this would be important for future female tract immune responses to semen, as well as for pregnancy, since the conceptus shares paternal antigens with those in semen. Early in the post-mating inflammatory cascade, these cells accumulate subjacent to the surface epithelium in the endometrial stroma,[2,3] then engulf and process paternal ejaculate antigens, before trafficking to para-aortic lymph nodes (PALN) draining the uterus, the mesenteric lymph nodes and spleen.[16,46]

These events result in activation and proliferation of lymphocytes in draining lymph nodes. The PALN of mice enlarge after allogeneic insemination,[47] as T-lymphocyte proliferation commences and expression of cytokines and activation antigens becomes evident.[48] Matings with vasectomised males indicate that lymphocyte activation occurs independently of sperm but in contrast, males from which seminal vesicle glands have been surgically removed fail to stimulate PALN cell proliferation or cytokine synthesis.[48] Amongst the responding cells are T-lymphocytes reactive with paternal MHC antigens, which are aggressively immunostimulatory in graft–versus-host assays for the first two days after insemination and then show evidence of suppressive regulation.[49] Similarly, paternal specific alloreactivity is strongly suppressed in para-aortic lymph node cells recovered on day 3 of pregnancy from rats.[50] Similar kinetics are evident in the time course of expansion of TGFβ-producing suppressor cells identified within the para-aortic lymph node from the time of implantation,[51] and in the appearance of T regulatory cell populations, which have recently been shown to increase in the blood, lymph nodes and spleen of mice within three days after mating.[52]

Induction of Maternal Immune Tolerance for Implantation

Activation of maternal lymphocytes after mating raises the possibility that exposure to semen can impact on the phenotypes or abundance of lymphocyte subsets regulating implantation and placental morphogenesis. In rodents, specific populations of lymphocytes appear within the decidua to promote placental growth and development during the first days after implantation. These lymphocytes include α/β and γ/δ T-cells,[53,54] NKT cells[55] and NK cells.[56] Based on the kinetics of their induction and the similarity in phenotypes between decidual and PALN lymhocytes, we have hypothesised that the appearance of these cells in the implantation site might be causally linked with the female immune response to ejaculate antigens.[48,57] The

possibility that lymphocytes activated and induced to proliferate at insemination might be selectively recruited into uterine implantation sites after recirculation via the blood has been evaluated by passive transfer experiments in pregnant mice. Lymphocytes recovered from the PALN after insemination and radiolabelled prior to passive transfer into pregnant recipients can indeed be shown to home to implantation sites in the uterus.[48]

The lymphocytes present in the implantation site are largely antigen nonspecific T-regulatory cells and NK cells. Uterine NK cell precursor cells are known to originate in tissues other than the uterus, with the spleen identified as the richest source and a lesser contribution from peripheral lymph nodes,[58] so it is unlikely that PALN-derived cells add substantially to this lineage. In contrast, it is possible that NKT cells activated in PALN at insemination home to the uterus and contribute to the dramatically (40-fold) expanded NKT population evident in the implantation site by day 6 of pregnancy.[55] Uterine NKT cells are T-cell receptor $V\alpha14^+$ but express a novel Vβ repertoire reactive with a class I/Ib molecule other than CD1 expressed by placental cells.[59] If semen were to provide or induce in female tissues the yet to be identified class I/Ib molecule, this could provide a mechanism for NKT activation in early pregnancy. This possibility is supported by the presence of MHC class Ia and Ib molecules in semen[60] and of α-galactosylceramide and other glycolipids in sperm.[61]

The smaller contingents of antigen-specific lymphocytes present in the implantation site might reasonably recognise paternal antigens present in semen and shared by the conceptus. Semen contains abundant major and minor histocompatability and other antigens,[62] as well as somatic cells such as leukocytes and desquamated genital tract epithelial cells,[63] and soluble HLA.[64]

The quality of any lymphocyte response raised to seminal antigens would need to be consistent with maternal tolerance of the conceptus at implantation. Seminal plasma contains several powerful immuno-regulatory molecules that can dampen potentially destructive Type-1 (cell-mediated) immune responses and drive immune outcomes of the quality required for functional immune tolerance. Both the PGE and TGFβ present in semen have well described immune-deviating properties in other tissues.[65,66]

Consistent with this, changes in T-lymphocyte status in draining lymph nodes and peripherally in the female after insemination are accompanied by evidence of a transient state of hypo-responsiveness in paternal alloantigen reactive lymphocytes. That semen can induce functional immune tolerance to male antigens was first suggested by experiments showing that mated mice are unable to reject syngenic skin grafts of paternal origin.[67] Subsequently it was demonstrated that protection is similarly conferred to major histocompatibility antigens,[47] but only when sperm is delivered in the context of seminal plasma. Washed sperm, but not whole semen, was shown to elicit transplantation immunity to paternal skin graft challenge, despite both immunisation events leading to lymph node hypertrophy. Likewise, immunisation with washed sperm but not natural insemination primed mice for generation of cytotoxic T-lymphocytes against H-Y antigen.[68] The potential beneficial effect of this immune response for pregnancy outcome has been identified in experiments showing that uterine 'priming' with semen can promote implantation and fetal growth in subsequent pregnancies, in a partner-specific manner.[47,69] Consistent with an immunological mechanism, removal of lymph nodes draining the uterus after exposure to semen revoked the effect and led to a decrease in litter size and fetal and placental weight.[70,71]

Contribution to Tissue Remodelling

In evaluating the impact of the post-mating inflammatory cascade it is important to recognise that leukocytes exert effects in their local milieu other than through activating immune responses. Macrophages and granulocytes secrete an array of potent enzymes and signalling molecules that can elicit proliferation, differentiation or other functional changes in the status of adjacent nonhemopoietic cells (see Chapter 6). Through influencing the structure of the extracellular matrix and the behaviour of endothelial cells, epithelial cells and fibroblasts comprising

the endometrium, tissue remodelling roles which assist in the preparation for pregnancy can be envisaged.

