



# An update on the aspects of Zika virus infection on male reproductive system

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## Abstract

Zika virus (ZIKV) is mainly transmitted through *Aedes* mosquito bites, but sexual and post-transfusion transmissions have been reported. During acute infection, ZIKV is detectable in most organs and body fluids including human semen. Although it is not currently epidemic, there is a concern that the virus can still reemerge since the male genital tract might harbor persistent reservoirs that could facilitate viral transmission over extended periods, raising concerns among public health and assisted reproductive technologies (ART) experts and professionals. So far, the consensus is that ZIKV infection in the testes or epididymis might affect sperm development and, consequently, male fertility. Still, diagnostic tests have not yet been adapted to resource-restricted countries. This manuscript provides an updated overview of the cellular and molecular mechanisms of ZIKV infection and reviews data on ZIKV persistence in semen and associated risks to the male reproductive system described in human and animal models studies. We provide an updated summary of the impact of the recent ZIKV outbreak on human-ART, weighing on current recommendations and diagnostic approaches, both available and prospective, with special emphasis on mass spectrometry-based biomarker discovery. In the light of the identified gaps in our accumulated knowledge on the subject, we highlight the importance for couples seeking ART to follow the constantly revised guidelines and the need of specific ZIKV diagnosis tools for semen screening to contain ZIKV virus spread and make ART safer.

**Keywords** Zika virus · Male reproductive tract · Assisted reproduction · Semen · Metabolomics

## Overview

ZIKV is a positive sense single-stranded RNA virus belonging to the Flavivirus genus. Its molecular structure is very similar

to other flaviviruses, like West Nile and dengue (DENV); however, cryoelectron microscopy analysis revealed that ZIKV is distinctively more stable at different temperatures than DENV [1].

ZIKV is mainly transmitted by female *Aedes aegypti* mosquitoes [2], but reports show that *Aedes albopictus* and *Culex quinquefasciatus* may also act as vectors [3, 4]. Besides transmission by vector and nonvector routes (blood transfusions, breastfeeding, etc.), ZIKV has also been shown to be transmitted by sexual contact, predominantly from male-to-female [5]. Female-to-male, as well as male-to-male transmission, has also been reported, and while female-to-female has not, it is considered biologically plausible [6–8].

The incidence of infection is highly variable, ranging from 10,510 cases per 100,000 in Saint Barthelemy epidemic outbreak to 65% of a major urban center population in Brazil [9, 10]. The most common symptoms are rashes, fever, arthralgia, arthritis, and conjunctivitis. Any combination of one of the first two with any of the latter three constitutes a suspected ZIKV case according to the World Health Organization (WHO) guidelines. A confirmed case is diagnosed by the presence of viral RNA or antigen

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on serum or other fluids, or a positive immunoglobulin M (IgM) test against ZIKV with exclusion of other flaviviruses [11].

Besides the common symptoms, other major complications have been associated with ZIKV. In adults, Guillain-Barre syndrome (GBS) [12–14], myelitis, and meningoencephalitis [15, 16] following ZIKV infection was observed. In newborns, GBS, congenital microcephaly and hydranencephaly, and stillbirth have been reported [17–19]. During the 2015 outbreak in the Northeast region of Brazil, the incidence of microcephaly was 20 times higher than in other periods, and viral RNA was found in fetal brain tissue of microcephalic babies as well as in the amniotic fluid and placentas of pregnant mothers [20]. It is estimated that 10% of babies from infected mothers may develop birth defects [21]. A recent case-report study confirmed that congenital Zika syndrome can be the result of sexual transmission [22].

In November 2016, the WHO ended its global health emergency over the spread of ZIKV and, in the following year, the number of new cases dramatically decreased [23, 24]. The change in designation and the drop in clinical cases, however, do not represent a downgrading of ZIKV importance. Specialists say that epidemics of ZIKV and other arboviruses are likely to continue to emerge [25]. Continents/countries at risk of ZIKV outbreaks include Africa, Asia, the Caribbean, Central America (including Mexico), the Pacific Islands, and South America (including Brazil) [26]. A recent study confirmed the presence of natural ZIKV infection in neotropical non-human primates in urban areas of Brazil, suggesting they might have a role in establishing a sylvatic cycle in the region [27]. Therefore, ZIKV infection continues to be a serious threat, especially to pregnant women and their fetuses, and immune-naive newborns [24].

## Introduction

ZIKV infection produces a short viremia in the blood leading to systemic infection [28]; however, over 80% of infected people are asymptomatic, making them potential reservoirs [29]. Through December 2018, 36 cases of probable or confirmed sexual transmission of ZIKV were registered in over 10 different countries [30]. In fact, researchers stated that the magnitude of person-to-person ZIKV transmission is substantially underestimated, and the role of sexual transmission is potentially much larger than previously identified [31].

