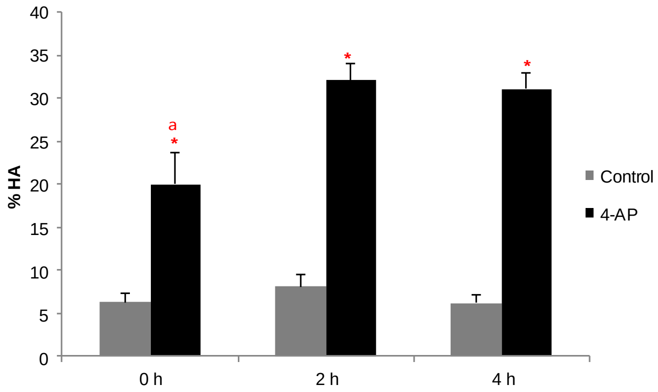
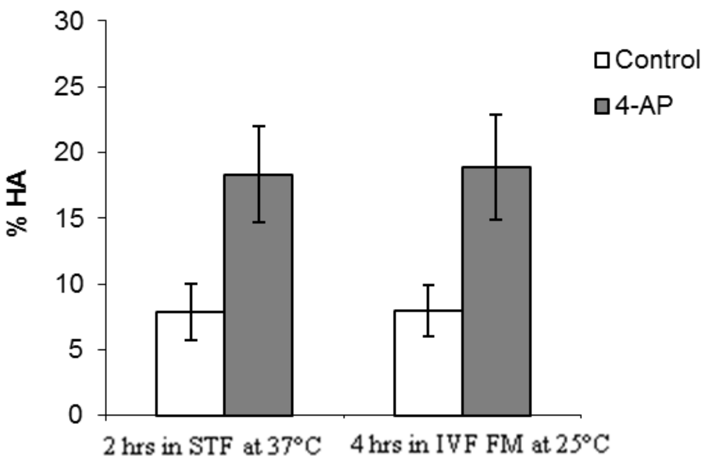
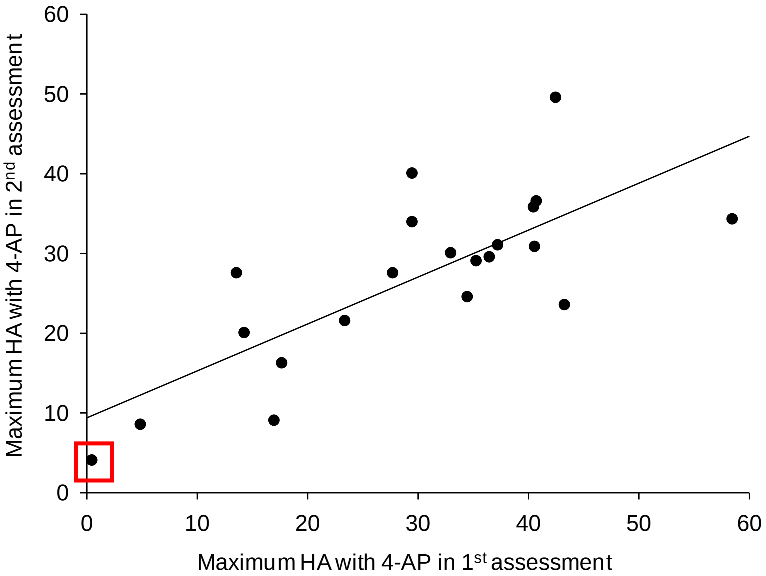


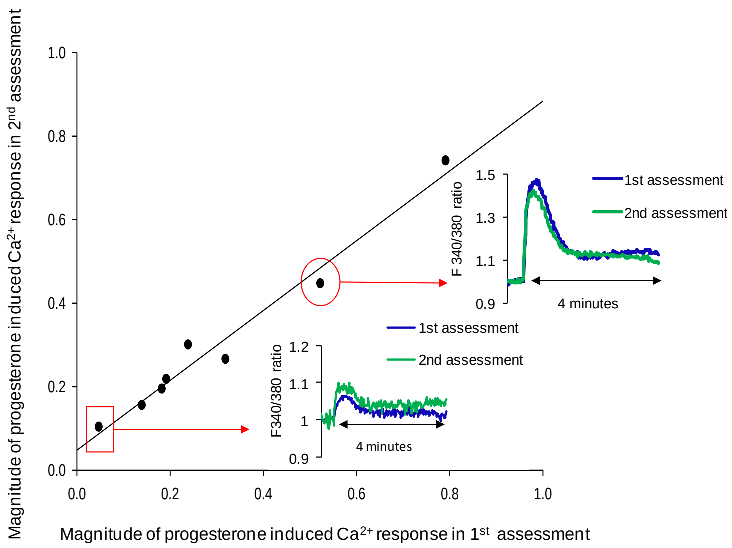
Supplementary Figure 1a. Effect of 4-AP on %HA in relation to the incubation time in CM. Time course study for the effect of 4-AP on HA from samples re-suspended in CM. All values are the mean ± SE (n=7 experiments performed with 7 different donor samples). ***-** indicates a significant difference from the control, **a**-indicates a significant difference between % HA with 4-AP at 0h and other time intervals (2hrs and 4hrs). Significance was considered as P<0.05 assessed by one way ANOVA and non-parametric analysis of variance on ranks Kruskal-Wallis test.



