Supplementary Appendix

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Acute ZIKA Infection in Men: Deleterious Effects on Sperm and Virus Clearance in Body Fluids. Results of a

Prospective Study

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A. Methods

Semen analyses

Semen analysis was performed according to WHO guidelines¹ on an aliquot (200 μ L) of semen. The characteristics considered were sexual abstinence (days), ejaculate volume (ml), pH, sperm concentration (SC, 10⁶ spermatozoa/ml), round cell concentration (RC, 10⁶/ml), spermatozoa vitality (V, %) forward motility (M, a+b: %), total sperm count (volume × SC = TSC, 10⁶ spermatozoa/ejaculate), and total motile sperm count (TMSC = TSC × M, 10⁶/ejaculate). Sperm cell morphology was assessed according to Auger's modification of the classification of David *et al.*¹ All semen analyses were performed by three trained technicians and underwent external quality control.

Sperm Processing to Isolate Spermatozoa Fractions

At days 7, 11 and 20, in order to isolate spermatozoa-enriched fraction whole semen was submitted to differential density gradient centrifugation over 40, 70 and 90% of PureSperm[®] (JCD S.A., Lyon, France) according to a previously published method.² The sperm pellet (90% PureSperm[®] layer) was washed twice with FertiCult IVF medium (JCD S.A., Lyon, France). The 40% fraction contains mostly low motile, dead, or abnormal spermatozoa and seminal non-spermatozoa cells (NSCs).

As the sperm pellet from the 90% fraction could contain rare non-sperm cells, motile spermatozoa from this fraction were further separated by the swim-up method according to a previously published method.² Briefly, the sperm pellet was overlaid with 1·1 mL medium and incubated at 37°C, 5% CO2 for 60 min in a tube at an angle of 45 degrees to allow the motile spermatozoa to swim up. The swim-up fraction contains only viable motile spermatozoa.

The seminal plasma, the whole semen cells and the different fractions obtained after density gradient preparation and after the swim-up method were collected and stored at -80°C until ZIKV isolation and genome analyses.

ZIKA Virus Isolation

Samples from a patient (P13) with positive ZIKV RNA detection in seminal plasma and cell fractions including motile spermatozoa were tested for infectivity. Seminal plasma (200 μ L) or cell pellets from 90% and swim-up fractions were diluted in 2 ml Dulbecco's modified Eagle medium (DMEM), 1% glutamine, 1% penicillin-streptomycin, 5% fetal calf serum (all from Gibco Invitrogen, Carlsbad, USA), and placed in contact with subconfluent Vero E6 cells for 24 h. Cell culture medium was collected (inoculum) and replaced with 8 ml of fresh medium.

At day 6 of culture, a second passage was performed by adding 750 μ l of supernatant diluted in 8 ml of culture medium to fresh subconfluent Vero E6 cells. Viral growth was assessed by daily checking of cytopathic effect (CPE) under a light microscope and confirmed by RT-PCR quantification of RNA copies/ml in supernatants (140 μ l) collected at different culture time points (inoculum, 6 hours, day 5, day 6 of first passage, and inoculum, day 4, day 5 of second passage).

B. Figures

Figure S1. Design of the Study.

Biological ZIKV Diagno	osis						
							
Acute per	iod			Follow	-up		
<u> </u>	1	1	1	1	1	1	\uparrow
D0 ZIKV clinical manifestations	D7 ±1	D11 ±2	D20 ±2	D30 ±3	D60 ±5	D90 ±5	D120 ±5
Blood (n)	+ (15)	+ (15)	+ (15)	+ (15)	+ (7)	+ (12)	+ (13)
Urine (n)	+ (15)	+ (15)	+ (15)	+ (14)	+ (7)	+ (12)	+ (13)
Semen cells (n)	+ (15)	+ (15)	+ (15)	+ (15)	+ (13)	+ (14)	+ (13)
Seminal plasma (n)	+ (15)	+ (14)	+ (15)	+ (15)	+ (13)	+ (14)	+ (13)
Semen preparation (n)	+ (14)	+ (15)	+ (15)				

Figure S2. Anti-ZIKV IgM and IgG Antibodies in Serum at Different Time Points Post Symptom Onset.