ZIKV replication has been detected in most organs so far, including the brain [32–35], placenta [36–38], ovaries, spleen [39], spinal cord [40], and testes [40, 41]. Although the major host cells sustaining ZIKV replication *in vivo* are of myeloid lineages found in the blood and tissues [42], other vulnerable cell types await further investigation. Cells of the male reproductive tract, namely interstitial Leydig cells, Sertoli cells, and germ cells in the testes have been found to be targeted by

ZIKV in mice [43]. Importantly, the male genital tract might harbor persistent reservoirs of ZIKV that could facilitate viral transmission over extended periods to uninfected hosts [44].

In response, health authorities have published guidelines for women and couples planning pregnancy. Women wishing to become pregnant and who are carrying out ART treatment in ZIKV endemic areas are encouraged to protect themselves from mosquitoes and from their partner's potentially infected semen as well. Other recommendations include counseling on the risk of infection during pregnancy, delaying pregnancy attempts until the risk is minimal, and undergoing ZIKV nucleic acid testing (NAT) and immunity testing before proceeding with ART [45].

The duration of excretion of virulent particles in semen needs further evaluation, but it is known that ZIKV RNA can persist in the semen up for 188 days (range 3–188 days) after the acute viremic phase in humans; infectious ZIKV was isolated in semen up to 69 days after symptom onset [46]. It is unknown, however, what impact this long-standing presence of the virus can have on male fertility and on the risk of sustained sexual transmission. This data and some specificities of ZIKV infection in the male genital tract represent a challenge to both public health and reproductive medicine including *in vitro* fertilization treatments, namely, the persistence of the virus in semen after the acute viremic phase, the teratogenicity of the virus, as well as the possible risk of infertility. Thus, an updated summary on the cellular and molecular ZIKV mechanisms and host cell-virus interactions related to the infection of the male reproductive system along with the description of identified damage observed in humans and animal models are presented. The potential impact of the virus on human ART and insights on the most recent treatment and diagnostics methods focused on the presence of the virus in semen, including an analytical approach based on lipidomics and its potential applications in prospective ZIKV diagnosis, will also be discussed in this review.

## ZIKV replication mechanisms on male reproductive tract

Viruses intricately interact with and modulate hosts cells at structural and molecular levels at several stages of their replication. ZIKV infection of skin cells showed receptor-mediated virus entry and increased virus replication upon stimulation of autophagy [47]. ZIKV seems to induce autophagosome formation to promote replication and may trigger apoptosis to foster viral dissemination. Similarly to DENV, ZIKV appears to use the C-type lectin receptor DC-SIGN and members of the TIM and TAM (two receptor families that mediate the phosphatidylserine (PtdSer)-dependent phagocytic removal of apoptotic cells) on host cell surface to gain access to the cytoplasm via receptor-

mediated endocytosis [47]. Widespread remodeling of intracellular membranes upon ZIKV infection have been observed during this process, including structural rearrangements of the endoplasmic reticulum associated with RNA genome replication and budding of virions inside it, as well as viruses in transit through the Golgi apparatus for viral maturation and subsequent cellular exit [48]. These findings show a major role for the phosphatidylinositol-3-OH kinase Axl as a ZIKV entry receptor and for cellular autophagy in enhancing ZIKV replication in permissive cells [47]. Further genomic analyses revealed that many antiviral response-related genes are induced by ZIKV and upregulation of genes related to immune response, and apoptosis pathways were also observed. Conversely, many genes involved in cell proliferation, differentiation, migration, and organ development were downregulated [49].

### ZIKV infection and male fertility: spermatogenesis and endocrine abnormalities

Axl receptors are thought to be the main access route of ZIKV to immune-privileged sites, like the human male reproductive tract. Similarly to what has been observed in the brain, it is hypothesized that ZIKV particles persisting in the blood could enter the endothelium cells of capillaries in the testis, which are known to have several receptors for flaviviruses (including Axl) replicate inside them and be released in the interstitial space in contact with Leydig cells, macrophages, dendritic cells, and fibroblast [50].

A physical blood-testis-barrier (BTB) formed by tight junctions between adjacent Sertoli cells, associated with mechanisms that suppress immune responses, aids developing sperm to remain protected from an autoimmune attack [51]. However, *in vitro* studies showed that Sertoli cells are very permissive to ZIKV infection, possibly because they, unlike Leydig cells, highly express the AXL gene. A breach of the immune privilege of the testis could be a serious threat to spermatogenesis as it would compromise the function of key cells in the process (e.g., Sertoli and Leydig cells) [51]. Infection of Sertoli cells led to the dysregulation of several mRNA transcripts, notably the upregulation of fibroblast growth factor (FGF2) that could possibly lead to lower fertility [52].