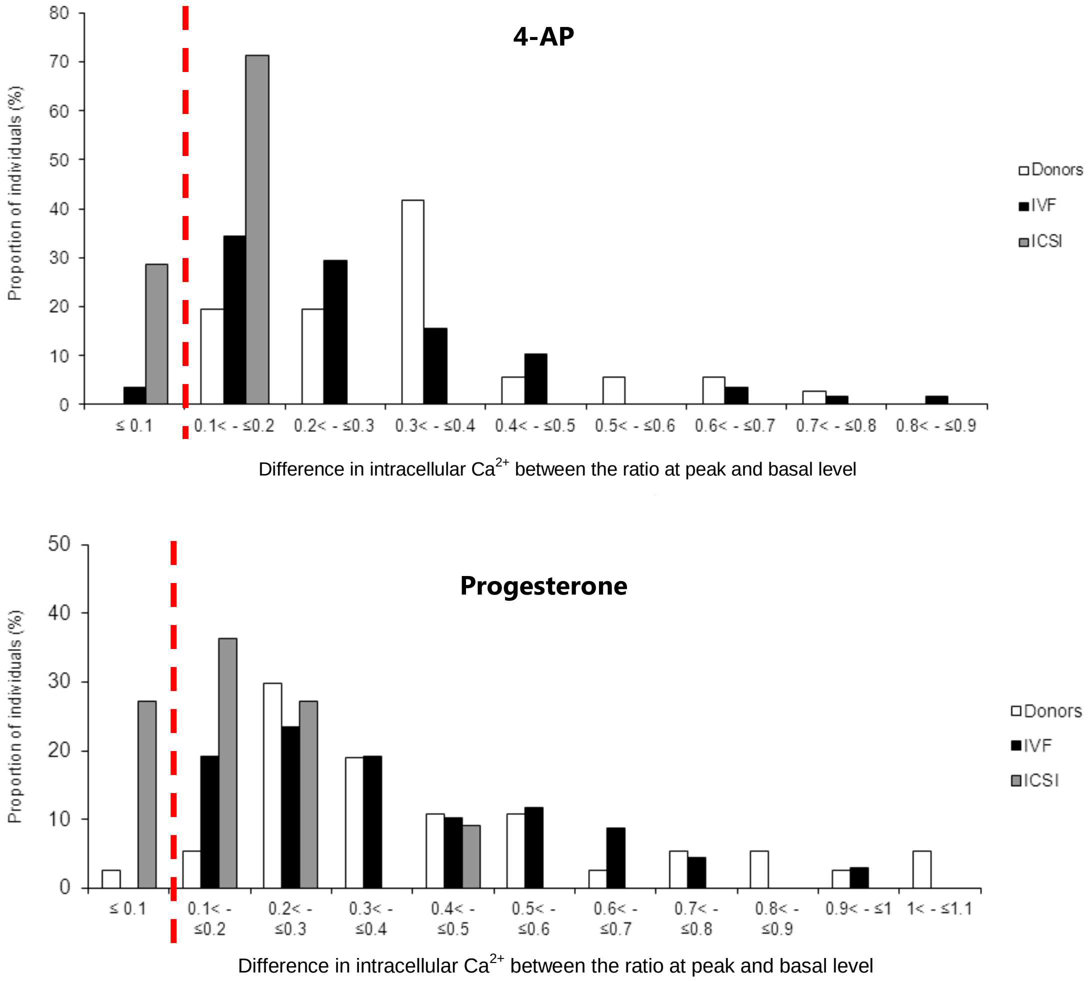
Supplementary Figure 1b. Effect of 4-AP on HA in relation to the incubation time in capacitating media used. Comparison between the effect of 4-AP on HA in two aliquots from the same sample. One portion was re-suspended in STF and the other portion was re-suspended in (IVF FM). All values are the mean ± SE (n=5 experiments performed with 5 different donor samples). * indicates a significant difference from the control. Significance was considered as P<0.05 assessed by one way ANOVA and non-parametric analysis of variance on ranks Kruskal-Wallis test.



