# The Cellular and Subcellular Environmental Entities confronted by Spermatozoa and Ova migrating along the Reproductive Tract of the Human Body §

Shu-dong Yin

Cory H. E. R. & C. Inc. Burnaby, British Columbia, Canada
Email: sdyin@fimpology.com

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## Abstract

According to traditional biology and physiology, our understanding of human reproduction at the cellular level is the fertilizing combination of spermatozoa and ova, and the growth and development of the fertilized egg (zygote) from an embryo into a fetus occur within a sterile environment within the reproductive system of the human body. However, in recent theories of evolution suggest that male sperm and female ova, as two different eukaryotic cells, exist in their own unique environment before fertilization. The relationship between these cells and their environments can be understood as the interaction between them and their environmental evolutionary entities at the same and/or different evolutionary levels. [1-3] It has been hypothesized that evolutionary entities at lower evolutionary levels serve as the evolutionary background entities of those at higher levels.[1,2] Over the past decades, evidence has accumulated indicating that fertilization between spermatozoa and ova, as well as the growth and development of the embryos and fetuses, do not occur in a completely sterile environment. Instead, certain bacterial species and subcellular entities, including viruses and extracellular vesicles, have been found normally in human semen, follicular fluid, and the reproductive tracts of men and women. From a fimpological perspective, the complex interactions between germ cells and their environmental entities at the same and/or different evolutionary levels [1-3] determine the fate of a fertilized egg, embryo, and fetus during pregnancy, leading to either "evolvamity" (survival) or "evoclash" (aborted).[1] Since the successful reproduction of any given macroorganism at the individual level is the prerequisite for the survival of its species at the population level, the male and female reproductive tracts should be considered a "microecological protection zone" in the human body. Recently, host-associated microentities, or evolutionary background entities (EBEs), including mono-cellular eukaryotic and prokaryotic entities (bacteria, archaea, and fungi), and subcellular entities (viruses/phages and extracellular vesicles) in vertebrate and invertebrate animals have been partially summarized.[4-7] In this paper, I attempt to briefly review the cellular and subcellular environmental entities encountered by spermatozoa and ova as they migrate through the

 

human reproductive tract, in order to supplement our understanding of the evolutionary background entities at the cellular and subcellular levels in the human body.[8]

Key words: Microecological protection zone; Evolvamity; Evoclash; Evolutionary background entities (EBE); Evolution; Diversity; Animals; Symbiosis; Eukaryote; Prokaryote; Vertebrate; Mammals; Bacteria; Archaea; Fungi; Virus; Phage; Archaealphage; Bacteriophage; Membrane-enclosed microentities; Extracellular vesicles; Evolvamity

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# 1. Background

Spermatozoa and oocytes play their unique roles in reproducing offspring for the survival and continuity of animal species, and have attracted most research attention for a long time. It is well known that semen, as the environment of spermatozoa, consists of a cellular component and a non-cellular component. The seminal cellular component normally includes host cells such as spermatozoa, macrophages, lymphocytes, immature germ cells, and epithelial cells. The cell-free phase of semen contains mainly various bioactive factors, such as enzymes, cytokines, prostaglandins, and hormones, and other substances including fructose and trace elements.[9,10] During the past decades, increasing attention has been paid to the environmental non-spermatozoa entities including bacteria, fungi, viruses, and extracellular vesicles in semen.[9,11-31] Moreover, bacteria, fungi, viruses, and extracellular vesicles have been found to exist normally in the female reproductive tract, a natural channel of sperm transportation.[32-46] However, the role played by these environmental non-spermatozoa entities at the cellular, subcellular, and molecular levels in the sperm transport and storage in the female reproductive tract and the sperm-epithelial interaction in the uterine and Fallopian tubes is rare in the existing literature, and therefore, their physiological and pathological roles need to be elucidated in the future. Pathological changes in the female cellular environment have been shown to influence the physiological sperm-endosalpingeal interaction directly or indirectly, and may be the reason for infertility in some clinical cases.[47,48] Reeve and colleagues, for the first time, revealed that human sperm–endosalpingeal interaction in vitro can be disturbed by tissues of endometriosis.[48] Considering the fact that spermatozoa have to migrate from the male genitourinary tract to the female reproductive tract and ova have to migrate from folliculi to the oviduct and uterus before and after fertilization, it is imperative to have a whole picture about the environmental entities of spermatozoa and ova in the male genitourinary tract and the female reproductive tract.

In fimpology, the host-associated microentities including bacteria, archaea, and fungi, subcellular entities such as viruses/phages, and extracellular vesicles, and molecular entities are called evolutionary background entities (EBEs). The EBEs in vertebrate and invertebrate animals have been partially summarized.[5-7] In addition, EBEs in physiological hominal liquids such as breastmilk of humans and nonhuman mammals have also been recently reviewed.[4,5] In this paper, I try to briefly review the environmental cellular and subcellular entities confronted by spermatozoa and ova migrating along the reproductive tract in the human body, as a supplement to the evolutionary background entities at the cellular and subcellular levels in the human body.[8]

# 2. Semen

To date, existing literature dealing with bacteria in human semen mainly concerns their pathological roles in various clinical disorders. However, the isolation or detection of bacteria and viruses in seminal samples from healthy individuals in control groups has

 

strongly suggested that their appearance in semen has much to be uncovered. During the 19th century, while modern physiology and pathology were emerging in Europe, the concept that cells are the basic unit of life and diseases was recognized. In 1883, German biologist August Weismann (1834-1914) first proclaimed that genetic material within the nuclei of germ cells carried heritable information that could be transferred to the next generation through reproduction.[49] Weismann's theory established the association between germs cells (spermatozoon and ovum) and biological heredity. Currently, in healthy fertile men, their semen quality is determined by various parameters, such as a sperm count of at least 48.0 million per milliliter, at least 63% motility, and at least 12% of sperm with normal morphology,[50] despite disparities in the standard semen parameters.[51] Moreover, since the 1990s, some authors have attributed the low birth rate to male infertility caused by poor semen quality, including significant decline in mean sperm count at the population level,[52-55] which was partially associated with sexually transmitted infections.[56-58] Semen, a type of body fluid, is produced by the sex organs of males, in which sperm bear the most important role in reproduction. According to the fimpological entity-environmental theory,[2] if sperm are assigned to be the entity in semen, then the other seminal cellular, non-cellular, and molecular entities are the sperm's environmental entities; therefore, complex interactions occur between sperm and its environmental entities at the cellular, subcellular, and molecular levels. The following content will focus on seminal bacteria, viruses, and extracellular vesicles normally existing in semen.