Foremost amongst macrophage- and granulocyte-derived enzymes are the matrix metalloproteinases (MMPs), a family of zinc-containing endo-proteinases that share structural domains but differ in substrate specificity and regulation of synthesis. Macrophage production and secretion of large quantities of MMPs is regulated by local cytokine environment, with induction after exposure to factors including GM-CSF, tumor necrosis factor alpha (TNF)α and IL-1. The catalytic activity of these enzymes is pivotal for cyclic endometrial breakdown and regrowth, and for the remodelling underlying embryo implantation and decidualisation.[72] Precise spatial and temporal patterns of expression of the MMP family and its regulatory component, the tissue inhibitors of metalloproteinases (TIMPs), are characteristic of the pre and peri-implantation period in rodents.[73] The significance of leukocytes recruited in response to seminal factors in regulating MMPs during early pregnancy has not been evaluated but consistent with such a role is observations in rats that expression of MMP-7 (matrilysin) is highest on the first day after mating,[74] with MMP-2 also induced prior to embryo implantation.[75] In golden hamsters, induction of pregnancy in the absence of male accessory gland fluids is associated with reduced expression of MMP-2 in the implantation site.[76]

Inflammatory leukocyte regulation of endothelial cells in the angiogenic response provides another potential avenue for seminal factor effects on implantation. Vasodilation and oedema are associated with the inflammatory response to semen in mice and several other species.[6,77] Vascular endothelial growth factor (VEGF) as well as other key angiogenic factors IL-1, TNFα and basic fibroblast growth factor (bFGF) are identified as products of activated macrophages and clearly are candidate mediators of the endothelial changes induced by semen.[3,78] That VEGF mRNA expression in the implantation site is diminished when pregnancy is initiated by accessory-gland deficient males in golden hamsters suggests that the consequences of semen-induced angiogenic changes perpetuate beyond the acute inflammatory period.[76]

A further target for the actions of macrophage-secreted products in early pregnancy are the luminal epithelial cells involved in embryo attachment during the initial phases of embryo implantation. The 'window of implantation' or opportunity for embryo adhesion is defined by specific changes in the expression of epithelial integrins and mucins, allowing close apposition between the blastocyst and the luminal surface, and finally adhesion of the two cell surfaces.[79] While ovarian steroid hormones clearly have an overarching role in regulating these changes, macrophages are closely juxtaposed with processes indigitating between epithelial cells in the endometrium, and this spatial association affords a potential role in influencing integrin expression at the paracrine level. That leukocytes may directly regulate the adhesive properties of epithelial cells has been demonstrated with human uterine epithelial cells in vitro, using membrane spheroids from the choriocarcinoma cell line BeWo.[80] The ability of macrophages to alter transport properties and maintenance of epithelial barrier integrity[81] might further contribute to implantation through facilitating trophoblast breaching of the epithelial surface.

Activation of Embryotrophic Cytokines

The cytokines induced after exposure to semen target not only maternal leukocytes in the endometrial stromal tissue, but also are secreted into the luminal space to potentially interact with the developing embryo as it traverses the oviduct and uterus prior to implantation. Several cytokines activated by semen are amongst those attributed with regulating proliferation, viability and differentiation of blastomeres in embryos.[82,83] GM-CSF, a major component of the post-mating cytokine response in mice,[28,84] is identified as essential for normal blastocyst development and subsequent viability. Expression during early pregnancy also occurs in the uterus and oviduct of women[85] and other mammalian species.[86,87] GM-CSF targets the preimplantation embryo to promote blastocyst formation, increasing the number of viable blastomeres through inhibiting apoptosis and facilitating glucose uptake.[88] Human embryos cultured in GM-CSF are twice as likely to reach the blastocyst stage of development, blastulate earlier and

have increased cell numbers both in the inner cell mass and trophectoderm.[89] Other cytokines targeting the developing blastocyst including IL-6 and LIF are similarly induced after exposure to semen[11,90] (SAR unpublished data).

Summary and Conclusions

A significant body of evidence now exists to link exposure to semen with pregnancy success in human, rodents and several additional mammalian species, and the studies reviewed herein are beginning to provide explanations for the underlying molecular and cellular mechanisms. Seminal plasma can thus no longer be considered simply a sperm transport medium, but instead must be recognised as a means for communication between the male and female reproductive tissues. This function of seminal plasma presumably has its evolutionary origins in benefiting the likelihood of a pregnancy after insemination by a given male. From the female perspective the opportunity for activation of immune events prior to implantation may facilitate sperm selection and discrimination between competent and suboptimal embryos.

To date, research in this field has focussed largely on rodent and livestock species, and for obvious reasons the significance of seminal factors in humans have been more difficult to explore. While direct extrapolation from the rodent to the human may not be justified, clearly these findings have implications for the association between semen exposure and the incidence of pathologies of human pregnancy. We speculate that the aberrant Type 1 immunity associated with 'shallow' placentation in preeclampsia and recurrent miscarriage[26,91] can be initiated by insufficient or inappropriate immune responses to seminal antigens following intercourse, perhaps linked with seminal plasma cytokine deficiency or female incapacity to respond to seminal signals. There are additional implications for assisted reproductive technologies, where pregnancies are routinely initiated in the absence of natural intercourse. A better understanding of the physiological significance of semen in human implantation requires further detailed exploration of the cellular and molecular events within the female reproductive tract at insemination, and may eventually yield novel therapies for infertility and pathologies of pregnancy.

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