Concrete information on the fertility of men who have been exposed to ZIKV is still scarce. In a prospective observational study, Joguet et al. [53] studied 15 male volunteers (mean age 35 years) with acute ZIKV infection and positive ZIKV RNA detection in blood or urine. Blood, urine, and semen were collected at days 7, 11, 20, 30, 60, 90, and 120 after symptom onset, and semen characteristics (such as total sperm count, sperm motility, vitality, and morphology) and reproductive hormone concentrations (testosterone, inhibin, follicle-stimulating hormone, and luteinizing hormone) were assessed. The authors demonstrated that total sperm count has decreased from median  $119 \times 10^6$  spermatozoa at day 7 to  $45.2 \times 10^6$  at day 30, and

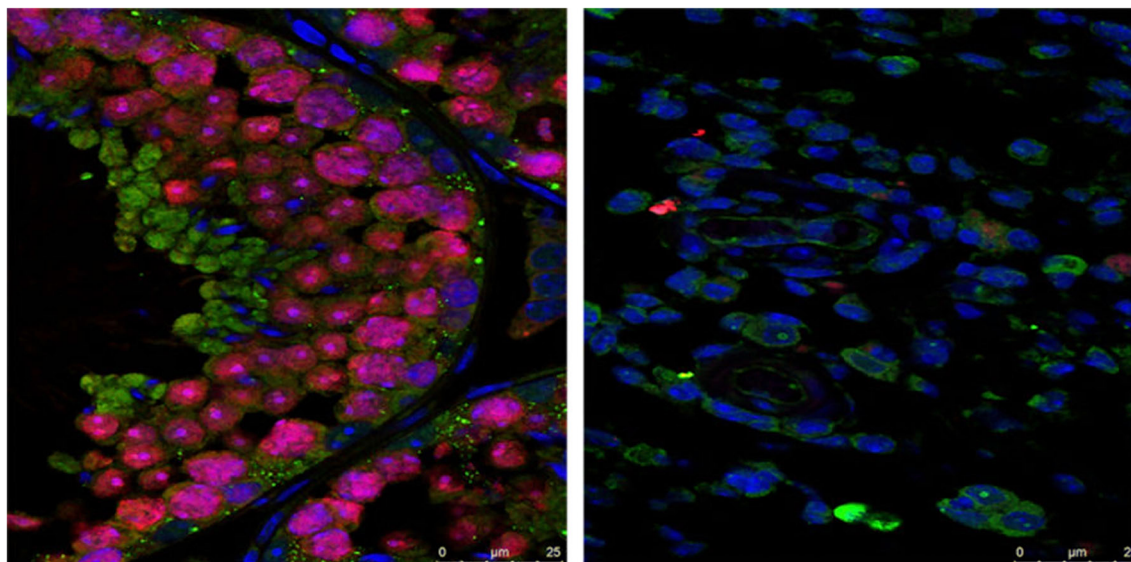
$70 \times 10^6$  at day 60, respectively, after ZIKV infection. Inhibin values increased from 93.5 pg/mL (55–162) at day 7 to 150 pg/mL (78–209) at day 120 when total sperm count recovered [53]. Taken together, these findings demonstrated harmful effects of ZIKV on human spermatozoa production. The authors concluded that semen alterations occurring early after acute ZIKV infection might affect fertility and could be explained by virus effects on the testis and epididymis, which needs further investigation. Clinical implications of ZIKV compartmentalization in whole semen are still currently not understood. This is a matter of concern as ZIKV seems to be the only arbovirus that causes such severe symptoms of infertility in humans.

### Insights from ZIKV infection of the male reproductive tract from animal models

Since the characterization of a novel murine model to study ZIKV by Rossi et al. [54], many experimental infection studies reported that ZIKV severely damages the testes of mice [41, 43, 55].

A longitudinal study in wild-type C57BL/6 mice infected with ZIKV (strain H/PF/2013—French Polynesia 2013), a mouse-adapted Dakar 41,519 (Senegal 1984), or DENV serotype 2 (strain D2S20) was recently conducted [43]. The persistence of ZIKV in semen was associated with testicular damages from both Sertoli and Leydig cells infection, oligospermia, and severely impaired male fertility [43]. Three weeks after infection, both testicles had shrunk to one-tenth of their normal size and the internal structure was completely destroyed [43]. The infected cell types found in this study included spermatogonia, primary spermatocytes, Sertoli cells, and, to a lesser extent, Leydig cells [43]. The symptoms affected the mice ability to produce two important sex hormones, including testosterone and inhibin B, both of which are important in sperm production [43]. This made their sperm counts fall and greatly reduced their fertility (Fig. 1). In addition, fecundity showed a 75% decrease in female mice mated with ZIKV-infected male mice than in female mice mated with uninfected controls. In contrast, male mice infected with DENV showed no evidence of acute or persistent infection in their reproductive systems [43].