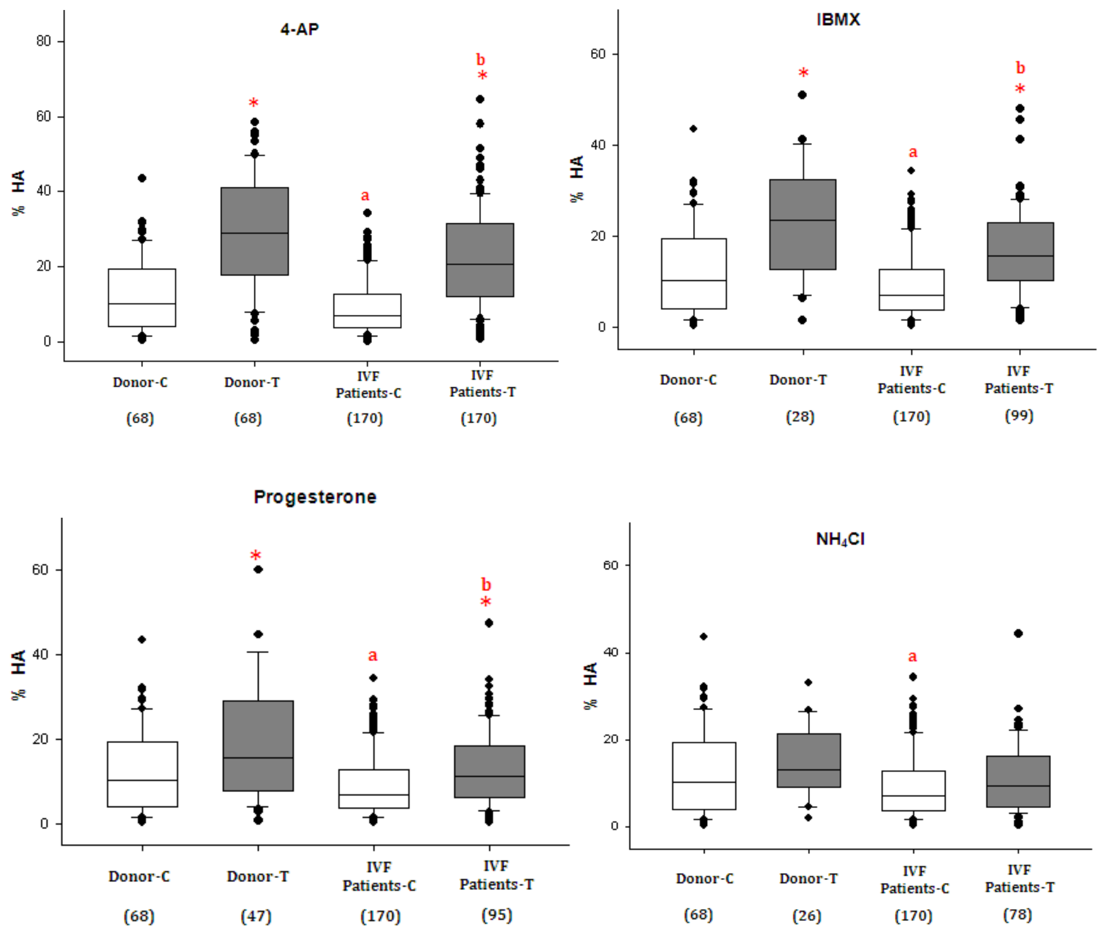
Supplementary Figure 2a Correlation between maximal HA in first and second assessments in 21 donors. Importantly, the HA response to 4-AP was consistent between the assessments in all 21 donors with 20/21 donors showing a normal significant response in both assessments and 1/21 showing a failed response in both samples (donor 1 highlighted with red box). R^2 =0.579 P<0.001



