

Supplementary Material

Effects of two environmental endocrine disruptors di-2-ethylhexyl phthalate and mono-2-ethylhexyl phthalate on human sperm function *in vitro*

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Fig. S1. An example for CTC staining patterns.

Table S1. Semen characteristics of the healthy donors

Donors	Age	Volume (mL)	pH	Concentration (10⁶ cell/mL)	Vitality (%)	TM (%)	PR (%)	Morphology (%)
1	22	2.5	7.5	75	85.6	73.2	56	12
2	23	2.0	7.8	60	83	69.3	54	8
3	24	3.0	7.6	30	80	60	43	6
4	24.5	3.5	7.4	35	85	70	54	10
5	25	2.0	7.2	55	85	73	56	8
6	27	2.5	7.8	52	75	43	32	7
7	27.5	2.3	7.9	48	79	59	41.2	4.5
8	27.5	2.2	7.5	40	81.7	61.2	48	8
9	28	2.6	7.4	44	79	58	39	8
10	28	3.2	7.5	46	78	55	35	10
11	28.5	3.5	7.6	52	79	59.4	42	12
12	29	3.1	7.3	70	81.7	62.8	53	6
13	29.5	3.6	7.8	25	75.8	45	34.1	8
14	31	2.5	7.5	35	76.1	48	35	7
15	35	2.4	7.5	30	89.4	74.2	60	9
Ref	-	> 1.5	7.2-8.0	> 15	> 58	> 40	> 32	> 4

The reference limit (Ref) is according to WHO laboratory manual for the examination and processing of human semen. Routine semen analysis was carried out immediately after liquefaction. TM, total motility; PR, progressive motility.

Table S2. Effects of DEHP on viability and motility of human sperm

	Test	0.1 % DMSO	20 nM DEHP	200 nM DEHP	2 µM DEHP	4 µM DEHP	8 µM DEHP
1h NC	Viability (%)	83.5±2.3	83.2±2.6	83.6±3.2	83.8±1.3	85.5±1.4	82.1±2.1
	Total motility (%)	59.0±4.2	57.1±5.5	58.2±4.8	58.8±4.1	59.7±5.3	59.6±4.6
	Progressive motility (%)	44.5±3.3	44.1±4.6	43.2±4.0	42.2±5.0	45.2±4.1	43.9±4.3
2h NC	Viability (%)	80.6±3.5	81.9±4.5	82.1±2.3	80.9±3.6	81.3±4.2	79.8±3.3
	Total motility (%)	56.5±2.5	56.4±1.9	56.2±2.2	56.1±2.8	55.9±2.9	56.4±2.6
	Progressive motility (%)	42.3±1.2	41.9±2.2	41.0±1.8	40.5±1.1	42.9±2.3	40.8±3.3
4h NC	Viability (%)	78.6±4.5	77.9±3.4	80.2±1.9	79.5±2.3	79.4±2.9	77.8±4.0
	Total motility (%)	53.1±2.3	53.6±3.5	52.4±2.9	54.2±3.6	53.7±2.5	52.9±3.3
	Progressive motility (%)	40.3±2.6	39.5±2.8	39.9±2.2	38.9±1.7	41.0±2.5	37.6±2.2
1h CAP	Viability (%)	83.4±1.9	82.6±2.2	84.1±2.6	83.6±2.2	86.1±2.2	83.7±2.3
	Total motility (%)	63.1±2.3	60.8±4.2	61.5±4.1	62.5±3.3	63.4±2.9	63.4±3.3
	Progressive motility (%)	46.1±1.9	46.8±2.2	45.1±1.9	43.9±2.2	46.7±1.9	44.3±5.2
2h CAP	Viability (%)	83.6±2.3	82.2±1.6	83.9±4.1	82.9±3.5	85.4±1.9	82.9±1.6
	Total motility (%)	65.5±4.5	63.8±2.3	65.4±4.4	66.1±3.3	65.9±3.3	66.5±2.2
	Progressive motility (%)	51.5±5.1	52.1±4.6	50.3±2.9	50.3±3.3	51.2±3.1	50.3±1.8
4h CAP	Viability (%)	82.5±1.9	80.9±1.6	79.3±3.1	79.5±1.6	81.1±2.1	80.0±1.5
	Total motility (%)	71.4±4.1	71.7±3.4	70.7±4.2	70.9±3.6	69.9±4.6	70.2±3.9
	Progressive motility (%)	58.0±4.2	58.4±5.1	60.3±5.7	56.8±6.3	57.5±6.1	56.4±5.4

Human sperm were treated with different doses of DEHP in 0.1% DMSO in HS medium for 1, 2 and 4 h (non-capacitating status, NC) and in HTF++ medium for 1, 2 and 4 h (capacitating status, CAP). Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay. Bar: mean ± SEM.

