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Oral tolerance and the materno-fetal relationship

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ABSTRACT

Oral administration of antigen is a potent route for induction of systemic tolerance. Regulatory T cells and TH3 cellsare generated at intestinal mucosal sites. Oral exposure to antigens in seminal plasma has been suggested to be ableto generate 'tolerance' at the materno-fetal interface that may reduce the risk of pre-eclampsia and recurrent miscarriage. Issues relating to assessing the applicability of oral exposure to seminal plasma antigens in recurrentmiscarriage are discussed. Such tolerance could represent an unappreciated confounder in immunotherapy trials.

This issue of the JRI contains two letters with respect to the recent publication from Meuleman et al. (2019a) implicating lack of oral ± upper gastrointestinal tract exposure to semen in the pathogenesis of recurrent spontaneous miscarriage (RSM). The first letter from Dr. Smits et al. raise a number of important issues with respect to this paper, and the second letter is a reply from the authors.

The first issue raised by Smits et al. (2019) is lack of rationale for the hypothesis that oral exposure and/or ingestion of semen might affect the risk of recurrent spontaneous miscarriage (RSM). A report that seminal plasma peptides could play a role in facilitating induction of tolerance in CBA/J mice that could prevent abortions (resorptions) when mated to histoincompatible DBA/2 males (the CBAxDBA/2 model) is deemed irrelevant to humans. However, it is well known that data obtained using mice has been very helpful in understanding and treating human disease, and if data obtained using mice were not relevant for humans, such studies would not be funded (Clark, 2014). Whilst one cannot directly extrapolate from a mouse model to humans, one can determine if findings in studies of the CBAxDBA/2 model have homologous findings in humans! Human RSM is poorly understood by clinicians, and so called 'causes' are, in most cases, merely associations which have not proven causal since to be causal, correcting such problems must prevent further RSM and result in live births. What is known that in primary RSM (3 consecutive losses and no live births with that same partner), 55% of abortus tissue is karyotype abnormal with a proclivity to repeat in subsequent pregnancies (Coulam et al., 1996), and this is very different from mice were the abnormal karyotype embryo rate is about 2–3%. [Interestingly, a parental karyotype abnormality does not seem to be of much value absent evidence that the parental chromosome abnormality is present in abortus tissue (Carp et al., 2004, 2006).] Secondary RSM (an initial live birth followed by consecutive losses) has a lower % abnormal karyotype embryos and has a well researched immunological basis of normal embryo losses where the first born was male (Christiansen et al., 2012). Considerable research has been done to try to identify the subset of primary RSM patients with immunologically-modifiable RSM, and there is a body of evidence indicating certain types of immune therapy may be efficacious, but identifying those who are likely to benefit cannot be done using obstetrical history alone. Studies done using inbred strains of laboratory mice have been helpful in shaping research studies in humans. In mice, resorptions occur because aborting a failing embryo would compromise the remaining otherwise normal fetoplacental units, but even so, occasionally the pregnant female may expel dying embryo tissue. Conversely, in humans, resorptions occur (e.g. the vanishing twin), and in missed abortion embryo death is not necessarily accompanied by symptoms such as pain, vaginal bleeding, and expulsion of the feto-placental tissue (Nakashima et al., 2010). So the external expulsion of dead embryonic tissue is not necessarily the essential event the pathogenesis of embryonic death in recurrent pregnancy loss. The CBAxDBA/2 model has been found to manifest many of the elements in human RSM particularly with respect to the key role of innate immune system effector cells such as NK cells producing pro-inflammatory cytokines, the essential role of complement and a novel direct prothrombinase in the pathogenesis of death of the feto-placental unit, and immunological mechanisms countering abortogenic cells including generation of tolerance-related Treg cells and suppressor cells producing IL-10 and TGF-β-related molecules (Knackstedt et al., 2001; Clark, 2014, 2016a, 2016b). In both mice and women, trophoblast cells form endovascular plugs to prevent oxidative damage during the first trimester after a distinct fetus and placenta have formed and this process is compromised in pregnancies destined to spontaneously abort (Clark, 2016a; Burton and Jauniaux, 2004). It is also clear that there can be recurrent pregnancy loss occurring at earlier stages of pregnancy manifest as occult loss or chemical pregnancies, and this also occurs in certain mouse models where it has been shown that T cell-mediated inflammation causes embryonic death and Treg cells counter this process (Clark, 2016a, 2016b). In the CBAxDBA/2 model which manifests RSM but not occult losses, DBA/2 seminal plasma was found to be lacking in putative peptides needed to induce protective anti-abortive