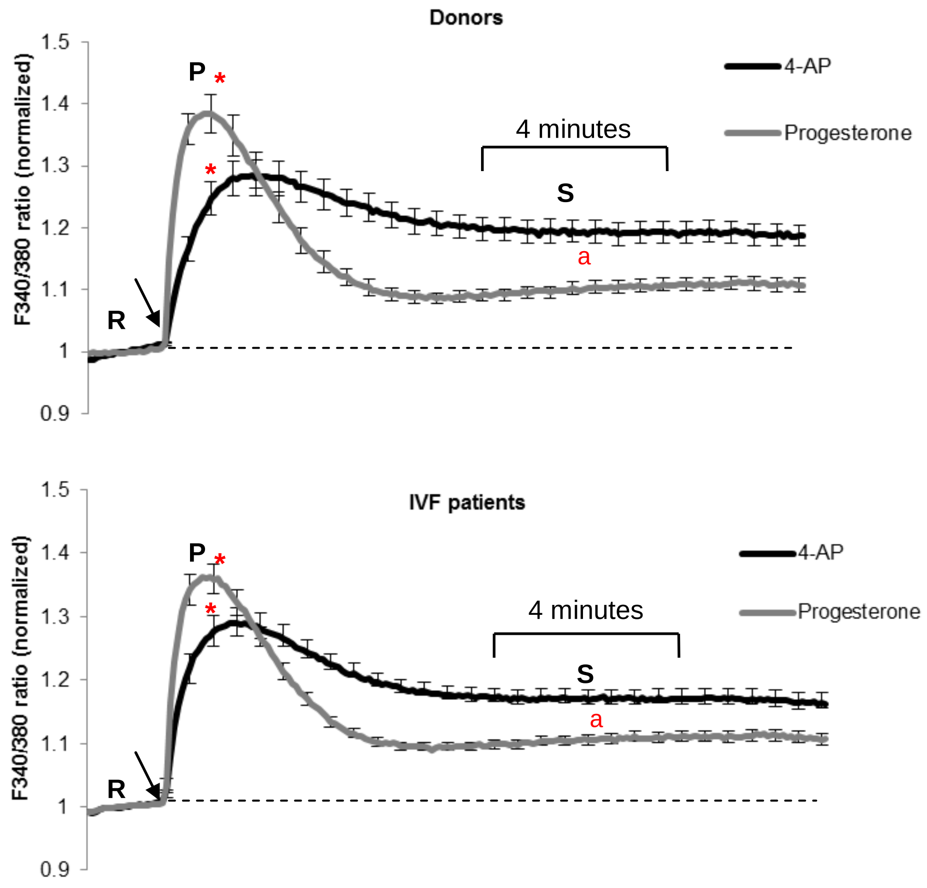
Supplementary Figure 2b. Minimal inter-ejaculate variability in the magnitude of Ca^{2+} response to progesterone between two assessments. There was a significant correlation between response to progesterone (absolute difference between peak and basal level) in two ejaculates from the same donor collected at least 1 month apart (n=8 donors) (R= 0.986, R^2= 0.972, P<0.001). Insets show example records from two donors. In each case the two records (green and blue) were obtained using samples from the same donor obtained ≥1 month apart. The intracellular Ca^{2+} response to progesterone was consistent between the assessments in all 8 donors with 7/8 donors showing a normal Ca^{2+} response in both assessments and 1/8 showing a failed response (increment of ≤0.1 in both samples - donor highlighted with red box).



Supplementary Figure 3. The frequency distributions of the Ca^{2+} response in donor cells (absolute difference in ratio between peak and basal levels) for 4-AP (n=36 donors; upper panel) and progesterone (n=37 donors; lower panel). The cut-off value defining a failed Ca^{2+} response to 4-AP or progesterone (increment of ≤0.1) is indicated by dotted red lines.



Supplementary Figure 4. Comparison between different groups for HA in control cells (baseline level) and in the presence of agonists. Box and whisker plots show the data distribution for HA in the baseline (control, C- white boxes) and in samples treated with 4-AP, Progesterone, IBMX and NH_4Cl (T- grey boxes) from 68 donors (first sample from each donor) and 170 IVF patients. The boxes represent the inter quartile range and lines within them are the medians. Number in brackets is sample size. ***-** indicates that the response to an agonist is significantly different from basal level (C control), **a-** indicates a significant difference of the basal HA between groups, **b-** indicates a significant difference of maximum HA between groups. No difference was found between donors and IVF patients upon addition of NH_4Cl. Significance was considered as $P<0.05$ assessed by one way ANOVA and non-parametric analysis of variance on ranks Kruskal – Wallis test.



Supplementary Figure 5. Effect of 4-AP and progesterone on intracellular Ca^{2+} in spermatozoa from donors and IVF patients.

