Evaluation of fertility outcome as a consequence of intravaginal inoculation with sperm-impairing micro-organisms in a mouse model

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The concept of infertility as a result of asymptomatic microbial colonization of the female reproductive tract has been neglected to date. However, increasing incidence of infertility and advanced research has drawn attention towards this idea. Many of these micro-organisms have been reported to bring about adverse changes in sperm parameters in vitro, but their in vivo potential to cause infertility is still a controversy. The present study was carried out to observe what effect the intravaginal inoculation of sperm-agglutinating *Serratia marcescens* and sperm-immobilizing *Candida albicans* had in the reproductive tract and consequently in fertility outcome.

When these strains were intravaginally inoculated into female BALB/c mice at $10^4$, $10^6$ and $10^8$ c.f.u. in 20 µl PBS for 10 consecutive days, with mating of mice on day 12, the results showed 100% decrease in fertility in all groups as compared with control mice receiving PBS alone. Furthermore, no clinical or histopathological changes were observed in the reproductive organs (i.e. ovary, uterus and vagina), suggesting that colonization of the genital tract with sperm-impairing micro-organisms could be a feasible reason for female infertility.

INTRODUCTION

Infertility can be defined as the lack of conception after at least 1 year of constant, unprotected sexual intercourse and impacts 10% of couples in the general population (Habbema *et al.*, 2004). It is a life crisis with invisible losses and diverse consequences. It can be due to a variety of diseases and medical conditions that affect one or both members of a couple. Genital infections are one of the most important causes of infertility worldwide, affecting anatomical urogenital sites in both males and females. Many micro-organisms seem to be involved in reproductive failure in different ways and to different degrees of statistical association (Pellati *et al.*, 2008). These agents disturb female fertility as they lead to obstruction of parts of the reproductive system, pregnancy loss, and substantial effects on perinatal mortality and morbidity (Günaylı *et al.*, 2011).

The clinical significance of sexually transmitted micro-organisms such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Treponema pallidum* has been implicated in infertility (Comar *et al.*, 2012). *Chlamydia trachomatis* infection is generally confined to the lower genital tract; however, it may involve the upper genital tract, and can result in ectopic pregnancy and infertility (Pal *et al.*, 1998). Similarly, *N. gonorrhoeae* can also lead to pelvic inflammatory disease resulting in infertility. Infertility in these cases is a recognized consequence of inflammation.

However, what about other micro-organisms, such as *Escherichia coli* (Huwe *et al.*, 1998; Diemer *et al.*, 2000), *Ureaplasma urealyticum* (Núñez Calonge *et al.*, 1998), *Mycoplasma hominis*, *Staphylococcus aureus* (Jiang & Lu, 1999) and *Candida albicans* (Tian *et al.*, 2007), that have been known to impede sperm motility and alter morphology in vitro, and whose role in vivo is yet to be elucidated? Well-designed studies on infections and fertility are still lacking. In our laboratory we have previously seen infertility as a result of vaginal colonization with sperm-impairing *Staphylococcus aureus* and *E. coli*. The present study was carried out with the aim to investigate the role of other sperm-impairing micro-organisms in female infertility.

METHODS

Micro-organisms. The standard strains of *Serratia marcescens* (MTCC 7641) and *Candida albicans* (MTCC 1637) used in the present study were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Sector 39, Chandigarh, India. The strains were grown in brain heart infusion (BHI) and Sabouraud’s dextrose agar, and maintained as glycerol stocks at −80 °C.

Animals. Sexually mature, 5–6-week-old male and 4–5-week-old female BALB/c mice used in the present study were kept in polypropylene cages and housed in the animal room of the Department of Microbiology, Panjab University, Chandigarh, India. The animals were maintained under standard laboratory conditions (12 h light/12 h dark). Water and feed were available *ad libitum*. The experimental protocols to be carried out were approved by the Institutional Animal Ethics Committee of Panjab University (IAEC/504, dated 2 April 2014). The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

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**Extraction of spermatozoa from mice.** Mouse spermatozoa were collected from 6–7-week-old male mice. The mice were sacrificed by cervical dislocation using a glass rod, and sperm from the vas deferens was collected in RPMI media by gentle teasing.

**Sperm/micro-organism interaction.** The standard strains were checked for their effect on sperm motility. For this, 100 μl bacterial culture was incubated with 100 μl sperm suspension at 37 °C. After different time intervals, a 10 μl aliquot of the mixture was placed on a clean glass slide, covered with a coverslip and observed under magnification using a bright-field microscope (Olympus). A control containing BHI/Sabouraud’s dextrose broth mixed with a semen sample was set up simultaneously.