Table S3. Effects of MEHP on viability and motility of human sperm

Test		0.1 % DMSO	1 nM MEHP	10 nM MEHP	100 nM MEHP	1 µM MEHP	20 µM MEHP
1h NC	Viability (%)	82.3±1.7	82.9±1.5	81.8±1.1	82.0±1.1	83.1±1.3	80.2±1.9
	Total motility (%)	60.4±3.2	60.2±3.0	59.6±2.7	60.8±3.7	60.3±2.5	60.0±2.7
	Progressive motility (%)	44.7±3.5	43.3±3.8	44.9±4.0	44.6±3.9	45.0±4.8	45.4±4.5
2h NC	Viability (%)	80.4±2.3	81.5±3.3	80.1±2.5	81.2±1.6	82.4±3.2	78.9±2.5
	Total motility (%)	55.6±2.4	55.4±2.9	55.3±3.4	55.9±3.8	54.9±2.1	55.3±2.7
	Progressive motility (%)	43.1±2.2	42.7±1.8	42.2±1.4	40.9±2.1	40.4±3.6	39.9±2.5
4h NC	Viability (%)	77.5±5.4	78.9±4.4	79.6±3.9	78.5±5.3	78.9±3.9	78.0±4.5
	Total motility (%)	52.1±4.3	52.4±2.6	52.8±3.5	53.9±4.6	51.8±4.4	50.7±4.1
	Progressive motility (%)	40.6±3.4	38.8±2.1	37.8±3.2	38.1±2.7	40.0±2.4	38.1±3.2
1h CAP	Viability (%)	84.0±5.9	83.6±4.3	83.8±3.6	84.6±4.5	85.6±3.8	84.1±4.3
	Total motility (%)	62.5±3.4	61.8±3.7	62.4±4.8	63.5±4.3	63.6±3.4	63.9±5.3
	Progressive motility (%)	45.8±2.9	45.4±3.2	46.1±3.9	44.3±3.2	45.6±2.9	42.3±5.3
2h CAP	Viability (%)	82.5±4.3	81.2±5.6	82.4±5.1	81.5±4.5	84.3±3.9	81.7±3.6
	Total motility (%)	64.2±4.9	63.4±5.3	65.6±5.4	64.1±6.3	66.9±4.3	65.9±4.2
	Progressive motility (%)	50.6±5.2	51.1±4.8	52.3±4.7	51.3±5.3	52.4±5.6	50.3±4.8
4h CAP	Viability (%)	80.9±1.1	80.9±1.0	79.1±2.2	79.3±2.6	78.2±1.9	78.6±1.8
	Total motility (%)	71.6±2.8	72.0±2.9	71.7±3.1	71.9±3.8	71.9±3.4	71.8±4.4
	Progressive motility (%)	58.2±4.0	56.1±4.7	55.4±3.9	53.4±5.4	55.2±5.4	55.5±4.8

Human sperm were treated with different doses of MEHP in 0.1% DMSO in HS medium for 1, 2 and 4 h (non-capacitating status, NC) and in HTF++ medium for 1, 2 and 4 h (capacitating status, CAP). Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay. Bar: mean ± SEM.

