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Long-term Zika virus infection of non-sperm cells in semen from infected men

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Zika virus (ZIKV) is a mosquito-borne teratogenic virus that was unexpectedly found to persist in semen. Viral RNA was detected up to 414 days post-symptoms onset (dpo)¹ and infectious virus rescued until 69 dpo². During the last outbreak, sexual transmission of ZIKV was reported in over 14 countries outside the epidemic zone³.

The cellular reservoirs of ZIKV and reasons for the sperm alterations we reported⁴ are unknown. ZIKV is present in seminal plasma, whole semen cell fraction and found associated with spermatozoa up to 56 dpo⁵. We showed that ZIKV infects the human testis *ex vivo* and that productively infected sperm progenitors (i.e. testicular germ cells) are shed in semen from acutely infected patients⁶. Here we performed the first characterization of the seminal non-sperm cells infected by ZIKV in 3 patients from our published cohort (P6, P13, P15)⁴, for whom we collected longitudinal semen smears up to 160 dpo. Using antibodies against cell markers and ZIKV antigens, we reveal the persistent shedding of ZIKV-infected DDX4+ testicular germ cells (TGCs) in the semen of all 3 patients. In P6, TGCs were infected up to 160 days and represented the bulk of infected non-sperm cells in longitudinal samples, as assessed by counting ZIKV envelope (E) positive cells on semen smears by fluorescence microscopy. An estimated 21.6 to 52% of the TGCs released in semen were positive for ZIKV-E across patients. In addition, detection of the non-structural viral protein NS1 in TGCs at 60 dpo (P6) demonstrated ongoing ZIKV replication. Up to 56.5 % of exfoliated Epcam+ epithelial cells (P15 dpo 10-20) and up to 72.7% of CD14/CD68+ monocytes/macrophages (P6 dpo 10-20) stained positive for ZIKV-E in the semen smears from these 3 patients. Infected epithelial cells and monocytes/macrophages were detected up to 90 dpo (P13). We searched for ZIKV E+ FSH receptor+ Sertoli cells but only rare events at very early time points (7 dpo) were found in one of the two patients tested (P6 and P15), and none

at later times (20, 60 and 90 dpo). In leukocytospermic samples, a small proportion of MPO+ neutrophils harbored ZIKV-E up to 60 dpo.

Altogether, these results reveal that human testicular germ cells are a persistent cell reservoir for ZIKV, in which the virus actively replicates. Exfoliated infected epithelial cells and leukocytes additionally contribute to prolonged virus shedding in semen. These two cell types may originate from several sites within the genital tract such as the epididymis, vas deferens, seminal vesicles, prostate or urethra. The persistent infection of non-sperm cells participates to the long-term shedding of ZIKV in semen from men and could explain the prolonged ZIKV excretion reported in semen from vasectomized men³. These data are important and highlight the need for further studies to decipher ZIKV localization within the human male genital tract and their consequences for reproductive function and sexual transmission.

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Figure

Characterization and quantification of ZIKV-infected non-sperm cells in longitudinal semen samples from 3 ZIKV-infected patients for whom semen characteristics were published in a previous paper⁴.

The table shows viral loads, cell concentrations in semen and percentage of infected non-sperm cells within each cell type (first value) or related to the total number of infected cells (second value in blue italic) counted on the semen smears using NDPview software. Slides were immunolabeled as we described⁶, using the following combinations of antibodies: ZIKV E (envelope)/ DDX4 (germ cells marker)/ Epcam (epithelial cells marker) or ZIKV E /CD14/CD68 (monocytes/macrophages marker) /MPO (neutrophils marker). For P6 d60, we performed additional staining with ZIKV NS1/DDX4. The percentage of infected cells within each cell type was obtained by quantifying cells double-labelled for ZIKV E and a specific cell marker *versus* cell single-labelled for this marker. The percentage of infected germ cells, epithelial cells, monocytes/macrophages and neutrophils related to the total number of

infected cells/slide was obtained by quantifying cells double-labelled with ZIKV and the cell marker of interest *versus* cells single-labelled with ZIKV. The picture shows examples of immunofluorescence detection on semen smears of ZIKV E (red) or NS1 (red, here for P6 60 days) together with specific cell markers (green or light blue). Nuclei are stained in blue with Dapi. Scale bars = 10µm.

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