Traces show averaged data ± SE (normalised to pre-stimulus level **R**, the initial resting calcium level) for increase in $[Ca^{2+}]_i$ in response to 4-AP (black) and progesterone (grey) in sperm from donors (upper panel) and IVF patients (lower panel). 4-AP and Progesterone were added to suspensions 100 seconds (indicated by black arrow) after acquisition of 20 readings at resting level (R). Both stimuli induced a biphasic elevation of intracellular Ca^{2+} level in spermatozoa from both groups, but progesterone increased calcium levels rapidly and transiently, while the response to 4-AP was of lower amplitude and more sustained. **P** shows the fluorimetric calcium ratio at peak; **S** shows the period used for measurement of the sustained response.***** Significant difference between the ratio at peak ($P \leq 0.001$ [donors], $P=0.02$ [IVF]) between 4AP and progesterone. a Significant difference between the ratio at sustained ($P \leq 0.001$ [donors], $P=0.002$ [IVF]) between 4AP and progesterone. Data assessed by non-parametric analysis of variance on ranks Kruskal – Wallis test. N=36 donors and IVF patients (4-AP, n=61, progesterone, n=68).

Supplementary Table I. Effect of 4-AP and progesterone on fluorimetric calcium ratio compared to basal level in spermatozoa from donors, IVF and ICSI patients.

		Mean ± SE	Median	Range
Donors treated with 4-AP	Untreated basal	1.03 ± 0.02	1	0.8-1.33
	Ratio at peak	1.39 ± 0.04*	1.38	1-2.14
Donors treated with Progesterone	Untreated basal	1.1 ± 0.035	1.06	0.8-1.67
	Ratio at peak	1.58 ± 0.07*	1.45	0.98-2.8
IVF Patients treated with 4-AP	Untreated basal	1 ± 0.02	1	0.8-1.4
	Ratio at peak	1.33 ± 0.03*	1.2	1-1.9
IVF Patients treated with Progesterone	Untreated basal	1.04 ± 0.02	1	0.7-1.7
	Ratio at peak	1.47 ± 0.04*	1.4	0.9-2.3
ICSI Patients treated with 4-AP	Untreated basal	0.89 ± 0.04 $	0.9	0.8-1.1
	Ratio at peak	1.06 ± 0.04* a, b	1	0.9-1.3
ICSI Patients treated with Progesterone	Untreated basal	0.84 ± 0.03 $	0.8	0.6-1.2
	Ratio at peak	1.06 ± 0.05* a, b	1	0.7-1.7

All values are from raw data prior to normalisation * Significant difference between the ratio at peak (P) and the initial resting intracellular calcium l (R) a significant difference of ratio at peak between donor and ICSI patients, b significant difference of ratio at peak between IVF and ICSI patients, $ basal calcium was significantly lower in the ICSI patients compared to donors and IVF patients (*P* < 0.05) assessed by non-parametric analysis of variance on ranks Kruskal – Wallis test. N=36 donors, 65 IVF and 11 ICSI patients**.**)

Supplementary Table II. CASA parameters in 17 IVF patients with a failed HA response to 4-AP.

Data expressed as mean ± SD for kinematic parameters and HA. *- Values are significantly different from control ($P < 0.05$) by one way ANOVA and Kruskal – Wallis one way analysis of variance on ranks. n/a (not available)