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Tregs mediating so-called maternofetal tolerance (as cited by Smits et al., 2019a) and BALB/c seminal plasma peptides were required. That makes the CBAxDBA/2 model somewhat different from the human situation where an induced immune response to the husband's antigens appears able to prevent further losses. However, in the B10xB10A mouse model of RSM, immunisation with B10A male cells *was found to be* protective (Chaouat et al., 1988). From studies of mice, one knows that the manner in which antigen is presented determined the type of immune response, that antigen presented at a mucosal surface such as the intestine can cause 'oral tolerance', and there is a common mucosal immune system such that Treg cells activated at one mucosal surface enter the circulation and may, like intestinal B cells, may home specifically to all mucosal surfaces (McDermott et al., 1980; Siewert et al., 2007). This is a feature of antigenic tolerance mediated by Tregs in peanut allergy which is currently being effectively prevented in humans using oral immunisation methods in early post-natal life (Nelson, 2018; Palomares et al., 2018). Indeed, as Meuleman et al. (2019b) point out in their rebuttal letter, there are a very large number of publications documenting that presenting antigens via the oral-intestinal route is a powerful and effective way to induce 'tolerance'. It makes sense that antigens at such mucosal sites should result in tolerance rather than a Th1-cytokine generated inflammatory response if only because continuous exposure to foreign antigens, whether in food or in flora cannot be allowed to compromise mucosal function that would arise if there were induction of continuous inflammation, and this protection must work from mouth to rectum. Finally, it may not be necessary to ingest antigens such as seminal plasma peptides to induce tolerance. Koelman et al. (2000) reported those who had oral exposure to semen had a 33% risk of preeclampsia versus 74% absent oral exposure, and among those who had oral exposure and swallowed, the risk or preeclampsia was only 25% versus 61% in controls. Sublingual induction of tolerance to antigens causing allergy is known to be effective although it requires continuing frequent exposure to antigen (Palomares et al., 2018; Nelson, 2018). The placenta is, of course, a source of paternal antigens, and in some species maternal consumption of the fresh placenta may have extra-nutritional benefits via induction of 'tolerance'. That could be important in preventing harmful maternal immunity to the male H–Y antigen that has been implicated in secondary RSM in humans where the HLA phenotype of the mother may predispose to generation of a harmful rather that helpful immune response (Christiansen et al., 2012).

In humans with RSM, Coulam and Stern (1995) studied vaginal application of seminal plasma capsules and improved implantation rates, and similarly and independently, Tremellen et al. in Robertson's group showed in a prospective RCT that exposure to male seminal plasma per vaginum improved implantation rates in IVF failure women. A 2019 systematic review and meta-analysis by Saccone et al. (2019) confirmed that vaginal exposure to seminal plasma improved the clinical pregnancy rate after IVFET. However, that has not yet translated into improved live birth rates or reduced miscarriage rates. A reasonable hypothesis could be the need for a larger dose of antigen and/or delivery by a more effective route. As discussed above, the oral-intestinal mucosal route may be far more effective than the vaginal-cervical mucosa. There are now many controlled studies showing that exposure to paternal mononuclear cells antigens at systemic sites can significantly reduce miscarriage rates and improve live birth rates, so one may suspect the route of exposure and/or absorbed dose of antigen and co-presence of the CD200 immune check-point inhibitor may be quite important. Oral immunisation with epididymal spermatozoa (minus seminal plasma) was found to decrease fecundity in a rat model (Allardyce, 1984) consistent with the idea that seminal plasma may promote tolerance given that oral sex in humans does not seem to compromise fecundity. Since seminal plasma peptides were shown in the CBAxDBA/2 model and by Robertson's group to promote protective Treg responses as mentioned by Meuleman et al. (2019b), so the Allardyce (1984) finding may reflect removal of immunoprotective seminal plasma peptides. We know from the Guadeloupe study by Robillard and Hulsey (1996) that multiparous women who take a new partner had a 50% risk of developing preeclampsia if they conceive within a month but that dropped to 5% if conception occurs after a year to exposure to that partner's seminal plasma, but this merely shows that physiological dosing by the genital route can take a long time to induce a protective response in the future mother. The Meuleman et al. (2019a) cited the JRI paper of Koelman et al. (2000) as reporting women with oral + intestinal exposure to semen had a lower risk of pre-eclampsia (discussed above). Mechanisms underlying preeclampsia are not dissimilar to immunological mechanisms in RSA, and both conditions can be prevented by exposing the woman to paternal antigens on blood mononuclear leukocytes delivered at other than genital sites (Clark, 2012). Therefore, it was entirely reasonable for Meuleman et al. to seek *any* evidence that exposure to seminal plasma at a different mucosal surface than the genital tract might induce an anti-abortive response having a direct impact on the outcome of a conception, and their study was approved by the local Ethics Committee.