## 2.1 Bacteria

It had been assumed for a long time that the human reproductive process, including sexual intercourse, seminal ejaculation, and fertilization between spermatozoa and oocyte, could be completed normally within a "sterile" environment in the female reproductive tract. Therefore, due to the limitations of existing medical theories, the presence of bacteria in the male genital tract and seminal fluids was mainly explained from a pathological perspective,[28,57,59-61] and interpretations of the seminal bacteria of fertile and infertile men were bogged down in the concept of "contamination" or "infection."[19,62-69] The multifaceted interactions between sperm and their environmental entities, including seminal and vaginal bacteria, protists, viruses, extracellular vesicles, and molecular entities in both the male and female genital tracts, hace been ignored for a long time. In fact, since the 1990s, the question of the effect of bacteria on spermatozoa and oocyte/embryo during in vitro fertilization has received increasing attention,[70,71] leading us to ask two questions: "Is the normal genital tract and ejaculated semen of a healthy man sterile or not?" and "When did bacteria first appear in the reproductive tract of healthy, fertile men?" and to reconsider the roles of bacteria in the genitourinary tract of healthy, fertile men from the perspective of reproductive physiology and reproductive medicine.[59,62]

Clinically, 4.8% to 69% of semen bacterial cultures were reported to be "sterile." [11,19,62,66,72-74] Bacterial species belonging to many genera, including Actinomyces, Arthrobacter, Corynebacterium, Enterococcus, Lactobacillus, Peptostreptococcus, Pseudomonas, Staphylococcus, Streptococcus, and Ureaplasma are commonly found in the seminal fluid of healthy men.[14,17-20,75-77] In fact, the high negative rate may be

 

the result of the low sensitivity of traditional culture methods, as clinical routine methods are unable to detect all microorganisms.[11,12] as has also been proved by newly emerged molecular analysis techniques.[13-15] Eggert-Kruse and colleagues reported that anaerobic bacteria were cultured from the semen in 99% of males in asymptomatic subfertile couples.[16] Stovall and colleagues reported that the positive rate of bacterial culture for semen samples from asymptomatic couples undergoing in vitro fertilization and embryo transfer was 80%, and most isolated bacteria were normal skin flora and had no effect on either fertilization or pregnancy rates.[78] Krissi and colleagues reported that 94.2% of manually obtained semen contained bacteria, and most isolated bacteria were those usually found on the skin. A positive bacterial culture result had no effect on fertilization rate and embryo quality.[74] Cottell and colleagues showed that microorganisms were detected in 51% of semen samples of asymptomatic men by culture, and most microorganisms were gram-positive microbes that were common to both urine and semen samples.[66] Rehewy and colleagues [72] showed that 54% of semen samples from fertile men were positive bacterial cultures, while 73% were positive in infertile men. Staphylococcus epidermidis, Staphylococcus aureus, Corynebacterium species, Mycoplasma hominis, and Ureaplasma urealyticum were isolated from semen samples of fertile men. In contrast, the bacterial flora in semen of infertile men showed more variation and higher colony counts. Besides the bacterial species isolated from semen samples of fertile men, other bacterial species were also isolated from the semen of infertile men, including Streptococcus pneumoniae type III, Streptococcus pyogenes group A, Strep. feacalis, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Candida albicans, Bacteroides species, Peptostreptococcus species, and Eubacterium species.[72] Rodin and colleagues showed that Staphylococcus species was the most common isolate in semen of asymptomatic men, followed by Streptococcus viridans and Enterococcus faecalis.[79] In addition, the presence of Gram-negative microorganisms in semen was found to be more associated with the expression of T-helper 1 cytokines than T-helper 2 cytokines.[80] While seminal bacteria can be separated from sperm effectively by the use of buoyant density centrifugation and seminal viruses can be inactivated by the addition of a protease,[81-83] in in vitro fertilization or artificial insemination, the total clearance of bacteria was not achieved [83] and the in vitro culture-obtained embryo still needs to grow and develop within the maternal uterus,[84-86] an environment containing bacteria, viruses, extracellular vesicles, and many molecular entities. [8]

### 2.1.1 The interaction between seminal bacteria and sperm

The pathological consequences of the interaction between seminal bacteria and sperm include sperm agglutination, impairing sperm-oocyte interaction, decreased sperm motility, [63] and sperm apoptosis, [87,88] whichc have been showed not only in humans, but also in nonhuman animals, such as pigs.[89] Some bacterial stains and species have been described as being associated with male infertility in vitro and in vivo, including Escherichia coli, [60,89] Chlamydia trachomatis,[57,64,90-95] Mycoplasma,[96-100] and Trichomonas vaginalis.[64] For example, Chlamydia trachomatis infection was shown in vitro to kill human spermatozoa, [88] and this spermicidal effect was attributed to chlamydial lipopolysaccharide (LPS).[91,101-103] Moreover, this pathogenic effect

 

depends on the concentration of bacteria in semen,[64,104] which may be the consequence of the dysregulation of normal aerobic and facultative bacteria in the male genital tract.[11] In fact, fertile men had significantly fewer positive cultures in their semen than infertile men.[66,72,105] Berktas and colleagues in an in vitro study on the interaction between semen specimens from healthy donors and bacteria Enterobacter aerogenes, Staphylococcus epidermidis, S. aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli revealed that coincubation for up to six hours with lower microorganism concentrations did not cause impairment of sperm function.[104] A critical question here is "how is the seminal bacterial community controlled and maintained at a pathogenic level?" because, in most cases, men and women carrying seminal microorganisms, such as chlamydial species, are asymptomatic.[94,106] This, from a fimpological perspective, should depend on the interactions among seminal entities, including host eukaryotic cells, prokaryotic cells, viruses, and various molecular entities, but the discussion of this question will not be further developed in this article. In addition, recently, Helicobacter pylori, a common bacterial species in the gut, was found to be correlated with alteration of sperm quality and early pregnancy loss.[107,108] Collodel and colleagues revealed that some bacterial strains expressing CagA of Helicobacter pylori were shown to be associated with increased systemic levels of IL-6 and TNF-alpha and decreased sperm quality.[107]

### 2.1.2 The intra- and inter-individual variation

Although studies specifically on the intra- and inter-individual variation of seminal microorganisms are rare, the indirect information in the existing literature strongly suggests a high intra- and inter-individual variation of bacterial communities in semen.[16,59,62,66,72,76,77,110] Recently, Mandar indicated that there was a high inter-individual variability of microorganisms in the male urogenital tract between sexually transmitted disease-positive and -negative men and between circumcised and uncircumcised men.[111]

## 2.2 Fungi

Studies on the fungal species in semen are rare. Two decades ago, Krzeminska-Jaskowiak, Cybulski and Pietkiewicz reported that fungal species in the genus Candida were isolated from semen.[21] Onemu and Ibeh, using culture methods, showed that pathogenic microorganisms, including Staphylococcus aureus, Klebsiella species, Escherichia coli, and Candida albicans, were isolated in 47.1% of semen samples.[112] In an experimental study on Candidda albicans, Burrello and colleagues showed that Candida albicans was associated with human sperm apoptosis.[22]

## 2.3 Viruses

The recognition of the existence of viruses in humans began near the end of the 19th century, in research on diseases, just as the association between diseases and bacteria was discovered, because at that time, some diseases in humans and non-human animals or