Table S4. Effects of DEHP plus MEHP on viability and motility of human sperm

	Test	0.1 % DMSO	20 nM DEHP 1 nM MEHP	200 nM DEHP 10 nM MEHP	2 µM DEHP 100 nM MEHP	4 µM DEHP 1 µM MEHP	8 µM DEHP 20 µM MEHP
1h NC	Viability (%)	82.3±1.5	82.9±1.1	81.8±1.1	82.0±1.3	83.1±1.9	80.2±1.5
	Total motility (%)	59.3±3.3	59.2±2.6	59.7±3.0	60.1±3.0	59.2±2.8	59.8±3.1
	Progressive motility (%)	46.3±2.8	45.9±3.7	45.1±4.3	44.6±4.4	44.8±5.0	47.4±5.1
2h NC	Viability (%)	80.6±2.4	81.1±2.9	80.6±2.2	81.5±2.6	81.9±2.4	78.5±3.5
	Total motility (%)	55.6±3.5	55.6±3.9	54.2±4.7	53.1±4.1	53.9±5.0	54.7±2.9
	Progressive motility (%)	44.3±3.2	43.2±4.5	42.5±3.8	42.6±4.1	42.1±4.3	41.4±5.3
4h NC	Viability (%)	79.4±4.4	78.9±3.8	78.5±4.9	79.8±2.3	79.8±3.9	76.9±4.1
	Total motility (%)	52.5±5.3	51.6±4.9	52.8±5.9	51.2±4.6	52.5±4.5	51.5±4.3
	Progressive motility (%)	41.5±3.6	38.5±5.8	39.6±5.2	38.4±5.7	40.6±4.5	38.6±4.2
1h CAP	Viability (%)	83.9±2.9	82.7±3.2	83.5±3.4	83.1±3.2	84.1±4.2	82.1±5.3
	Total motility (%)	60.4±4.3	60.1±4.5	62.5±3.1	62.4±4.3	63.1±3.9	61.4±4.9
	Progressive motility (%)	48.9±3.5	47.4±3.2	46.1±4.9	45.9±4.2	46.2±3.9	48.3±5.5
2h CAP	Viability (%)	82.6±5.3	81.2±4.6	82.4±4.8	82.0±4.5	83.0±3.9	81.9±4.2
	Total motility (%)	64.5±4.3	63.9±5.3	62.9±4.8	65.1±4.3	66.4±5.3	65.4±4.8
	Progressive motility (%)	52.6±3.5	51.9±5.8	50.4±4.2	50.8±5.0	51.6±5.2	52.3±5.8
4h CAP	Viability (%)	80.9±1.1	80.9±1.0	79.1±2.2	79.3±2.6	78.2±1.9	78.6±1.8
	Total motility (%)	71.8±3.7	71.1±2.7	71.1±3.7	72.4±3.9	71.7±4.2	70.7±4.9
	Progressive motility (%)	56.4±4.8	59.0±4.8	56.7±5.8	58.1±5.6	57.8±5.7	59.8±5.0

Human sperm were treated with different doses of DEHP plus MEHP in 0.1% DMSO in HS medium for 1, 2 and 4 h (non-capacitating status, NC) and in HTF++ medium for 1, 2 and 4 h (capacitating status, CAP). Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay. Bar: mean ± SEM.

Table S5. Effects of DEHP on membrane integrity (MI), reactive oxygen species (ROS) and mitochondrial activity (MA) of human sperm

Test		0.1 % DMSO	H ₂ O ₂	20 nM DEHP	200 nM DEHP	2 µM DEHP	4 µM DEHP	8 µM DEHP
1h NC	MI (%)	73.0±5.9	38.3±3.8 ^a	74.0±2.9	73.7±7.2	75.0±7.0	75.3±6.4	74.0±10.7
	ROS (%)	100.0±3.0	122.0±3.9 ^a	99.9±1.2	100.6±2.8	102.4±3.0	100.7±2.5	100.1±2.3
	MA (%)	69.0±2.1	48.3±6.7 ^a	69.3±2.6	68.3±5.2	68.7±1.0	66.7±3.5	69.3±7.2
2h NC	MI (%)	72.1±4.9	39.6±4.0 ^a	75.5±4.1	71.5±6.8	73.3±6.4	72.5±5.1	72.9±8.8
	ROS (%)	100.0±2.5	133.4±5.5 ^a	98.4±3.9	99.2±4.2	96.1±2.8	100.9±2.1	99.4±2.4
	MA (%)	68.3±4.2	45.1±5.5 ^a	67.9±2.8	71.0±3.4	70.2±5.1	65.9±2.8	70.4±3.5
4h NC	MI (%)	70.6±4.1	37.7±5.6 ^a	70.9±3.8	71.2±4.9	70.5±4.3	72.5±3.9	71.8±5.0
	ROS (%)	100.0±2.3	144.5±5.6 ^a	95.6±3.5	101.4±3.9	104.7±3.9	103.7±2.1	104.9±4.3
	MA (%)	65.3±5.6	42.0±5.5 ^a	61.5±4.8	59.9±3.2	58.9±4.7	61.0±4.5	57.6±2.9
1h CAP	MI (%)	73.4±1.9	36.8±2.2 ^a	72.6±5.2	74.4±3.6	73.2±5.2	72.1±4.2	73.7±2.8
	ROS (%)	100.0±2.8	133.2±4.0 ^a	100.8±5.2	101.3±4.7	102.1±5.3	103.5±2.2	103.1±4.6
	MA (%)	70.1±3.1	49.5±2.6 ^a	70.8±3.2	70.9±2.9	69.9±3.2	68.7±4.9	70.7±5.3
2h CAP	MI (%)	73.6±2.3	37.9±2.6 ^a	71.2±3.6	72.9±4.1	72.9±1.5	70.4±5.9	71.9±3.6
	ROS (%)	100.0±3.5	140±5.6 ^a	100.8±2.9	101.4±2.9	106.1±4.3	105.9±3.8	101.5±4.2
	MA (%)	71.6±4.4	51.3±3.5 ^a	71.9±3.1	70.5±2.6	71.1±2.8	70.5±3.5	71.3±3.5
4h CAP	MI (%)	75.7±4.1	39.7±7.2 ^a	73.0±6.8	71.0±6.7	71.3±9.8	73.7±4.2	71.3±6.8
	ROS (%)	100.0±2.7	153.2±7.0 ^a	103.9±4.2	102.9±4.3	104.2±2.9	101.6±2.1	103.3±1.5
	MA (%)	75.7±4.1	54.7±3.5 ^a	71.7±4.3	71.0±6.0	70.7±5.9	71.0±3.8	72.7±4.8