Patient	VCL (μm/sec)	LIN (%)	ALH (μm)	HA (%)
1 – Control	76.7 ± 6.7	57.3 ± 4.5	3.4 ± 0.4	1 ± 0.5
4 AP	66.4 ± 7.2	44.5 ± 0.6	3.9 ± 0.3	0.7 ± 0.8
– Control	86.7 ± 7.6	70 ± 6.1	3 ± 0.2	0.5 ± 0.6
4 AP	95.5 ± 2.5	64.3 ± 1	4 ± 0.1 *	2 ± 2.1
– Control	92.4 ± 2.4	57.3 ± 3.5	3.7 ± 0.1	1.3 ± 0.5
4 AP	94.3 ± 3	52.5 ± 2.3	4.8 ± 0.6 *	1.5 ± 1
– Control	87.5 ± 5.1	67 ± 2.6	2.9 ± 0.3	2.3 ± 1.3
4 AP	96.8 ± 7.6 *	54.5 ± 5.9 *	3.8 ± 0.4 *	4 ± 1.8
– Control	119 ± 3.4	70.3 ± 1	3.9 ± 0.1	3.7 ± 1.3
4 AP	122.5 ± 3.3	67.3 ± 1.3	4.4 ± 0.2 *	3 ± 1.5
– Control	103 ± 2.3	66.8 ± 3.9	3.5 ± 0.2	2.8 ± 1.3
4 AP	119 ± 5.7 *	63 ± 2.9	4.3 ± 0.2 *	4.3 ± 1.9
Progesterone	113.2 ± 1.7 *	68.3 ± 3.3	3.7 ± 0.14	3.5 ± 0.5
IBMX	122.1 ± 4.7 *	60.8 ± 3.3	3.7 ± 0.14	7.4 ± 2.7 *
7 – Control	96.7 ± 2.8	66. ± 1.7	3.7 ± 0.1	2.5 ± 0.6
4 AP	106 ± 7.5	56.5 ± 4 *	4.8 ± 0.6 *	3.9 ± 2.5
8 – Control	96.7 ± 7.1	48.8 ± 1.7	5 ± 0.4	6.5 ± 1.3
4 AP	105.8 ± 4.3	53.8 ± 1.9	4.7 ± 0.2	6.5 ± 2.9
Progesterone	99.9 ± 1.4	53 ± 2.2	5 ± 0.1	4.5 ± 0.6
IBMX	106.3 ± 2.9	57.8 ± 1.7	4.8 ± 0.2	3.8 ± 1.3
9 – Control	108.3 ± 4.5	56.5 ± 0.6	4.5 ± 0.3	5.5 ± 2.6
4 AP	105.8 ± 4.5	50.3 ± 2.5 *	5 ± 0.3	6 ± 2.4
Progesterone	115.1 ± 3.9	58.8 ± 1	4.6 ± 0.3	7.2 ± 0.6
IBMX	109.3 ± 3.9	60.3 ± 2.6	4.3 ± 0.2	5.7 ± 3
10 – Control	128 ± 5.6	52 ± 1.8	5.4 ± 0.3	9.9 ± 3.7
4 AP	121 ± 2.8	49.8 ± 1.7	5.3 ± 0.1	8.1 ± 1.7
11 – Control	155 ± 3.3	49.8 ± 0.5	6.7 ± 0.1	25.5 ± 2.4
4 AP	142.6 ± 10	43 ± 3.2 *	6.8 ± 0.6	22.8 ± 5.4
Progesterone	141.4 ± 5.9 *	46 ± 2.9 *	6.9 ± 0.3	21.4 ± 3.2
IBMX	147.5 ± 6.1	47.8 ± 5.8	6.3 ± 0.6	22.3 ± 3.4
12 – Control	170 ± 5.4	50.8 ± .1	7 ± 0.3	27.3 ± 1.5
4 AP	151 ± 6.5 *	44 ± 2.9 *	7 ± 0.3	27.5 ± 3.4
13 – Control	141.5 ± 8.5	51.3 ± 1.7	5.6 ± 0.3	23.5 ± 5.9
4 AP	138.5 ± 6.7	53.8 ± 0.5 *	6 ± 0.4	20.3 ± 2.9
Progesterone	144.7 ± 6	41.5 ± 1.9 *	6.6 ± 0.5 *	27.6 ± 3.4
IBMX	152.6 ± 6.1	50.5 ± 1.7	6 ± 0.4	27.2 ± 3
14 – Control	162 ± 11.1	61.8 ± 2.6	5.8 ± 0.5	8.9 ± 2.4
4 AP	166 ± 11.1	55 ± 2.2 *	6.2 ± 0.4	16.5 ± 5.9
15 – Control	104.7 ± 4.9	60 ± 4.1	4.3 ± 0.4	6.75 ± 3.4
4 AP	116.8 ± 3.7 *	47 ± 1.4 *	5.7 ± 0.2 *	10.5 ± 2.4
Progesterone	108 ± 3.9	55.8 ± 1	4.8 ± 0.4	7.3 ± 3
IBMX	116.3 ± 3.4 *	52 ± 6.1	5.2 ± 0.3 *	11.8 ± 1.3 *
NH_4Cl	96.7 ± 12.2	53.5 ± 0.6 *	4.5 ± 0.6	5 ± 1.2
16 – Control	n/a	n/a	n/a	2.3 ± 0.5
4 AP	n/a	n/a	n/a	3.5 ± 2.4
17 – Control	n/a	n/a	n/a	3.8 ± 2.1
4 AP	n/a	n/a	n/a	4.2 ± 2.3