Using C57BL/6 murine infected with American-derived ZIKV (MEX2-81 strain), Uraki and colleagues [41] showed that Leydig cells were able to support ZIKV replication, possibly acting as a reservoir for ZIKV, and that persistent infection may lead to hypofertility. The weight and length of testes were also significantly affected when compared to uninfected mice (Fig. 2), indicating progressive testicular atrophy. In addition, a high amount of antigen associated with the sperm mass within the epididymal duct lumen was observed [41]. In type I interferon receptor-deficient (IFNAR<sup>-/-</sup>) mice, highest levels of ZIKV RNA ( $10^7$  FFU equivalents per gram) were also observed in the testes, which could explain male-to-female sexual transmission of ZIKV observed in humans [41].



**Fig. 1** Immunofluorescence staining showing that the testis of ZIKV-free mice (left) are full of developing sperm (pink), while the testes of ZIKV-infected mice (right) contain very few sperm. Font: Reprinted from

Govero et al. [43], with permission from Macmillan Publishers Ltd.: [Nature Publishing Group], advance online publication, Oct 31, 2016 (<https://doi.org/10.1038/nature20556>)

A few studies have also been carried in non-human primates model. A summary of the main outcomes of previously cited and other key studies on the effects of ZIKV infection on the male reproductive tract of different models, including humans, are presented in Table 1. Collectively, these data will allow further investigation of ZIKV pathogenesis and persistence in the male reproductive tract but, although alarming, these findings have to be analyzed with caution.

Immunocompetent mice are generally resistant to ZIKV infection, so, many groups (including the ones cited above) opted for using immunocompromised strains to carry out studies about the disease [58]. In humans, ZIKV antagonizes type I interferon (IFN) response through its NS5 protein leading to degradation of a transcription factor (STAT2) that mediates signaling by the type I IFN receptor, IFNAR. However, ZIKV NS5 did not promote degradation of mouse STAT2 which may explain their immunity. Therefore, mice strains lacking the *Ifnar1* gene, including A129 and *Ifnar1*<sup>-/-</sup> C57BL/6, were used and developed severe disease [58].

Meanwhile, it is known that ZIKV infection of rhesus and cynomolgus macaques share many key clinical findings with humans [27]. The recent finding that New World primates, like the common marmoset in Brazil, are naturally infected by ZIKV points to a possible new and easier to manage experimental model for the study of arboviral infections [27].

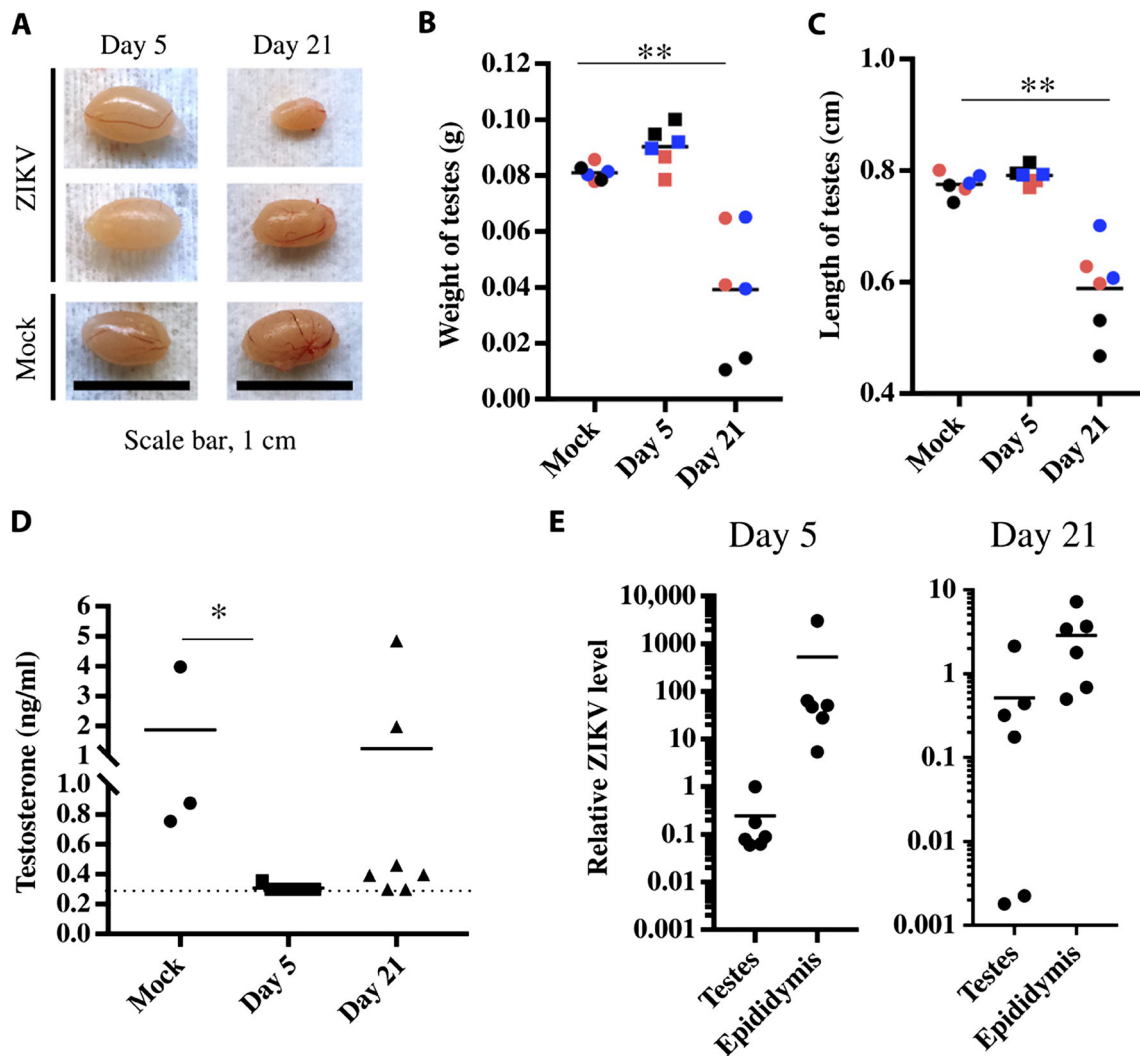
### Detection and diagnosis, impact on ART, and protective approaches