Blue line: IgG; red dash: IgM. Values above line 1 indicate positive results. d=days; mo=months. Mean and SD were represented.



Figure S3. ZIKA Virus Isolation from Seminal Plasma and Spermatozoa Fractions of Patient 13 at Day 7 Post Symptom Onset. (A) Quantitative RT-PCR analysis on supernatants from Vero E6 cells inoculated with the semen fractions indicated. (B) Cytopathic effect on Vero E6 cells (day 4 of the second passage).



C. Tables

Table S1. Age and Clinical Signs Reported by the 15 ZIKV-Infected Patients.								
Age, years mean (SD)	34 (6)							
	n (%)							
Fever	13/15 (87)							
Eruption	14/15 (93)							
Conjunctivitis	12/15 (80)							
Muscle pains	13/15 (87)							
Asthenia	14/15 (93)							
Headache	9/15 (60)							

Table S2. Semen Characteristics of the 15 ZIKV-Infected Patients According to Time Points after ZIKV Infection Symptom Onset, and ZIKV RNA Native Semen Status.

	ZIKV RNA detection in native semen (seminal plasma or semen cells)							
			gative	Positive				
		n	median	[Q1–Q3]	n	median	[Q1–Q3]	
Volume (mL)*	D 7	4	3.1	[2.4-4]	11	2.2	[2-3.6]	
	D 11	6	2.55	[2.4-4.2]	8	3.4	[1.8-4.2]	
	D 20	8	3.2	[2.05-4.55]	7	2.5	[1.4-3.9]	
	D 30	12	2.6	[2.05-3.95]	3	2.2	[1.5-4]	
	D 60							
	(2 mo)	9	3.4	[2·2-4]	4	1.7	[1.6-3.6]	
	D 90	11	2.4	[2, 4, 7]	2	2.5	[1.7.4]	
	(3110) D 120	11	5'4	[2-4.7]	5	2.2	[1.7=4]	
	(4 mo)	12	2.8	[1.9-4.3]	1	1.6	[1.6-1.6]	
	D7	4	168·95	[1209-258.55]	11	50.4	[7.92–234]	
Total sperm count* (millions per ejaculate)	D 11	6	42·54	[33–52]	8	64·35	[47·37–236·8]	
	D 20	8	99.7	[60.86-121.45]	7	28.6	[7.54-281.4]	
	D 30	12	53·84	[20.12-131.75]	3	16.5	[2.42-45.2]	
	D 60							
	(2 mo)	9	76.76	[68-81.4]	4	24.025	[10.66-146.25]	
	D 90	11	го		2	10.75		
	(3 110) D 120	11	56	[17:0-307:5]	3	19.75	[3·37-80]	
	(4 mo)	12	91·75	[33·96–210·18]	1	13.76	[13.76–13.76]	
Total motile sperm count* (millions per	D 7	4	68·96	[57.27-90.49]	11	17.64	[3.4–134.46]	
ejaculate)	D 11	6	15.609	[8·4–20·8]	8	22.755	[15.91–136.98]	
	D 20	8	40.61	[24.29-48.86]	7	6.118	[1.89-126.63]	
	D 30**	12	17.362	[6·97–57·18]	3	3.3	[0·36–6·78]	
	D 60							
	(2 mo)	9	28.105	[23.8–35]	4	9.08	[2·4–79·73]	
	D 90	11	17.15	[4.75 02.25]	2	2.0625	[1.20.27]	
	(3 110) D 120	11	17.15	[4.75-92.25]	3	2.9025	[1.30-32]	
	(4 mo)	12	39.115	[11.97-88.29]	1	4·128	[4.13-4.13]	
Normal sperm (%)	D7	4	24.5	[14–33]	11	21	[15-33]	
	D 11	6	16	[12-21]	7	23	[19–37]	
	D 20	8	21.5	[12.5-32]	7	14	[13–16]	
	D 30	12	11	[6.5-24.5]	3	18	[13-42]	
	D 60							
	(2 mo)	9	16	[8–22]	4	19	[13-23.5]	
	D 90	10	14 5	[0, 10]	2	10	[12, 21]	
	(3 110) D 120	10	14.2	[9-19]	3	15	[12-21]	
	(4 mo)	10	20	[12–26]				
MAI	D 7	4	1.93	[1.78-2.14]	11	1.85	[1.71–2.17]	
	D 11	6	2.05	[2.01-2.12]	7	1.97	[1.84–2.2]	
	D 20	8	1.99	[1.67-2.19]	7	2.06	[1.83-2.35]	
	D 30	12	2.09	[1.9-2.38]	3	1.74	[1.72–2.13]	
	D 60			-			-	
	(2 mo)	9	2.18	[1.92-2.31]	4	2.08	[1.84–2.3]	
	D 90	10	2 105	[2.02 2.26]	n	2 22	[1.01 2.20]	
	(5 mo) D 120	10	2.192	[2.02-2.30]	3	2.27	[1.31-5.23]	
	(4 mo)	10	2.085	[1.96-2.13]				