^a $P < 0.05$, Two-way ANOVA. Bar: mean ± SEM. NC, non-capacitating status; CAP, capacitating status. Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay.

Table S6. Effects of MEHP on membrane integrity (MI), reactive oxygen species (ROS) and mitochondrial activity (MA) of human sperm

Test		0.1 % DMSO	H ₂ O ₂	1 nM MEHP	10 nM MEHP	100 nM MEHP	1 μM MEHP	20 μM MEHP
1h NC	MI (%)	74.2±7.7	39.1±6.6 ^a	72.2±10.2	71.9±9.5	71.9±8.1	70.9±7.9	70.8±7.1
	ROS (%)	100.0±2.7	126.5±5.8 ^a	98.2±8.8	103.4±7.1	99.7±6.7	104.7±7.0	114.8±5.9
	MA (%)	69.7±5.6	49.7±4.9 ^a	70.7±8.8	69.3±7.1	69.0±6.7	70.0±7.0	72.0±5.9
2h NC	MI (%)	72.6±3.4	38.6±4.2 ^a	71.9±8.4	70.8±7.6	72.1±5.5	68.7±7.5	69.2±7.2
	ROS (%)	100.0±6.2	134.2±7.2 ^a	102.4±3.2	100.2±4.5	106.2±2.1	102.7±3.7	100.4±4.6
	MA (%)	68.5±4.1	46.4±5.2 ^a	65.9±3.8	64.7±3.4	66.9±5.0	65.9±3.0	66.4±4.4
4h NC	MI (%)	71.1±4.5	37.1±5.5 ^a	70.3±3.3	69.2±4.2	69.5±4.7	69.3±3.0	68.8±5.2
	ROS (%)	100.0±5.0	143.5±5.2 ^a	102.6±3.1	101.5±3.7	102.0±3.9	102.4±2.2	103.9±4.5
	MA (%)	64.7±5.4	40.0±4.5 ^a	60.5±5.8	61.9±3.5	60.3±5.4	61.8±4.8	58.6±2.7
1h CAP	MI (%)	75.4±2.9	42.8±2.9 ^a	72.1±5.0	70.4±3.1	70.2±3.2	72.5±4.6	73.1±3.8
	ROS (%)	100.0±5.8	135.4±4.9 ^a	102.8±4.2	103.3±4.3	102.4±6.3	101.5±5.2	102.1±6.6
	MA (%)	70.4±3.6	50.5±2.9 ^a	69.2±3.7	67.9±3.9	68.4±3.5	70.7±4.1	70.3±6.4
2h CAP	MI (%)	74.5±5.3	41.9±3.6 ^a	73.2±3.5	73.9±5.1	72.9±4.5	71.4±4.9	70.9±3.0
	ROS (%)	100.0±4.9	142±6.5 ^a	102.8±4.9	101.8±2.0	102.8±5.3	102.4±5.8	105.5±4.0
	MA (%)	71.0±3.4	51.7±4.5 ^a	70.6±6.6	71.72±5.5	70.2±4.5	72.2±5.5	70.9±6.6
4h CAP	MI (%)	76.2±5.7	41.5±5.7 ^a	76.6±5.6	73.0±8.2	72.6±13.9	71.9±8.2	70.8±11.1
	ROS (%)	100.0±1.9	147.8±7.5 ^a	99.1±1.9	100.3±2.4	100.2±2.9	102.6±3.0	105.6±3.5
	MA (%)	71.0±7.6	53.0±2.5 ^a	71.0±7.5	71.3±7.2	73.3±8.0	70.7±8.0	71.0±5.5