The human immune response to ZIKV is complex and can be both protective and pathogenic. Also, there are cross-reactions

among different viruses such as DENV and chikungunya (CHIKV) serotypes [59]. This fact has implications not only for the understanding of clinical manifestations but also for diagnosis strategies. So far, no diagnostic tests have been adapted to low-resource countries, and there are no rapid tests specific for semen. Currently, there are at least 14 nucleic acid detection lab-based tests (NAT), and 5 serology tests that have received authorization for emergency use from the U.S. Food and Drug Administration (FDA) and likely more from other regulatory agencies around the world [60]. In addition, there are several companies with kits at various stages of product development. [61].

Theel and Hata [60] reviewed the main studies regarding the efficiency of the serological and molecular tests available in the USA. Of the five serological tests available at the time, only three had peer-reviewed studies assessing their efficiency beyond those performed by their manufacturers. The tests based on the detection of IgM class antibodies to ZIKV use mostly serum as a specimen and, in general, have similar results to molecular assays. Comparison of serological and molecular tests showed high correlation between positive results, however, negative results agreement was inconsistent between the two assays [60]. This reinforces the recommendation of the Centers for Disease Control (CDC) to use its plaque-reduction neutralization testing (PRNT) for the confirmation of presumed positive, equivocal, or inconclusive tests. To further demonstrate active infection, either viral proteins or viable infectious viral particles would need to be recovered from a patient's samples [62].

As for molecular diagnostics approaches for ZIKV, the tests are based mainly in virus RNA detection in serum or urine samples collected during the first 2 weeks of symptom onset. Due to DENV, CHIKV, and ZIKV comorbidity, a



**Fig. 2** Testicular abnormalities after ZIKV infection. The testes of *Ifnar1*<sup>-/-</sup> mice infected with 10<sup>5</sup> plaque-forming units (PFU) of ZIKV<sup>MEX</sup> via subcutaneous route and of mock-infected mice were harvested at 5 and 21 days post-infection (dpi). **a** The photographs compare the testis of infected mice. Scale bar, 1 cm. **b, c** Weight and length of whole testis from infected animals were measured (*n* = 6 testes comprising three mice; *\*\*P* < 0.01). Same color indicates the same mice.

**d** The levels of testosterone in blood of infected mice investigated by enzyme-linked immunosorbent assay (*\*P* < 0.05). The detection limit is 0.3 ng/ml. **e** The relative viral RNA levels in testis and epididymis at 5 and 21 dpi were examined by qRT-PCR. Data are normalized to mouse *b*-actin. Font: Reprinted from Uraki et al. [41], with permission from Science Advances

laboratory test to sort out the three infections in emergency situations, named Triplex RT-PCR was the first to be approved by the FDA and showed highly accurate results, although, a false-positive has been reported, urging the CDC to release a statement about it. At least three other molecular assays had similar results [60]. The challenges for the current methods being developed include the lack of specificity for antibody-based assays [6, 63], especially in semen [64], and a very narrow time window for PCR (< 1 week) [65]. Serum and urine are the still the most used samples for practical reasons; however, the recent report of detection of ZIKV RNA in semen of asymptomatic blood donors by RT-PCR [66] highlights the need for readily-available, cost-effective methods of detection of viral load in semen.