 

plants of economic value could not be explained by bacterial infection. Therefore, it was initial research for medical purposes that opened the window to the world of viruses; in fact, even today, major efforts of current studies on virus and bacteria are still working towards the goal of one day eradicating all harmful microorganisms, like smallpox virus. Naturally, our knowledge is mainly limited to the medical associations between viruses and human health, nonhuman animal health, and plant health. Since the time of Pasteur, bacteria and viruses have habitually been treated as external "invaders" or "enemies" that cause our diseases and death and should be controlled or eradicated. However, since the 1990s, as molecular biology has developed, accumulating evidence has suggested that microorganisms, such as bacteria and viruses, may play an important role in biological evolution. The presence of viruses in the mammalian male genital tract and semen has long been an ignored research topic mainly because of limitations in techniques and its unknown significance. The AIDS epidemic, which began in the 1980s in Western countries and later became pandemic, has attracted worldwide attention and has led to extensive studies on the human immunodeficiency virus (HIV), raising thought on the relationship between virus and the reproductive system. It is generally accepted that seminal viruses exist in two forms: (i) cell- associated viruses and (ii) cell-free viruses.[113] During the past 15 years, several reviews have thoroughly examined viruses in the mammalian male reproductive system and their pathological significance in clinical medicine.[9,23-28,114]

### 2.3.1 Three sources of seminal viruses

Semen, as a vector of viruses, plays an important role in the transmission of viruses into the vaginal tract,[115,116] rectal intestine,[117-121] or oral cavity,[122-124] where seminal viruses, as invading entities, interact with various originally-harbored entities at the cellular, subcellular, and molecular levels, such as host mucosal cells, prokaryotic cells, viruses, and extracellular vesicles. For example, Tang, Roan and Yamamura recently showed that in vitro amyloids enhance cytomegalovirus infection through interactions between semen amyloid fibrils and viral particles.[125] Liu and colleagues found that there was a reversed association between HIV infection and seminal microbiome diversity, which can be restored by antiretroviral therapy.[126] One interesting question about viruses in semen is whether seminal viruses are a consequence of genital tract shedding, transmission from blood, or viral release from spermatozoa. In my opinion, viruses in a given seminal sample obtained naturally from an individual should be the collection of these three sources in both physiological and pathological conditions. It is not difficult to understand the mechanism for viral transmission from blood.[127-129] Evidence for the viral shedding from the genital tract has been obtained from animal studies on macaques.[113] The most important question is whether spermatozoa release viruses, or even transfer integrated foreign DNA. Although there is evidence indicating that there is no germ cell-mediated viral gene transfer in HIV-1 [130] and adenovirus, [131,132] germ cells-mediated viral gene transfer has been proved in hepatitis B virus in both clinical[133-135] and experimental studies.[136-138]

### 2.3.2 Human herpesvirus in the family Herpesviridae

Human herpesviruses are common in normal semen.[26] The prevalence of herpesviruses in semen of Danish sperm donors was 27.2%.[139] Some herpesvirus species in semen may associate with impaired human reproduction.[26]

#### 2.3.2.1 Herpes simplex virus 1and 2 (HSV-1 and HSV-2), or Human herpesvirus-1 and -2 (HHV-1and HHV-2)

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), also known as human herpesvirus 1 and 2 (HHV-1 and HHV-2), were detected in the semen of asymptomatic men with normal semen parameters. The prevalence of HSV-1 was 0.4% to 2.1%.[140,148] DNAs of HSV-1 was detected in semen of 25% human male infertile patients.[141] HSV-2 was found to exist in semen in a low concentration in healthy fertility men [9] and the prevalence of HSV-2 in semen of Danish sperm donors was 0.1%.[139] Monavari and colleagues showed that seminal HSV was correlated with lower sperm count, but not with sperm motility and morphological defects.[142] Kapranos and colleagues found that herpes simplex virus (HSV) was significantly associated with low sperm concentration and poor motility.[143] Kotronias and colleagues using in situ hybridization technique showed that HSV DNA (HSV-1 or HSV-2) was found in the nuclei of spermatozoa in 80 men attending a maternity center for their fertility problems.[144]

#### 2.3.2.2 Varicella zoster virus (VZV), or Human herpesvirus-3 (HHV-3)

The prevalence of VZV in the semen of asymptomatic men with normal semen parameters was 3.2%.[140]

#### 2.3.2.3 Epstein-Barr Virus (EBV), or Human herpesvirus-4 (HHV-4)

The prevalence of EBV in the semen of asymptomatic men with normal semen parameters was 45%.[140] Kaspersen and colleagues reported that the prevalence of EBV in semen of Danish sperm donors was 6.3%.[139] EBV seminal shedding was found to associate with higher HIV-1 loads in blood and seminal plasma.[145]

#### 2.3.2.4 Human cytomegalovirus (HCMV), or Human herpesvirus-5 (HHV-5)

The prevalence of CMV in the semen of asymptomatic men with normal semen parameters was 62.5%.[140] Kaspersen and colleagues reported that the prevalence of HCMV in semen of Danish sperm donors was 2.7%.[139] Eggert-Kruse and colleagues showed that CMV in semen was not associated with the presence of CMV in the endocervical material of the female sexual partners.[146] Rinaldo and colleagues found that the increasing prevalence of cytomegalovirus in semen was associated with HTLV-III seropositivity in asymptomatic homosexual men.[147] The intracellular localization of

human cytomegalovirus (HCMV) in male germ cells was confirmed recently by Naumenko and colleagues using real time PCR, rapid culture method and PCR in situ.[148] Lupton and colleagues found that cytomegalovirus DNA was detected in semen from 48% of the HIV-infected men and cytomegalovirus in semen was not accurately predicted by serology.[149] Increasing CMV seminal reactivation was found to associate with HIV-1 infection.[145,150-154] Moreover, the presence of CMV in semen may also be the consequence of intermittent release,[9] and without recent contamination.[155]

2.3.2.5 Human herpesvirus-6 (HHV-6)

The prevalence of HHV-6 in the semen of asymptomatic men with normal semen parameters was 70%.[140] Kaspersen and colleagues reported that the prevalence of HHV-6A/B in semen of Danish sperm donors was 13.5%.[139] Godet and colleagues detected HHV-6 DNA in 1.7% spermatozoa, which was implied to be chromosomally integrated HHV-6.[156]

2.3.2.6 Human herpesvirus-7 (HHV-7)

Kaspersen and colleagues reported that the prevalence of HHV-7 in semen of Danish sperm donors was 4.2%. [139]

2.3.2.7 Human herpesvirus-8 (HHV-8)

Danish researchers reported that the prevalence of HHV-8 in the general population was low and under the detectable level in semen from healthy Danish sperm donors. [139,157]

### 2.3.3 Hepatitis viruses

2.3.3.1 Hepatitis B Virus (HBV)

Some authors using molecular hybridization showed that integrated hepatitis B virus (HBV) DNA sequences were detected in the seminal plasma of patients with acute hepatitis,[133] spermatozoa,[133-136,158] human ova at different stages,[159,160] peripheral blood leucocytes, [134] and seminal mononuclear cells.[134] Fei and colleagues showed that 43.0% patients with serum HBV DNA-seropositive were HBV DNA-positive in seminal plasma.[161] In vitro studies revealed that the interaction between Hepatitis B virus S protein and human spermatozoa can induce an apoptotic cascade in the latter[162] and result in loss of sperm membrane integrity and dysfunctions.[163,164] HBV DNA was also detected in hamster oocytes fertilized with human spermatozoa carrying HBV DNA,[137,138, 165] which strongly suggests the possibility of vertical transmission of HBV to the next generation through the male germ line.[133,136,138] Spermatogenetic alterations including higher apoptosis and necrosis, and lower fertility index have been observed under light and transmission electron

 

microscopy (TEM) in HBV infected patients while comparing with that in the control groups.[166,167] However, the association between seminal HBV and impaired human infertility needs deeper studies.[57,168,169]