^a $P < 0.05$, Two-way ANOVA. Bar: mean ± SEM. NC, non-capacitating status; CAP, capacitating status. Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay.

Table S7. Effects of DEHP plus MEHP on membrane integrity (MI), reactive oxygen species (ROS) and mitochondrial activity (MA) of human sperm

	Test	0.1 % DMSO	H ₂ O ₂	20 nM DEHP 1 nM MEHP	200 nM DEHP 10 nM MEHP	2 µM DEHP 100 nM MEHP	4 µM DEHP 1 µM MEHP	8 µM DEHP 20 µM MEHP
1h NC	MI (%)	73.3±9.7	41.6±6.1 ^a	67.8±9.6	68.9±12.3	69.4±14.5	69.9±11.4	71.1±16.1
	ROS (%)	100.0±3.3	129.3±3.1 ^a	103.3±1.6	100.8±2.7	101.3±3.5	101.5±2.3	103.9±5.0
	MA (%)	71.2±5.7	49.3±9.7 ^a	71.3±2.7	72.0±5.7	69.3±5.4	70.3±7.3	70.1±5.2
2h NC	MI (%)	71.8±5.3	40.2±4.2 ^a	70.9±5.0	71.5±6.4	70.9±7.9	70.5±8.5	71.2±9.0
	ROS (%)	100.0±4.1	135.1±6.5 ^a	109.4±5.9	105.3±4.9	106.1±8.8	103.2±4.1	109.8±10.0
	MA (%)	68.9±5.2	46.5±6.5 ^a	67.9±3.2	68.0±7.4	65.2±7.1	65.0±8.8	64.4±7.5
4h NC	MI (%)	69.7±5.1	38.7±5.6 ^a	68.9±4.8	67.2±5.9	66.5±4.7	68.5±6.9	65.4±5.5
	ROS (%)	100.0±5.3	142.6±4.6 ^a	101.6±3.4	102.4±4.9	104.7±3.1	105.0±2.9	105.9±7.3
	MA (%)	64.3±5.2	43.0±5.2 ^a	61.0±4.7	61.9±3.5	58.7±4.5	59.0±5.5	57.8±4.9
1h CAP	MI (%)	72.5±4.9	38.8±5.2 ^a	72.0±5.0	70.4±1.6	71.2±5.5	70.1±5.2	70.0±5.8
	ROS (%)	100.0±5.0	138.0±4.9 ^a	102.7±5.0	104.3±5.7	106.1±8.3	106.5±6.2	105.1±4.1
	MA (%)	70.5±4.2	50.5±5.6 ^a	70.8±2.2	69.9±5.9	67.2±3.4	66.7±8.0	65.7±9.8
2h CAP	MI (%)	73.0±5.1	39.5±5.6 ^a	71.0±5.6	71.9±4.0	70.9±8.5	70.4±6.9	69.9±8.6
	ROS (%)	100.0±6.7	144±7.8 ^a	101.8±5.9	105.4±8.1	106.7±6.3	106.9±7.8	108.4±8.2
	MA (%)	71.4±8.2	51.9±4.5 ^a	68.3±5.5	69.8±5.3	68.7±4.6	68.5±5.0	67.8±4.3
4h CAP	MI (%)	74.9±8.9	41.6±8.9 ^a	73.8±13.9	72.7±7.6	69.9±10.4	68.3±6.3	67.8±13.9
	ROS (%)	100.0±3.7	152.8±6.9 ^a	104.6±1.0	102.8±1.1	104.1±1.3	100.8±1.8	104.0±4.5
	MA (%)	72.7±4.1	52.0±3.5 ^a	69.3±6.6	71.3±6.4	69.7±6.8	71.0±3.6	70.3±6.4

^a $P < 0.05$, Two-way ANOVA. Bar: mean ± SEM. NC, non-capacitating status; CAP, capacitating status. Sperm from 9 individuals (3 technical replicates for each individual) were analyzed for each assay.