### Detection of ZIKV in semen

Recently, Joguet and colleagues [53] demonstrated that replicative ZIKV can persist in semen after clinical remission despite its clearance in other fluids, such as blood and urine. It is important, however, to note that long-term persistence of ZIKV in the male genital tract does not necessarily reflect infectivity [31].

The time between onsets of symptoms in the partner after presumed sexual transmission range from 4 to 44 days, the average reported to be between 9.5 and 13 days [31, 67]. Viral load in the semen was roughly 100,000 times that of blood or urine 2 weeks after symptom onset [68]. Recent data reported the detection of ZIKV in the spermatozoa head obtained in the

**Table 1** Main outcomes of key studies on the effects of ZIKV infection on the male reproductive tract of different models

| Publication (year/authors) | Model   | Main outcomes  |
|----------------------------|---|--|
| <b>Mice</b>                |   |  |
| 2016/Ma et al. [55]        | Ifnar1 <sup>-/-</sup> mice (C57BL/6 background)                                 | <ul style="list-style-type: none"> <li>• ZIKV caused orchitis-epididymitis that led to infertility;</li> <li>• Peritubular cell and spermatogonia could be the potential repositories for ZIKV.</li> </ul>   |
| 2016/Govero et al. [43]    | Wild-type C57BL/6 mice (treated with a single dose of Ifnar1-blocking antibody) | <ul style="list-style-type: none"> <li>• Observed persistence of ZIKV in the testis and epididymis associated with tissue injury that caused diminished testosterone and inhibin B levels and oligospermia.</li> </ul>                                   |
| 2017/Uraki et al. [41]     | Ifnar1 <sup>-/-</sup> mice (C57BL/6 background)                                 | <ul style="list-style-type: none"> <li>• ZIKV replication persists within the testes even after clearance from the blood;</li> <li>• ZIKV infection caused a reduction in serum testosterone.</li> </ul>   |
| 2017/Sheng et al. [56]     | AG6 mice  | <ul style="list-style-type: none"> <li>• Observed marked atrophy of seminiferous tubules and reduction in lumen size accompanied by positive staining of ZIKV antigens on Sertoli cells.</li> </ul>  |
| 2018/Clancy et al. [57]    | Male Ifnar1 <sup>-/-</sup> mice and female AG129 mice                           | <ul style="list-style-type: none"> <li>• Confirmed the passage of virus from epididymal flush and seminal plasma to naïve females via artificial insemination.</li> </ul>  |
| <b>Non-human primates</b>  |   |  |
| 2016/Osuna et al. [42]     | Indian-origin rhesus monkeys and cynomolgus monkeys                             | <ul style="list-style-type: none"> <li>• Viral RNA was detectable in seminal fluids 3 weeks after the resolution of viremia in the blood;</li> <li>• Re-infection of animals 45 days after primary infection resulted in complete protection.</li> </ul> |
| <b>Humans</b>              |   |  |
| 2017/Joguet et al. [53]    | Humans  | <ul style="list-style-type: none"> <li>• Total sperm count decreased progressively after acute Zika virus infection for 30 days, at day 120 sperm count was recovered;</li> </ul>  |

**Table 1** (continued)

| Publication (year/authors) | Model  | Main outcomes  |
|----------------------------|--|--|
| 2018/Kumar et al. [52]     | Primary human Sertoli and Leydig cell cultures | <ul style="list-style-type: none"> <li>• Zika virus RNA was found in motile spermatozoa obtained after density gradient separation.</li> <li>• Sertoli but not Leydig cells are highly susceptible to Zika virus infection, this process is dependent on the TAM family receptor Axl.</li> </ul> |

semen of a 32-year-old man returning from French Guiana using immunohistochemical analysis [68]. The proportion of spermatozoa contaminated by ZIKV was estimated at 3.52%.

In motile spermatozoa obtained after density gradient separation, ZIKV RNA was found in three out of 14 patients at day 7, four of 15 at day 11, and four of 15 at day 20. Replication-competent virus was found in the tested patient, showing replication-competent virus in seminal plasma and in spermatozoa after sperm isolation by semen preparation methods [53, 69]. However, only one semen preparation protocol has been evaluated so far; therefore, more studies are needed to assess the effectiveness of different procedures.

In fact, several questions on the effect of ZIKV infection on human health remain open [70]. Further studies are needed to identify the precise locations in male genital tract targeted by ZIKV infection, to explain the longtime presence of the virus in semen in comparison to other body fluids, determine the maximal duration of ZIKV infectivity of semen, and the long-term impact of infection on male fertility [70]. It is also of great importance to investigate semen preparation protocols capable of eliminating ZIKV transmission through ART.