The second issue raised by Smits et al. (2019), concerns the study design and its analysis. A retrospective analysis was a feasible and necessary prerequisite for design and ethical approval of a prospective study. Retrospective studies have caveats due to incomplete reporting and fallibility of memory, but retrospective analysis of memory of perceived stress done by Arck et al. has been able to identify a subset of RSM women where stress may cause RSM (and stress also causes immunologically-modifiable RSM in mice where the molecular pathways have been worked out!). A recent systematic review and meta-analysis by Qu et al. (2019) provides robust evidence that stress may be aetiologic in susceptible women. Meuleman et al. (2019a) recognized the problem with missing data, and used a statistical technique to show that the anti-abortive effect of oral/upper GI exposure to semen was associated with a statistically significant lower RSM rate might not be statistically significant. Unfortunately, they did not explain exactly what data was missing and how the statistical interpolation using the chained equation algorithm was done, but they did they acknowledge the possibility that only a subset of RSM cases would likely benefit from an immune response to semen. An article by Van Buuren and Groothuis-Oudshoorn, 2011 in the Journal of Statistical Software stated, "The chained equation algorithm possess a touch of magic." As such, Meuleman et al.'s paper might have been more comprehendible by readers of a biostatistics journal, but that would have missed the more important target audience, clinicians and scientists trying to understand and remedy human RSM. Indeed, missing data in the RSA group using the non-RSA control group, as explained in the reply from Meuleman et al. (2019b), may have biased the result. It is therefore not possible to explain away a significant result (on which the Meuleman et al. paper's title was based). The putatively 'non-significant result' after statistical manipulation concerned the 97 cases and 137 controls that were in the matched analysis, and their power analysis had indicated 93 women in each group ***would be required to completely fill out the questionnaire to avoid missing a statistically significant effect 20% of the time. Not all women completed the questionnaire, and of those who did, 41/72 cases and 70/96 matched controls had oral sex (P = 0.04), so the end result was underpowered!*** An underpowered result does not allow one to accept the null hypothesis (Clark et al., 2001). Did the reduced P value of 0.21 allow the conclusion that oral sex *did not* reduce the risk of RSM? No, that P value meant that for every 50 cases, 40 out of 50 had a successful pregnancy due to oral sex but 10/50 were successful by chance alone (versus the initial result before adjustment where 48/50 did better due to oral sex and 2/50 did better due to chance. *A P value of ≤ 0.05 is arbitrarily imposed by accepted convention as the criterion for 'statistical significance'.* In a casino, a gambler is happy if his or her chance of winning is > 0.5. But Meuleman et al. (2019a) had a solution to the need for P ≤ 0.05. To make their study more robust, they employed a second control population, a Dutch Reference group of 1259 women, and 89.2% were affirmative about oral sex question

which when compared to the 57.1% of 77 RSA women who filled out the questionnaire was a statistically significant difference at $P < 0.001$, so of 1000 non-abortive pregnancies, 999/1000 could be a result of oral sex. So the weight of evidence supported the association of oral sex with fewer miscarriages. Even so, in the present medical-political environment, advocating a treatment requires proof of efficacy, and Evidence-Based Medicine requires controlled trials, preferably prospective double blind and randomized, more than one, and preferable with concomitant lab evidence of immune responses in the woman to the immune intervention and with quantitation of the potential confounder of stress reduction. A RCT is not necessarily the *sine qua non* proving clinical efficacy using the Grade criteria, a cohort-controlled trial (which is much easier to do given difficulties recruiting women to a study that is randomized) can be more reliable than a RCT (Clark, 2009). No prospective study is likely to be funded without a preliminary retrospective study. However, any prospective study whether a RCT or cohort-controlled stud seems likely to have a biased sample made up of those types of individuals who were willing to fill out a questionnaire for the Meuleman et al. (2019a) study. There will be the willing and the unwilling, and these populations cannot be assumed to be biologically identical. So the Meuleman et al. (2019a) study has potentially enabled identification of problems that informs for designing a better study given better understanding of subject characteristics.