#### 2.3.3.2 Hepatitis C Virus (HCV)

Hepatitis C virus RNA has been detected in seminal plasma and spermatozoa from men infected with HCV.[170-176] HCV RNA in semen was also found to be intermittently detectable.[173,177] The frequent rate of HCV RNA in the semen of men coinfected with HIV-1 was higher than that in the semen of those with only HCV infection.[173] But recently, Bradshaw and colleagues did not find the association between HIV positivity and an increased frequency of seminal HCV RNA detection.[176] Moreover, the risk for hepatitis C virus (HCV) transmission by sexual activity was shown very low among monogamous heterosexual couples; [178,179] and HCV infection had no affection on the outcomes of in vitro fertilization.[180]

### 2.3.4 Human Papillomavirus (HPV)

Human papillomavirus (HPV) DNA is commonly detected in semen of healthy men.[181-188] The prevalence of HPV DNA in semen samples varied from 0 to 100%.[27,186,188,189,190] To date, more than 100 human papillomavirus (HPV) species or types including HPV-16, HPV-18, HPV-53 HPV-84, HPV-CP6108 have been identified,[115,183,191-193] among which, HPV-16 in semen was most studied for its etiological agents of cervical cancer and other anogenital cancers.[27,194-197] Oncogenic HPV species were detected on spermatozoa from asymptomatic subjects.[198] Perez-Andino and colleagues observed that capsids of human papillomaviruses (HPVs) type 16 bound efficiently to live human sperm cells.[194] HPV DNA was detected in spermatozoa from healthy individuals [186,199] and there was no relationship between seminal HPV infection and sperm quality.[192,198]

### 2.3.5 Human Immunodeficiency Virus (HIV)

Human immunodeficiency virus (HIV) belongs to the genus Lentivirus of the family Retroviridae, and can be detected in semen of individuals with HIV viremia, which was considered to be the consequence of viral migration from blood to semen.[153,200] However, some individuals intermittently shed HIV RNA in semen despite suppression of viremia.[126,201] Spermatozoa were shown to play a role as the vector of HIV [202] and HIV-1 proviral sequences were detected in sperm from HIV-1-infected men.[203,204] Huang and colleagues [205] reported their finding that HIV-1 viral DNA was stably integrated into the genome of resting CD4 + T lymphocytes, and the HIV-1 infected T cells did not produce any viral proteins.[205] HIV was found to be shed intermittently into semen.[9]

### 2.3.6 Adeno-Associated Viruses (AAV)

Adeno-associated virus (AAV) belongs to the genus Dependoparvovirus of the family Parvoviridae.[206] Rohde and colleagues first found the presence of AAV in human semen in 1999.[207] Erles and colleagues showed that AAV DNA was detected in 4.6% of normal semen samples and 38% of ejaculates from men with oligoasthenozoospermia or asthenozoospermia.[206,207] Recently, Schlehofer and colleagues indicated that AAV DNA in semen was not significantly related to semen quality.[208]

## 2.4 Extracellular Vesicles

Extracellular vesicles have been detected in human semen from normal individuals [29-30,209,210] and seminal exosomes consist of heterologous populations of exosomes.[30,211-214] Mandison and colleagues revealed that seminal exosomes from healthy human donors possessed anti-HIV-1 activity.[30,215] Vojtech and colleagues suggested that seminal exosome could potentially play a regulatory role in the recipient mucosa via the release of exosominal small RNA molecules.[210,216,217]

# 3. The genitourinary tract

In this section, the cellular and subcellular evolutionary entities in the urine, prostate, bladder, and urethra will be reviewed. In addition, the cellular and subcellular entities in the vaginal tract and the non-pregnant uterus, which belong to the female reproductive system and in semen, are reviewed in other sections of this article.

## 3.1 Anatomic features of the male and female genitourinary tract

In men, both the genital tract and the urinary tract share a common anatomic part called “the urethral tract” for exit, and therefore, microorganisms that appear in naturally discharged urine or ejaculated seminal samples may theoretically come from any anatomic components of the reproductive system and the urinary system, including the urethral tract, bladder, prostate, spermatic ducts, seminal vesicles, testicles, ureter, and kidney, or even blood under physiological or pathological conditions. The female urethra is shorter than that of men, and the female urinary tract, reproductive tract, and the gut are separated anatomically, but their external exits are closely neighboring. The urine samples from females are more accurate in representing the urinary tract than those from males because of the potential influence from the male reproductive tract. It is worth to remind readers that some reseach results cited in this paper should be paid attention when to evaluate them, because the relevant authors did not release the sex information for their collected urine samples.

## 3.2 Bacteria

 

### 3.2.1 Urine

In the traditional culture-based notion, the urine of the healthy urinary tract was "sterile." [223-228] The clinical threshold for a positive culture result of urine specimens is 105 colony-forming units per ml (CFU/ml),[227] and urinary bacteria are usually associated with some pathological conditions, such as acute cystitis[229,230] and nosocomial infections.[231] However, over the past decade, researchers using culture-independent molecular approaches have revealed that there are diverse bacterial species in healthy human urine and that healthy urine is not sterile.[222,224,225,227,232-234] Wolfe and colleagues revealed that uncultivated bacteria in urine specimens obtained by suprapubic aspirate (SPA) and transurethral catheter (TUC) from the adult female bladder belonged to the genera Aerococcus, Actinobaculum, Escherichia, Shigella, Burkholderia, Ralstonia, Gardnerella, Streptococcus, Prevotella, Atopobium, Lactobacillus, Corynebacterium, and Aerococcus.[222] Fouts and colleagues, using 6S rDNA sequencing and metaproteomic analysis, showed that bacteria were also detected in the urine of healthy men and that the healthy urine microbiome of males and females was different, with the dominant bacterial species in male urine being superficial skin flora in the genus Corynebacterium and those in female urine belonging to the genus Lactobacillus.[225] Moreover, Fouts and colleagues also revealed that the urinary bacterial community compositions in normal bladders, spinal cord injury-related neuropathic bladders, and catheter utilized-bladders were varied.[225] Uropathogenic Escherichia coli was even found within the cytoplasm of superficial epithelium cells and so called intracellular bacterial communities (IBCs) in mice and human bladders.[235,236] Moreover, some uropathogenic Escherichia coli strains were able to survive within murine and human macrophages.[237] Some authors proposed that the intracellular bacterial communities may be a mechanism of recurrent urinary tract infections caused by uropathogenic Escherichia coli because this bacterium simultaneously enhances iron acquisition and avoids lysosomal degradation within bladder epithelial cells.[238,239] However, Robino and colleagues recently uncovered that intracellular bacterial communities were a common phenomenon in children with urinary tract infection[240] and Navidinia and colleagues revealed that uropathogenic and commensal E. coli isolates were found to be considerably different in their phylogenetic groups, presence of class 1 integron and hlyD gene, hemolysin activity, and resistance patterns.[241]