### Current and prospective diagnosis approaches for semen samples

So far, the only mention in literature of a method of detection of ZIKV in semen being developed is a single patent by Mariani and Stern [71] that describe “a highly sensitive and robust method for Zika detection in semen, as well as related compositions that include: (a) extracting nucleic acids from a human semen sample; (b) detecting Zika virus nonstructural protein 5 (NS5) mRNA using real-time reverse-transcription polymerase chain reaction (rRT-PCR); and (c) simultaneously, in the rRT-PCR, detecting human beta-actin mRNA as positive control.”

Another prospective diagnosis approach involves lipid screening in semen samples. A recent study indicated that lipids might be useful biomarkers for identifying the infection

by ZIKV in *Aedes* mosquitoes [72], and the same might be speculated for humans. In the past few years, mass spectrometry (MS)-based strategies in combination with bioinformatics have been successfully applied to identify and quantify global changes in cellular proteins, lipids, peptides, and metabolites in response to viral infection. Structural lipids and proteins, the main components of biological membranes, play an important role in viral infection and, notably, Flavivirus replication induces invaginations in the membranes of the endoplasmic reticulum, forming vesicles where RNA replication occurs. The redistribution of cellular lipids to replication sites that follows appears to favor virus replication and survival but is not well understood [73]. Perera et al. [74] used high-resolution MS to evaluate global changes in lipid metabolism upon DENV infection. They found drastic alterations in the global lipid profile in the cells of both human (hepatic cells, Huh-7) and mosquito (*Ae. albopictus* derived C6/36 cells). Furthermore, genes associated with lipid metabolism were regulated after bacterial or DENV infection in mosquito cells, suggesting that lipid droplets have a role in mounting an antiviral response [73, 75]. Electron spin resonance studies suggest that the virus membrane lipid composition produces a more ordered membrane in the virus compared with the host cell plasma membrane from which it buds [76], such changes in the lipid profile of cells infected by DENV and HIV could also be present on sperm cells infected by ZIKV and readily detected by MS.

Initial exploratory results of our group indicate that informative lipid profiles can be obtained from small amounts of sperm. It seems likely that lipid markers can be characterized for semen infected with ZIKV using widespread metabolomics approaches [77] or using novel biomarker discovery methods such as the MRM-profiling, which has been reported for Parkinson's disease and polycystic ovarian syndrome [78, 79]. Specific panels of small molecules could become a diagnostic tool to support semen screening before the use of ART in humans and even a prognostic method if metabolic signatures related to infertility due to virus-induced damage can be found.

### ZIKV and assisted reproductive technologies

In September 2016, the American Society of Reproductive Medicine (ASRM) published a set of guidelines modified from combined CDC, FDA, and WHO publications, with a focus upon caring for patients with infertility and donors of sperm, oocytes, and embryos [80]. A copy of the table present in the updated ASRM document summarizing recommendations for diverse cases can be seen in Table 2.

As shown by those guidelines, ZIKV infection affects not only ongoing ART treatment but fertility preservation programs as well, and the handling of semen, a leukocyte-rich material and therefore highly susceptible to viral infection, is

given special attention. A recent study argued that ZIKV present in spermatozoa likely survives both the freezing and thawing processing of semen cryopreservation [82], thereby resulting in frozen sperm having the ability to transmit the virus at a later point in time. Regarding oocytes, there is only one case reporting the presence of ZIKV to date [83], and it is unknown if they will be susceptible to lasting ZIKV infection. Since there is no rapid test available for ZIKV IgM and IgG for semen sample, neither a standardized method to test human oocytes or embryos derived from women who underwent oocyte retrieval at a time of a positive serum ZIKV PCR result [84], the presence of ZIKV in semen and potentially in the female genital tract [85, 86] must be taken into account when reviewing all protocols used in ART.

As an effort to keep ZIKV out of sperm banks and clinics supply, organs and tissue donations, including reproductive tissues and gamete cells, were suspended to avoid infection of future patients. Other measures included the ineligibility of tissue and semen donors that have traveled to, or live in, an area of active ZIKV transmission, under current FDA guidance [87]. Patients must also be informed that direct viral RNA testing with RT-PCR could result in both false-negative and false-positive results. Testing outside of the time of treatment for infertility does not indicate viral infectious status at the time of treatment [80]. In addition, a negative serum RNA result cannot rule out infection in reproductive tissues [80]. More data regarding the maximal duration of ZIKV infectivity in the semen of men and new accurate diagnostic tools are needed in order to allow for different recommendations.