**Effect of sperm-agglutinating and sperm-immobilizing strains on fertility outcome**

**Screening of mice.** Female BALB/c mice vaginas were screened for the presence of micro-organisms that naturally inhabit the vagina. For this, sterile cotton-tipped swabs were introduced into the vagina and the swabs were gently rotated against the vaginal wall. Swabs were cultured at 37 °C on BHI agar plates. These strains were further checked for any sperm-agglutinating/immobilizing activity in vitro. Mice harbouring micro-organisms that had sperm-agglutinating/immobilizing activity were excluded from the study.

**Preparation of inoculum.** *Serratia marcescens* was cultivated in BHI broth at 37 °C for 24 h, whereas *Candida albicans* was grown in Sabouraud’s dextrose broth for 24 h. The cell culture was centrifuged at 6 700 g for 20 min and the pellet obtained was washed twice with PBS (50 mM, pH 7.2). The cells were then resuspended in the same buffer so as to produce 10⁴, 10⁵ and 10⁶ c.f.u. in 20 μl.

**Intravaginal inoculation.** Female BALB/c mice were divided into four groups (Groups I–IV) of five mice each. Three groups (Groups I–III) of female mice were inoculated intravaginally without any anaesthesia with 10⁴, 10⁵ or 10⁶ c.f.u. of either *Serratia marcescens* or *Candida albicans* per mouse in 20 μl PBS, respectively, for 10 consecutive days. Group IV served as a control and was inoculated intravaginally with 20 μl PBS alone for 10 consecutive days. Vaginal swabs were taken every third day so as to monitor vaginal colonization. The reisolated micro-organisms were confirmed as *Serratia marcescens* and *Candida albicans* by culture characteristics and biochemical identification, and checked for sperm-impairing activity. Vaginal cultures for sperm-imparing micro-organisms were always negative in the control group administered with PBS. The female mice were synchronized in their oestrous cycle by the Whitten effect. This was done by introducing the bedding of male mice soiled with faeces and urine into the cages of females. On day 12, these female mice were allowed to mate overnight with breeder male mice at a ratio of 2:1 to check the effect on fertility outcome. Mating was confirmed on the next morning by observing for the presence of a vaginal plug. The females that did not show a plug were excluded from the study. For the entire period of gestation (21 days), animals were examined for weight change or any other pregnancy-related changes, such as abdominal distension and delivery of pups. The control group mice receiving PBS showed consistent weight gain, and a string of ‘pearls’ could be palpated by day 14 and pups delivered at the end of the gestation period (Figs 1 and 2).

**Histopathological examination of reproductive organs after 10 days’ intravaginal administration of *Serratia marcescens* and *Candida albicans.** The histopathological examination was carried out to check any adverse effects of *Serratia marcescens* or *Candida albicans*.

**RESULTS**

**Effect of sperm-agglutinating and sperm-immobilizing strains on fertility outcome**

The standard strains of *Serratia marcescens* and *Candida albicans* were found to impede sperm motility by agglutinating and immobilization, respectively. Following intravaginal inoculation with different doses of *Serratia marcescens* and *Candida albicans* for 10 consecutive days, vaginal swabs taken every third day showed that the inoculated micro-organisms could efficiently colonize the mouse vagina and became the only isolate in subsequent cultures. Mating on day 12, post-inoculation female mice showed a 100% decrease in fertility when compared with the control group. All the mice were rendered infertile and failed to show any pregnancy-related changes, such as weight gain or abdominal distension and delivery of pups. The control group mice receiving PBS showed consistent weight gain, and a string of ‘pearls’ could be palpated by day 14 and pups delivered at the end of the gestation period (Figs 1 and 2).

**Histopathological examination of reproductive organs after 10 days’ intravaginal administration of *Serratia marcescens* and *Candida albicans.** Another set of experiments was carried out for the histopathological examination of the reproductive organs (i.e. ovary, uterus and vagina) following intravaginal inoculation with *Serratia marcescens* and *Candida albicans*. For this, female BALB/c mice were divided into four groups (Groups I–IV) of five mice each. Three groups (Groups I–III) of female mice were intravaginally administered with 10⁴, 10⁵ or 10⁶ c.f.u. of either *Serratia marcescens* or *Candida albicans* per mouse in 20 μl PBS, respectively, for 10 consecutive days. Group IV served as a control and was inoculated intravaginally with 20 μl PBS alone for 10 consecutive days. On day 12, mice were sacrificed and the reproductive organs were collected. They were fixed in 10% formaldehyde for 24 h and then embedded in paraffin according to standard histological methods. Serial paraffin sections were made, stained with haematoxylin/eosin and observed at × 400 magnification.