#### 3.2.1.1 Female urine microbial communities

Wolfe and colleagues pointed out that "urine specimens reported to clinicians as 'culture-negative' or 'insignificant growth' can contain varied bacterial communities that can be simple or extremely diverse and can be composed of typical uropathogens or of genera not identified by standard cultivation techniques."[222] The microbial species in normal female urine microbial communities belong to various genera, including Actinobacillus, Actinomyces, Aerococcus, Arthrobacter, Bifidobacterium, Corynebacterium, Lactobacillus, Gardnerella, Oligella, Prevotella, Staphylococcus, and Streptococcus.[224,227,242,243] Siddiqui and colleagues revealed that the microbial diversity and richness decreased and the bacterial genus Lactobacillus highly increased in

 

the composition of the bacterial community in the urine from interstitial cystitis female patients compared to that in healthy female urine.[242] In addition, intra- and inter-individual variations were described in the urine microbial community from healthy female individuals.[227]

#### 3.2.1.2 Male urine microbial communities

The urinary bacterial species in male urine belong to various genera, including Lactobacillus, Corynebacterium, Streptococcus, Sneathia, and Propionibacterium.[220,244,245] Nelson and colleagues, using 16S rRNA molecular approaches, revealed that all of the urine samples from male adults contained some human vaginal bacterial species, and some uncultivated bacterial species in the urine samples collected from men who had sexually transmitted infection were pathogenic in the female genital tract.[220] Furthermore, the male urinary microbiomes showed inter-individual variation.[220]

### 3.2.2 Prostate

Bacteria in normal prostates of healthy men have not been specifically studied yet, and our knowledge of the prostate-associated bacterial community is mainly from studies on prostatitis. It has been recognized that the etiological factors of prostatitis are associated with various pathogenic agents, such as bacteria, fungi, viruses, parasites, and some chemical substances.[246] Prostatic bacterial communities of prostatitis are found to be associated with the seminal bacterial community. For instance, seminal Enterobacteriaceae, enterococci, and Staphylococcus aureus are isolated only from patients suffering from chronic prostatitis syndrome ,[18] and patients with chronic prostatitis syndrome have a significantly higher proportion of seminal coagulase-negative staphylococci strains than healthy men.[247] Seminal microbial organisms of healthy men are different in composition and phenotype from those of patients with chronic prostatitis syndrome or persistent nonspecific urethritis.[17,18,244,247]

### 3.2.3 Bladder

Bacterial 16S rRNA genes have been detected in bladder biopsies from healthy individuals in control groups.[230,248] Moreover, in a pathological condition called interstitial cystitis, bacterial 16S rDNA is often detected in bladder biopsy specimens from patients.[230,248,249] However, like many other clinical disorders, interstitial cystitis is a syndrome caused by different etiological factors including bacteria and viruses.[230,248-250]

### 3.2.4 Urethra

Theoretically, bacteria normally inhabit in both the male and female urethra, despite most of evidence from studies on the male.[219-221,251] Naboka and colleagues showed that a wide spectrum of bacterial species were detected in urethra scrapings from healthy individuals in the control group, with the dominant bacteria being coagulase-negative

 

staphylococci and those in the genera Corynebacterium and Eubacterium.[252] Bowie and colleagues showed that significant aerobic species including lactobacilli, Haemophilus vaginalis, alpha-hemolytic streptococci (not Streptococcus faecalis), as well as anaerobic Bacteroides species were isolated in the anterior urethra of sexually active Caucasian males.[251]

## 3.3 Archaea

There have not been any specific studies on detecting archaea in human urine yet. Based on the fimpological theory about evolutionary background entities, I hypothesize that there may be naturally archaea occurring in normal human urine, and the investigations on detecting human urinary archaea are expected to appear in the future.

## 3.4 Fungi

Gutierrez-Fernandez and colleagues, using the Sysmex UF1000i system, reported that yeasts were detected in urine samples for clinical screening.[253]

## 3.5 Viruses

In the recent years, viral species have been uncovered in normal urine. Gupta and colleagues detected human papillomavirus DNA in the urine of healthy women.[254] Coelho and colleagues showed that John Cunningham virus DNA was detected in the urine of 37-41% of healthy individuals in the control group.[255] Most recently, Santiago-Rodriguez and colleagues revealed that there were viral communities normally in human urine, in which bacterial phages were predominant and eukaryotic viruses such as human papillomaviruses were non-pathogenic and detected in almost all of the individuals studied.[223] Csoma and colleagues revealed that human polyomaviruses were detected in the urine of 2-3% of women.[256] Human papillomavirus (HPV) DNA was detected in female urine.[257] Cytomegalovirus-DNA was detected in urine and saliva of preterm infants.[258] West Nile virus was detected in the urine of human individuals with acute infection.[252-261] Hepatitis A virus DNA was detected in the urine samples of hepatitis A patients.[262] BK virus and JC virus were detected in urine after renal transplantation, which is the consequence of polyomavirus reactivation.[263,264] However, BK virus and JC virus were also detected in pediatric healthy controls.[265,266]

## 3.6 Extracellular Vesicles

Urinary exosomes have attracted increasing research attention for their physiological and pathological roles and potential clinical application in the past decade.[267-275] Normal human urinary exosomes contain many bioactive molecules, including proteomic and miRNomic molecular entities.[276-278] For example, Hiemstra and colleagues showed that exosomal proteins of human urine were antimicrobial proteins and peptides and bacterial and viral receptors [277] Gildea and colleagues showed that urinary exosomal miRNAs were associated with an individual's blood pressure response to

 

sodium.[278] Moreover, the potential biomarkers of human urinary exosomes have been explored in prostate cancer,[272,279-283] and bladder cancer, [284] for cancer detection and progression. [274] They have also been studied in renal pathologies including polycystic kidney disease, kidney stones, and diabetic nephropathy for noninvasive evaluation.[275,285-293]

# 4. The vaginal tract

## 4.1 Bacteria

### 4.1.1 Advantage and disadvantage in cultivation and molecular analyses

The vaginal bacterial communities, as one of the most studied microbial communities in the human body, are anatomic site-specific and compositionally complex, dynamic, and fragile.[294] Although, the first microbiological study of the human vagina was completed by Doderlein over 120 years ago,[295-299] and most information about the composition of the microbial flora of the female genital tract was derived from qualitative and descriptive studies that relied on the traditional culture-based bacterial isolation techniques, which are useless for uncultured bacteria in the human genitosystem.[300-303] Therefore, our understanding of the importance of vaginal microorganisms with regard to vaginal health, reproductive health and diseases, and health of offspring is just beginning.[32,304] Cultivation-independent molecular techniques have already revealed many astonishing facts that are far beyond what we knew before, and have promoted us to reconsider their potential roles, which were once severely underestimated and simplified. It should be noted that cultivation-independent molecular methods usually use universal bacterial primers to identify 16S rRNA gene libraries prepared from total community DNA, and populations of bacterial species less than 1% in the communities may not be detected in this gene libraries.[38,39,305] Burton and Reid using polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE) techniques, found that bacterial species belonging to the genera Mobiluncus, Staphylococcus, and Corynebacterium known as normal members of the vaginal bacterial community, were not detected by sequencing of dominant DGGE fragments.[306] Primers bias is another factor that can influence amplification efficiency and specificity.[307] In contrast, in traditional culture-based methods, populations of any cultivable bacteria, regardless of whether they are abundant or limited in a complex bacterial community, can be identified if there are suitable media, environments, and biochemical identification methods. However, certain microorganisms from the vaginal tract are difficult to cultivate due to their anaerobic nature or nutritional requirements,[302] and can even be in a viable but nonculturable (VBNC) status; therefore, conventional culture-dependent methods often leave many bacterial strains or species uncultured and unidentified. It is clearly still too early to say we have thoroughly understood the structure and biological significance of the vaginal microorganism system.[308] In fact, it is just at the beginning of systematically describing and exploring the normal vaginal bacterial community, which mainly depends on the further development of methodology in both culture-based and cultivation-independent approaches, as well as the novel theoretical recognition.