In some cases, however, ART procedures may be urgent, (e.g., fertility preservation before cancer treatment or women with poor ovarian reserve) [88, 89]. For cases like this, the French Biomedicine Agency based its recommendations for ART management on the ZIKV infection status of the male patient, through combining ZIKV serology (IgM and IgG) and RT-PCR testing of a semen sample [90, 91]. If both ZIKV serology and RT-PCR testing of semen are negative, ART can be performed with no specific ZIKV-related measures. In case of positive results or previously diagnosed ZIKV infection, sperm preparation through selection on a density gradient and washing procedure is recommended. Importantly, ZIKV RT-PCR should be performed on both seminal fluid and selected sperm, and only ZIKV-negative sperm should be used for fertilization [92]. Extreme caution is still advised since the risks involve possible ZIKV transmission to a noninfected partner, to the couple's embryo, to other embryos and gametes, and to the laboratory team [89].

### Protection against ZIKV in the male reproductive tract

Recent studies described promising results utilizing a DNA vaccine to reduce the damage the male reproductive tract of

**Table 2** CDC recommendations for preconception counseling and prevention of sexual transmission of Zika virus among persons with possible Zika virus exposure

| Exposure scenario  | Recommendations (update status)  |
|--|--|
| Only the male partner travels to an area with risk for Zika virus transmission and couple planning to conceive   | The couple should use condoms or abstain from sex for at least 3 months after the male partner's symptom onset (if symptomatic) or last possible Zika virus exposure (if asymptomatic).<br>(Updated recommendation)  |
| Only the female partner travels to an area with risk for Zika virus transmission and couple planning to conceive   | The couple should use condoms or abstain from sex for at least 2 months (8 weeks) after the female partner's symptom onset (if symptomatic) or last possible Zika virus exposure (if asymptomatic).<br>(No change in recommendation)   |
| Both partners travel to an area with risk for Zika virus transmission and couple planning to conceive  | The couple should use condoms or abstain from sex for at least 3 months from the male partner's symptom onset (if symptomatic) or last possible Zika virus exposure (if asymptomatic). (Updated recommendation)  |
| One or both partners have ongoing exposure (i.e., live in or frequently travel to an area with risk for Zika virus transmission) and couple planning to conceive | The couple should talk with their health care provider about their plans for pregnancy, their risk for Zika virus infection, the possible health effects of Zika virus infection on a baby, and ways to protect themselves from Zika. If either partner develops symptoms of Zika virus infection or tests positive for Zika virus infection, the couple should follow the suggested timeframes listed above before trying to conceive.<br>(No change in recommendation) |
| Men with possible Zika virus exposure whose partner is pregnant  | The couple should use condoms or abstain from sex for the duration of the pregnancy. (No change in recommendation)   |

Source: Polen et al. [81]

mice. Griffin et al. [93] demonstrated that administration of a synthetic DNA vaccine encoding ZIKV pre-membrane and envelope (prME) was able to completely protect mice against the damage described by Govero and collaborators in 2016 [43] to the reproductive tract of immunocompromised mice and was also capable of preventing viral persistence in the testes. Zou et al. [94] reported similar results as well as the protection against maternal-to-fetal transmission in pregnant subjects after the administration of a single dose of a plasmid-launched live-attenuated Zika vaccine (DNA-LAV), suggesting that its simplicity and effectiveness might lead to the development of a universal vaccine platform for ZIKV and other RNA viruses.

## Summary and perspectives

While rodent models will keep helping to improve our understandings of tissue tropism and viral spread, their relevance to human infection and disease must be critically evaluated. Immunocompetent non-human primates infected with ZIKV produce clinical responses that closely resemble those of humans and, therefore, are likely more relevant.

There is an urgent need for more and better research focusing on prospective cohort study designs to determine the frequency and duration of viral shedding in the male genital tract. Equally important is determination of the origins of ZIKV in semen and the seminal components, and elucidating whether ZIKV could be present in semen as free particles or associated with leucocytes and epithelial cells in vasectomized/azoospermic men, whether ZIKV preferably adheres to the spermatozoa or could be present in both germ cells and the seminal fluid in normospermic men, and to look for specific methods to obtain ZIKV-free gametes in infected men undergoing ART. Overall, the observations reported by Joguet and colleagues have provided valuable baseline information about endocrine abnormalities and spermatogenesis commitment in ZIKV-infected men. Studies in areas with high rates of ZIKV occurrence, however, are still lacking to determine the impact of ZIKV on men's reproductive health. Identification of fusexins, fusion proteins essential for both sexual reproduction, and exoplasmic merging of plasma membranes may contribute to our better understanding of how ZIKV infect their target hosts and could help developing diagnostic approaches. Altogether, these studies provide valuable insights into ZIKV infection and about strategies for helping to manage



the virus in ART procedures as new protocols to purify ZIKV-infected semen.

In the face of the gaps in knowledge around ZIKV persistence/infectivity in semen, the successively revised guidelines are important to both limit public health risks and deliver general information to couples planning to conceive, gamete donors, and patients requesting ART. Nonetheless, novel sensitive and specific ZIKV diagnosis tools for semen screening are urgent public health instruments to contain ZIKV virus spread and make ART safer.

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