The third issue raised by Smits et al. is expressed as concern for an "incredibly vulnerable population" which is desperate to try anything, and who might be induced to change their sexual practices (to that reported by 89.2% of Dutch women?) based on sensationalism by exploitative tabloid-type media, and so Meuleman et al. (2019a) should never have been published with a title claiming efficacy. But why should they be vulnerable if they are in the care of a compassionate clinician *able* to deliver effective TLC, who has *up-to-date expertise and in-depth knowledge based on published research*? Such a person would be able to explain the importance of proper diagnosis in order to determine if they are in a subset that might benefit from an immunological treatment, would be able to outline alternative options, and would be able to explain what is and is not known. There is a moral obligation for investigators to publish their research since without that stimulus, better studies that will lead to effective application in the clinic are unlikely to be done. Sensationalism by media, who take information out of context, is a problem, but one cannot limit free speech in non-autocratic Western democracies, even for religious reasons, and all that investigators (clinician scientists) can do is to try to educate the public. Even so, it would be unethical to decline publication based on devotion to political correctness and to prefer ignorance over knowledge, and the Meuleman et al. (2019a) study, as already mentioned, did have institutional ethics approval. Moralistic passion directed at this publication would do more good if it were directed instead at clinicians managing RSA patients since the "incredible vulnerability" of this group has been argued by some clinicians as a good reason for prohibiting testing products of conception for evidence of fetal or trophoblast karyotype abnormalities (which can be done with modern genetic technology without having to grow the cells in culture). Such reluctance to properly investigate to make a proper diagnosis (that may require additional sophisticated laboratory testing), and to ensure that clinical trial results get confirmed, does an incredible disservice to their patients since potentially effective alternative non-oral sex immunotherapies exist (e.g. Clark et al., 1996; Winger and Reed, 2008; Clark, 2011, 2012; Gomaa et al., 2014; Dakhly et al., 2016; Liu et al., 2016). It is not surprising they are desperate and will even risk bankruptcy to pay for IVF that is likely to fail without treatment of immune abnormalities (Winger et al., 2011; Dakhly et al., 2016).

It is a common caveat found at the end of preliminary studies to state that a further larger study needs to be done. But might there be a better approach than correlating oral exposure to semen with outcome? Indeed, that may be the case. Simple math indicates 56.9% of RSA women are having oral sex so 43.1% are not. Assuming oral tolerance is induced by oral sex and prevents immune-mediated abortion, those having oral sex are likely to be losing karyotype abnormal embryos (which occur 55% of the time from the Coulam et al., 1996 study and which is a recurrent problem), so the 43.1% (who are not having oral sex) may be predicted to be losing otherwise normal embryos (which occurs 45% of the time). Determining the karyotype of the losses in the manner described by Coulam et al. (1996) could be a good way to test the biological validity of the hypothesis, and circumvent the problem that the 4 groups (oral sex no RSM, no oral sex no RSM, oral sex RSM, and no oral sex RSM) may never be completely matched with respect to other parameters that may be relevant to the risk of RSM. Further, applying the Bradford-Hill criteria to assess causality, one would expect the efficacy of oral sex would depend on the dose and duration of exposure, and as LIT is more effective if started or continued during pregnancy, a similar timing issue would be found with oral sex. It is also important to determine in what way the RSM couples having oral sex differ from those not having oral sex. Whilst oral sex would be predicted to have a very high success rate in the RSM subgroup abstaining from oral sex by preventing immune rejection triggered by stress mechanisms, the decision to start having oral sex may cause additional stress that could abrogate protection. If this were the case, it would be the responsibility of the medical community to offer effective alternatives. Offering corticosteroids, is suggested in the reply from Meuleman et al. (2019b) but that data is conflicting, and the RCTs done to date indicate there are more effective alternatives of which Smits et al. (2019), and Meuleman et al. (2019b) appear unaware. Finally, if oral sex does promote generation of anti-abortive Treg cells, oral sex will be a confounding factor in immunotherapy trials which could obscure protective effects of treatment (Clark, 2012). But the "if" means the analysis of such studies needs to be repeated to determine if the subpopulation engaging in oral sex are, in fact, already tolerant of paternal antigens by determining the karyotype of subsequent miscarriages with and without immunotherapeutic treatment. Absent such information, any prospective study of immunotherapy would need to stratify subjects for effects of oral sex. Thus, a great deal has been learned from the Meuleman et al. (2019a) publication.

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David A. Clark
McMaster University, Hamilton, Ontario, Canada
E-mail address: clarkd@mcmaster.ca.