 

### 4.1.2 Normal bacterial community in the human vaginal tract

It was a traditional dogma that the fetal vagina was sterile and that the initial colonization by external microorganisms occurred at birth and/or after birth,[309] but this has been challenged by new facts and theories.[310,311] Culture-based studies showed that vaginal bacterial species belonged to the genera Lacrobacillus, Enterococcus, Peptococcus, Prevotella, Porphyromonas, Bacteroides, Clostridium, Staphylococcus, Corynebacterium, Peptostreptococcus, Weissella, and Eubacterium.[301,312-314] In healthy women, lactobacilli are thought to be the predominant members of the postpubertal vaginal microflora, [315] with various species of lactobacilli numbering approximately 108 cfu per ml (or g) of vaginal secretion.[38] Other bacteria such as coagulase-negative staphylococci, group B streptococci, Ureaplasma, Bacteroides, Bifidobacterium, Corynebacterium, Escherichia, Enterococcus, Mycoplasma, Prevotella, Peptostreptococcus, and Gardnerella can be isolated but in much lower numbers.[302,303,316] Many novel bacterial species such as those in the genera Atopobium, Dialister, Leptotrichia, Megasphaera, and Sneathia have been found in the vaginal tract,[39,294,317-320] which has enpanded our knowledge of microbial communities in the human vaginal tract. Some researchers have proposed that bacterial species in the genera Atopobium and Leptotrichia are usually members of the normal bacterial community in the human oral cavity and their isolation from vaginal specimens of several normal, healthy women may be associated with unusual sexual habits and behavior.[39,320,321]

#### 4.1.2.1 Vaginal anaerobic bacteria vs. aerobic bacteria

Vaginal aerobic bacteria are usually one tenth the number of anaerobic bacteria.[312,322] Evaldson and colleagues reported that the most prevalent vaginal bacteria were peptococci (107-108 cfu/ml).[322] Egwari and colleagues showed that the most frequently encountered aerobic bacteria included Escherichia coli and Staphylococcus epidermidis.[313]

#### 4.1.2.2 Hydrogen peroxide (H2O2.)-producing Lactobacilli

In the normal vaginal flora, lactobacilli are the predominant species and play an important role in the female genitomicroorganism ecosystem. The predominant vaginal lactobacilli include Lactobacillus crispatus, Lactobacillus jensenii, Lactobacillus gasseri, and Lactobacillus iners,[323-325] and majority of vaginal lactobacilli are able to produce hydrogen peroxide (H2O2.).[326,327,315] 70 to 95% of lactobacilli present in the vaginal flora of healthy women produce hydrogen peroxide.[328] Antonio and colleagues found that 94-95% strains of Lactobacillus crispatus and Lactobacillus jensenii produced H2O2, whereas only 7-9% strains of Lactobacillus iners and Lactobacillus gasseri produced H2O2.[315] A low pH and H2O2. have been shown to have a direct microbicidal, virucidal, and antimicrobial effect. However, H2O2.-producing vaginal lactobacilli do not protect against vulvovaginal candidiasis or vaginal trichomoniasis[329]

 

and non- H2O2 producing lactobacilli are usually anaerobic lactobacilli.[330] Vaginal bacterial species in the genera of Prevotella, Porphyromonas, Peptostreptococcus, Mobiluncus, and Gardnerella, and H2O2-nonproducing lactobacilli were believed by some researchers to be associated with bacterial vaginosis.[331]

### 4.1.3 Intra- and inter-individual variation in the composition of the vaginal bacterial community

Previous studies using traditional culture-based methods had already shown that there was individual variation in terms of both the types and abundances of vaginal bacteria species.[301,312,332] The intra-individual variation of the vaginal bacterial community described by Kim and colleagues was characterized by different anatomic locations of the vaginal tract, such as the cervix, fornix, and outer vaginal canal, where harboring site-specific bacterial species.[333] The inter-individual variation of the vaginal bacterial community was also shown by researchers using culture-independent methods in healthy non-pregnant women.[33-37,335,336] For example, although up to 91% of women had Lactobacillus-dominated vaginal microbiota,[337] there were no two women whose vaginal tracts were colonized by the same two Lactobacillus species [299,316] and some vaginal microbial communities of women were not Lactobacillus-dominated.[333,336,338]

### 4.1.4 Variations in the vaginal microbial composition among different racial groups

Variations in the vaginal microbial composition among different racial groups, such as Caucasian, black, and Asian women, have been described by several research groups.[34,39,294,336,339-343] Ravel and colleagues revealed that the vaginal bacterial community compositions of North American reproductive-age women representing white, black, Hispanic, and Asian ethnic groups varied with racial characteristics.[294] Studies from different research groups showed that American women of European ancestry and African American women exhibited racial difference in their vaginal microbial community composition. For example, Fettweis and colleagues found that the vaginal microbial communities of American women of European ancestry were more often Lactobacillus-dominated, whereas a diverse vaginal microbial profile was more often seen in African American women.[339] Zhou and colleagues showed that there was a difference in the vaginal bacterial composition between healthy Caucasian and healthy black women, with vaginal communities dominated by lactobacilli in 93% of Caucasian women and 67% of black women.[336] In addition, Goldenberg and colleagues revealed that such race-associated variation in vaginal bacterial composition was also found in the vagina during pregnancy in four ethnic groups and could not explained by differences in health behaviors.[343]

### 4.1.5 Dynamics and fragility of vaginal microbial communities

 

#### 4.1.5.1 Normal menstrual cycle

Studies have uncovered that the normal vaginal microbial flora varied in both abundance and species during the menstrual cycle.[35,36,312,344,345] Eschenbach and colleagues examined the vaginal microbial flora using traditional bacteria cultivation methods during the menstrual cycle in 74 asymptomatic white women aged 18-40 years and found that among those healthy women without bacterial vaginosis, the rates of recovery of any Lactobacillus, Prevotella species, and Bacteroides fragilis from the menstrual phase (days 1-5) to the postovulatory phase (days 19-24) changed to different degrees.[345] Chaban and colleagues showed that the vaginal microbial community of most women was relatively stable, but there were physiological fluctuations in species richness and diversity.[35]

#### 4.1.5.2 Vaginal acidity

The vaginal low pH environment is generated and maintained by lactic acid. The pH value of a healthy vagina is usually around 4 ± 0.5.[345-349] Ravel and colleagues showed that there was a variation in the vaginal pH among different racial groups, [294] with the vaginal pH in Hispanic and black women ranging from pH 5.74 and pH 3.66, compared to the vaginal pH of 3.81 to 4.99 in Asian and white women.[294] The origin of lactic acid is traditionally attributed to the vaginal epithelium, where glycogen is anaerobically metabolized to lactic acid.[350] However, base on the facts that human host eukaryotic cells can only produce L-lactate,[351] and that bacteria can produce both D- and L-lactate,[352-354] Boskey and colleagues found that more than 50% of vaginal lactic acid was D-isoform, indicating that vaginal bacteria, not host vaginal epithelial cells, are the main source of vaginal lactic acid.[350] It is known that hydrogen peroxide (H2O2) produced by lactobacilli and a low pH generated by lactic acid are two crucial protective factors for maintaining a healthy vaginal microenvironment.[329,350,355]