* p<0.05 between negative and positive detection of ZIKV RNA in native semen, for all specimens

** p<0.05 between negative and positive detection of ZIKV RNA in native semen, over time

D=days; mo=months

MAI : multiple anomalies index

	Day 7				Day 11				Day 20			
Patients	Semen cells	Seminal plasma	90% fraction	Swim-up fraction	Semen cells	Seminal plasma	90% fraction	Swim-up fraction	Semen cells	Seminal plasma	90% fraction	Swim-up fraction
1	0	0	0		0	0	0		0	0	0	
2	4·18	4.60	0		5.04	5.80	2.8	0	0	3.28	0	
3	0	2.81	0		0	2.72	0		0	2.76	0	
4	3.16	2.79	0		0	0	0		0	0	0	
5	3.79	0	0		0	3.76	0		0	3.41	0	
6	2.56	0	0		3.64	3.57	0		5.31	5.47	3.69	2.78
7	0	0	0		0	0	0		0	0	0	
8	5.43	6.12	3.10	0	8.48	8.06	4.70	4.00	6.06	5.10	3.21	0
9	0	2.71	ND		0	INH	0		0	0	0	
10	2.64	3.82	0		0	0	0		0	0	0	
11	2.43	3.20	0		0	3.05	0		0	0	0	
12	0	0	0		0	0	0		0	0	0	
13	9.12	10.16	6.88	7.21	8.39	8.87	5.38	4.92	6.92	7.11	4.92	0
14	0	0	0		0	0	0		0	0	0	
15	7.02	7.85	3.71	3.56	7.78	7.84	4.34	3.08	4.43	5.16	3.20	3.03

Table S3: ZIKV RNA viral load in semen samples (semen cells, seminal plasma) before and after semen preparation by density gradients (90% fraction) or swim-up methods at 7, 11 and 21 days of follow-up. The swim-up method was performed if the 90% fraction was ZIKV RNA positive. ND: not done, INH: inhibitor of RT-PCR.

Table S4. ZIKV in the Blood, Urine and Different Semen Fractions.

Number (%) of samples with detectable ZIKV RNA and viral load (means, standard deviation, median, quartile 1, quartile 3 and maximum) for specimens with detectable ZIKV RNA.

	n (%)	Mean±SD (log RNA copies/mL or /2×10 ⁶ cells)	Median [Q1-Q3] (log RNA copies/mL or /2×10 ⁶ cells)	Max
Whole blood	62/92 (67)	3.68±0.06	3.75 [3.40-4.00]	4.70
Serum	36/92 (39)	3·30±0·08	3.30 [2.93-3.61]	4.38
Urine	36/91 (40)	3.62±0.12	3.68 [2.79-4.19]	5.38
Semen cells	27/100 (27)	5·23±0·40	5.03 [3.60-6.91]	9.12
Seminal plasma	35/99 (35)	4·96±0·36	4.20 [3.19-6.12]	10.20
90% fraction	11/44 (25)	4·17±0·36	3.70 [3.20-4.91]	6.88
Swim-up fraction	7/11 (64)	4·08±0·58	3.55 [3.03-4.91]	7.21

D. References

1. WHO. WHO laboratory manual for the examination and processing of human semen; Fifth edition. Geneva: World Health Organization, 2010.

2. Bujan L, Daudin M, Matsuda T, et al. Factors of intermittent HIV-1 excretion in semen and efficiency of sperm processing in obtaining spermatozoa without HIV-1 genomes. *Aids* 2004; **18**: 757–66.