**Fig. 1.** Weight profiles of female BALB/c mice receiving different doses of *Candida albicans* (right columns) and *Serratia marcescens* (centre columns) as compared with controls receiving PBS (left columns) during the gestation period. Values represent mean ± SD.

<table>
<thead>
<tr>
<th>Day</th>
<th>Weight (g)</th>
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<tbody>
<tr>
<td>Day 0</td>
<td><em>P&lt;0.05</em></td>
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<tr>
<td>Day 5</td>
<td><strong>P&lt;0.01</strong></td>
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<tr>
<td>Day 10</td>
<td><em>P&lt;0.05</em></td>
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<td>Day 15</td>
<td><strong>P&lt;0.01</strong></td>
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<tr>
<td>Day 20</td>
<td><em>P&lt;0.05</em></td>
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*Candida albicans* on tissue morphology of the reproductive organs (i.e. ovary, uterus and vagina). The results revealed that these organs had normal morphology and there were no signs of inflammation following inoculation with $10^4$, $10^6$ or $10^8$ c.f.u. of either *Serratia marcescens* or *Candida albicans* for 10 days as compared with the control group (Fig. 3).

**Fig. 2.** Representative photographs of pregnancy-related changes in mice. (a) No abdominal distension after different doses of either *Serratia marcescens* or *Candida albicans*. (b) Abdominal distension after PBS. (c) Delivery of pups in controls after the end of the gestation period.

**Fig. 3.** Histopathological examination of reproductive organs (i.e. ovary, uterus and vagina). Representative photomicrographs showing normal tissue morphology of ovary, uterus, vagina of mice treated with PBS (a, b, c), *C. albicans* (d, e, f) and *S. marcescens* (g, h, i) respectively.
DISCUSSION

The greatest hope for lowering the occurrence of infertility related to infection lies in the prevention, detection and treatment of freshly acquired asymptomatic or mildly symptomatic infections (Novy et al., 2008). Sexually transmitted infections have long been linked with infertility. These infections are associated with inflammatory changes in the genital tract that lead to infertility. In females, Chlamydia trachomatis infection sometimes presents itself without symptoms, thereby remaining unnoticed and untreated for long durations. This chronic infection can lead to pelvic inflammatory disease, ectopic pregnancy and tubal infertility (Carey & Beagley, 2010). In men, chlamydial infection can affect sperm quality and function, leading to urethritis, prostaticitis and epididymitis (Cunningham & Beagley, 2008). Likewise, N. gonorrhoeae infection also has a negative effect on fertility outcome, by causing pelvic inflammatory disease in women. Apart from these sexually transmitted micro-organisms, the association of other urogenital tract organisms and infertility still remains speculative. In vitro studies have revealed the detrimental effects of various micro-organisms on human spermatozoa, but their role in vivo on fertility outcome is still controversial. Therefore, the present work aimed to carry out parallel studies under in vitro and in vivo conditions to provide a better understanding of sperm-imparing micro-organisms as a cause of infertility.

The standard strains of Serratia marcescens and Candida albicans were found to impede sperm motility in vitro by sperm agglutination and immobilization, respectively. These results are in concordance with earlier studies showing that Candida albicans had an inhibitory effect on human sperm motility and impaired the ultrastructure of human spermatozoa (Tian et al., 2007), and Serratia marcescens deteriorated the quality of boar spermatozoa (Ubedha et al., 2013). Furthermore, when in vivo studies were carried out to assess the role of these sperm-imparing micro-organisms, the results showed that all the female mice were rendered infertile after 10 days of intravaginal inoculation, whereas control mice delivered pups. Infertility in these mice could not be attributed to inflammation as no histopathological changes were observed in any of the reproductive organs (i.e. ovary, uterus and vagina). From the results it seems reasonable to conclude that the infertility caused by Serratia marcescens and Candida albicans may be due to their sperm-agglutinating and sperm-immobilizing activity, respectively. These results are in concordance with earlier studies performed in our laboratory wherein sperm-agglutinating Staphylococcus aureus and E. coli were shown to cause infertility without producing any adverse effects in female BALB/c mice, whereas non-sperm-agglutinating/immobilizing strains failed to do so (Kaur & Prabha, 2012, 2014).

Thus, it is tempting to conjecture that microbes with sperm-imparing activity can asymmetrically colonize the female genital tract and thus participate in the subsequent development of infertility.

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REFERENCES