#### 4.1.5.3 Pregnancy

The study of the alteration of the maternal vaginal microbial community during pregnancy is in its infancy. Despite the interference of variations in the sample size of the enrolled pregnant women, ethnicity, study design, and technical approaches, published papers on this topic have already presented a dynamic picture of vaginal microbial composition during pregnancy.[32,356-358] From a bacterial perspective, the fluctuation of the maternal vaginal microbial community composition during pregnancy has been confirmed in recent studies.[32,357-359] The dominant vaginal bacterial species in the genus Lactobacillus was shown to change from the first trimester to the second or third trimester.[297,358] Walther-Antonio and colleagues showed that the dominant Lactobacillus crispatus in the first trimester vaginal microbial community of two full-term uncomplicated pregnant women shifted to Lactobacillus iners in the second trimester.[358] However, the reasons and mechanisms for the pregnancy-associated alteration of the vaginal bacterial community composition are largely unknown.

#### 4.1.5.4 Sexual and non-sexual influencing factors

It has been revealed that some human sexual habits and behaviors are associated with destabilization of vaginal microbial communities, including early sexual experiences,[360] having more than one sex partner,[329,361-364] having sex with partners of a different race,[365-367] using vaginal microbicide or spermicides,[368-371] douching,[363,372-375] using diaphragm or cervical cap,[376,370] having frequency sexual intercourse,[32,377,378] and engaging in unusual sexual activities such as receptive oral sex, digital penetration, anal sex, and using sex toys.[340] Additionally, epidemiologic studies have shown that bacterial vaginosis can also occur in women who have never had sexual intercourse.[379] In fact, over the past decades, non-sex factors have also been found to play a role in destabilization of vaginal microbial communities, such as antibiotic use,[36,313,380,381] personal hygiene,[382] stress,[383,384] diet,[385,386] poverty,[363] and smoking.[362,387,388]

### 4.1.6 Role of vaginal microbial communities

There is accumulating evidence that normal vaginal microbial communities have physiological functions that directly affect the health of women. These communities play a role in colonization resistance against the invasion of external pathogens, and against the overgrowth and dominance by potentially pathogenic species among the normal flora. They also play a role in reproduction.[304,371,389-395] Disturbed vaginal bacterial composition in female sex workers has been found to be associated with Chlamydia trachomatis infection.[396] It is becoming a challenge in modern societies for a woman and her sexual partner to maintain a healthy vaginal microbial ecology before, during, and after pregnancy, considering the dynamics and fragility of vaginal microbial communities and the sexual habits and behaviors mentioned above.[397-406]

## 4.2 Archaea

To date, the question of whether there are normally any archaea in the human vaginal tract has not received much research attention, and there is a lack of literature on vaginal archaea.[407,408] In 1990, Belay and colleagues used traditional culture methods and a methanogenic enrichment medium and found Methanobrevibacter smithii existing in vaginal samples from patients with bacterial vaginosis.[407]

## 4.3 Fungi

Studies on fungal species in human vaginal microbial communities are rare. Recently, Gunther and colleagues indicated that vaginal fungal species in the genus Candida are a normal part of the vaginal microbial community in some women of the control group [409] and that diabetes is a risk factor associated with yeast colonization in the vaginal tract.[409,410]

## 4.4 Viruses

Despite the great interest on bacterial microorganisms in the vaginal tract, there is little literature on viruses in the female reproductive system. As a result, our understanding of viral communities in the female reproductive system is largely based on information from clinical studies, which are mainly from a pathological perspective.

### 4.4.1 Hepatitis C Virus (HCV)

Hepatitis C virus (HCV) has been detected in genital secretions such as cervicovaginal lavage fluid of HIV-1–infected women, which suggested that local HCV replication may be facilitated by local HIV-1 replication.[411,412]

### 4.4.2 Human Immunodeficiency Virus Type 1 (HIV-1)

Many studies have shown that HIV can exit in cervicovaginal secretions,[413-424] which is believed to be associated with sexually transmitted HIV infection.[425]

### 4.4.3 Human Papillomavirus (HPV)

Human papillomavirus (HPV) can be spread by skin-to-skin sexual contact[426] and in the female reproductive tract is divided into carcinogenic HPV types and noncarcinogenic HPV types[427] or low-risk and high-risk types.[428] Noncarcinogenic HPV types are mainly distributed in the vaginal epithelium, while the prevalence for any carcinogenic HPV type in the vaginal tract does not vary by anatomic site.[427] Carcinogenic HPV types are etiologically associated with cervical cancers.[429-430] Yamasaki and colleagues revealed that HPV genotypes including HPV 52, HPV 16, and HPV 31 were detectable in 35.8% of pregnant Japanese women on their first hospital visit.[431] Studies have also revealed an association between maternal human papillomavirus and childhood retinoblastoma.[432-434]

### 4.4.4 Cytomegalovirus (CMV)

Women with bacterial vaginosis were 4 times more likely to have vaginal cytomegalovirus detected than women without bacterial vaginosis.[498]

### *4.4.5 Herpes Simplex Virus type 2*

Herpes simplex virus type 2 was detected in vaginal secretions [435,436] and an increased frequency of HSV-2 in vaginal secretions was associated with the presence of bacterial vaginosis and high-density vaginal group B Streptococcus colonization.[437]

### 4.5 Extracellular vesicles

Madison and colleagues demonstrated that vaginal epithelial cells can internalize semen exosomes.[215]

## 5. The non-pregnant uterus

### 5.1 Bacteria

Although the presence of bacteria in the normal non-pregnant uterus of humans has been recognized for more than three decades,[40,438-442] our understanding of it remains within the traditional Pasteuranh theoretical system. This is because the traditional belief is that the endometrial cavity of a non-pregnant human uterus is "sterile," as supported by bacterial culture and histopathological studies.[439,443] Clinical investigations have found diverse bacterial species, including Enterobacteriaceae, Streptococcus agalactiae, and anaerobic bacteria in some disease-relevant non-pregnant uteri, such as those with uterine endometrial cancer,[444,445] irregular vaginal bleeding,[446] postpartum endometritis,[40,438,447] myoma uteri,[445,446] infertility,[440,448] acute pelvic inflammatory disease,[449] and preterm delivery.[442,450,451] Moreover, intracavitary radiotherapy cannot sterilize the endocervix or endometrium of patients with endometrial cancer.[444] Cicinelli and colleagues used culture-based methods to have shown that diverse acteria species, including streptococci, Enterococcus faecalis, Escherichia coli, Ureaplasma urealyticum, and Mycoplasma, were isolated from the endometrium in 69% of 404 women undergoing diagnostic hysteroscopy.[452] However, endometrial bacteria have also been isolated from the uterus of normal, non-pregnant women[40,438,440] and 3 months after term births.[442] Most recently, Mitchell and colleagues used molecular approaches to have shown that the uterine endometrial cavity of women undergoing hysterectomy between the age of 36 and 50 years is normally inhabited by low levels of diverse bacteria, including those in the genera Lactobacillus, Gardnerella, Atopobium, Megasphaera, Prevotella, and Leptotrichia/Sneathia, and some species are similar to those associated with bacterial vaginosis (BV).[41]