Figure S1

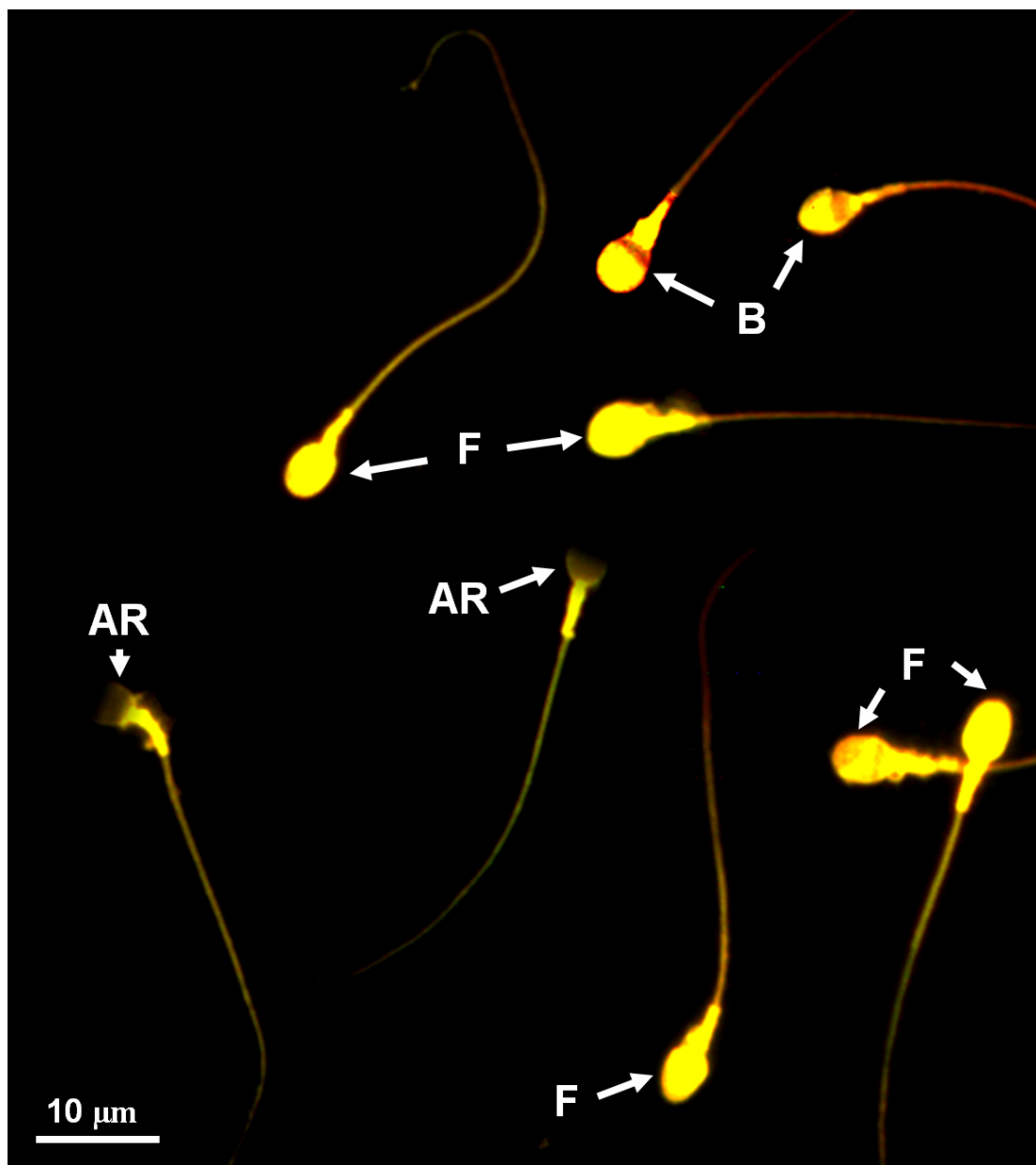


Fig. S1. An example for CTC staining patterns. 'F' is classified as uniform yellow fluorescence in the head, which is characteristic of uncapacitated cells; 'B' is classified by a fluorescence-free band in the post-acrosomal region, which is characteristic of capacitated but acrosome-intact cells; Capacitated sperm were calculated as the sum of "F" and "B"; 'AR' is classified as very weak or absent fluorescence, which is characteristic of acrosome-reacted cells.