### 5.2 Viruses

Our existing knowledge of viruses in the non-pregnant uterus is mainly from the pathological perspective, such as viral endometriosis,[42] and the possible role of human papillomavirus (HPV) in the pathogenesis of endometrial carcinoma.[43]

### 5.3 Extracellular vesicles

Recently, a few researchers have revealed that there are exosomes in the murine uterine fluid and some important bioactive proteins, such as plasma membrane Ca2+-ATPase 4a and glycosyl phosphatidylinositol (GPI)-linked proteins, have been suggested

 

to be transferred to sperm via uterine extracellular vesicles.[44,45] Ng and colleagues showed that maternal endometrial epithelial cells can release exosomes/microvesicles, which may play a role in the interaction between embryo and maternal endometrial epithelial cells during implantation.[46]

# 6. Human follicular fluid

Although the mechanism for the formation of the follicular antrum and follicular fluid is still unclear,[453-455] it is generally agreed that human follicular fluid constitutes the microenvironment for the growth and maturation of a healthy oocyte, which is one of prerequisites for later fertilization and embryonic development.[453,456-458] Studies have shown that follicular fluid is derived from plasma and secretions of granulosa and theca cells, and contains many bioactive molecular entities including carbohydrates, steroid hormones, immunoglobulins, adrenomedullin, growth factors, cytokines, lipids, and nucleotides.[454,458-469,471-473] In recent years, the cellular and subcellular components in follicular fluid have attracted increasing research attention. For example, it has been revealed that besides oocyte lineages, follicular fluid also contains other host eukaryotic cells such as granulosa, thecal, and ovarian surface epithelial cells,[474] and the interaction between oocyte and other host eukaryotic cells is critical for oocyte growth and maturation.[459,474] Moreover, some prokaryotic cells and subcellular entities such as viruses and extracellular vesicles have been found normally in follicular fluid, which is beyond our previous definition of their pathogenic role.

## 6.1 Bacteria

In the 1980s, the presence of bacteria in the genera Escherichia and Streptococcus was found in porcine follicular fluid, although Sluss and Reichert considered it to be the consequence of contamination by intestinally originated bacterial species.[475] On the other hand, Magata and colleagues reported that a low concentration of lipopolysaccharide (LPS) was detected in follicular fluid of dairy cows.[476] Despite the antimicrobial entities in human follicular fluid,[477] the presence of bacterial species in human follicular fluid collected at the time of trans-vaginal oocyte retrieval was described for the first time by Cottell and colleagues in 1996.[468,478,479] In recent years, Pelzer and colleagues provided evidence supporting the novel notion that human follicular fluid is not sterile and showed that the percentage of cultivable bacterial species in follicular fluids collected from fertile women was 27%, and from infertile women it was between 24% and 37%.[479,480] Bacterial species belonging to the genera Lactobacillus, Actinomyces, Corynebacterium, Fusobacterium, Peptinophilus, Peptostreptococcus, Propionibacterium, Prevotella, and Staphylococcus were detected in all follicular fluid specimens from asymptomatic women taken via transvaginal oocyte retrieval.[468,479] Pelzer and colleagues also revealed an interesting and puzzling phenomenon that there was a difference in bacterial profile between the left and right ovarian follicles of women.[479] Although the same bacterial species were not isolated from paired vaginal secretions, their clinical significance was mainly accounted for from a pathological perspective.[479,480] For example, generally, microbial species detected

 

in human follicular fluid were associated with adverse in vitro fertilization (IVF) outcomes, such as a decrease in the embryo transfer and pregnancy rates.[480] However, Pelzer and colleagues also found that bacteria in the genus Lactobacillus within the left ovary was associated with a positive pregnancy outcome.[480] Additionally, bacteria in human follicular fluid were found to persist for at least 28 weeks in vitro and their growth can be influenced by steroid hormones.[468] Therefore, the presence of bacteria in follicular fluid cannot be attributed simply to contamination, and their significance may not be limited to clinical pathology.

## 6.2 Fungi

Fungal species belonging to the genus Candida were detected in all follicular fluid specimens from asymptomatic women taken via transvaginal oocyte retrieval.[479]

## 6.3 Viruses

Studies on bovine indicated that viral species such as bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1) can be detected in follicular fluid of heifers.[481-486] Bielanski and colleagues revealed that BVDV can also be detected in embryos, oviductal epithelial cells, endometrium, and corpora lutea tissues.[483] In addition, torque teno virus (TTV) and porcine circovirus 2 (PCV2) were detected in ovaries, follicular fluid, and uteri of normal sows,[487] and Maedi-Visna virus (MVV) was detected in follicular fluid of ewes from breeding flocks.[488]

### 6.3.1 Hepatitis viruses

Hepatitis B virus DNA was detected in the nuclei of granulosa cells extracted from the follicular fluid of female carriers of hepatitis B.[489,490] Hepatitis C virus RNA was detected in the follicular fluid samples of HCV(+) women.[491,492] Papaxanthos and colleagues showed that there was a weak correlation between plasma and follicular fluid HCV RNA loads.[492] Mansour and colleagues showed that hepatitis delta virus (HDV) RNA was detected in the follicular fluid of a woman infected with HDV.[493]

### 6.3.2 Endogenous retrovirus

Nilsson and colleagues revealed that an endogenous retrovirus was detected in human oocytes within follicular fluids.[497]

## 6.4 Extracellular vesicles

In recent years, studies on mammals have indicated that there are extracellular vesicles in ovarian follicular fluid. For example, da Silveira and colleagues used transmission electron microscopy and flow cytometry to have reported that they identified and observed extracellular vesicles in ovarian follicular fluid from mares, and that the follicular fluid-isolated exosomes contained proteins and miRNAs.[457] Moreover, exosomal miRNAs in ovarian follicular fluid varied with the age of the

 

mare.[457] Exosomal miRNAs were also detected in bovine follicular fluid.[453] miRNAs-containing microvesicles and exosomes have been identified in follicular fluid from healthy women and were believed to involved in follicular maturation.[494-496]

## 7. Concluding Remarks

Anatomically, the human reproductive tract is a structure opened to the external environment, and it can easily be affected by various environmental entities at the cellular, subcellular, and molecular levels. During the past decades, studies based on culture-dependent and culture-independent approaches have indicated that the co-existence of spermatozoa and ova with various environmental entities, such as bacteria, viruses, extracellular vesicles, carbohydrates, hormones, growth factors, cytokines, lipids, and nucleotides within the reproductive tract of the human body is a physiological state. From a fimpological perspective, the complex interactions that occur between germ cells and their environmental entities at the same and/or different evolutionary levels [1-3] determine the final fate of fertilization, embryonic formation, and fetal growth and development during pregnancy to be either "evolvamity"—survival or "evoclash"—abortion.[1] Considering that successful reproduction at the macroorganism individual level is a prerequisite for the survival of any given macroorganism species, the male and female reproductive tracts should be treated as a "microecological protection zone" in the human body.

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