

## Supplementary Information

### Non-thermal plasma treatment improves chicken sperm motility via the regulation of demethylation levels

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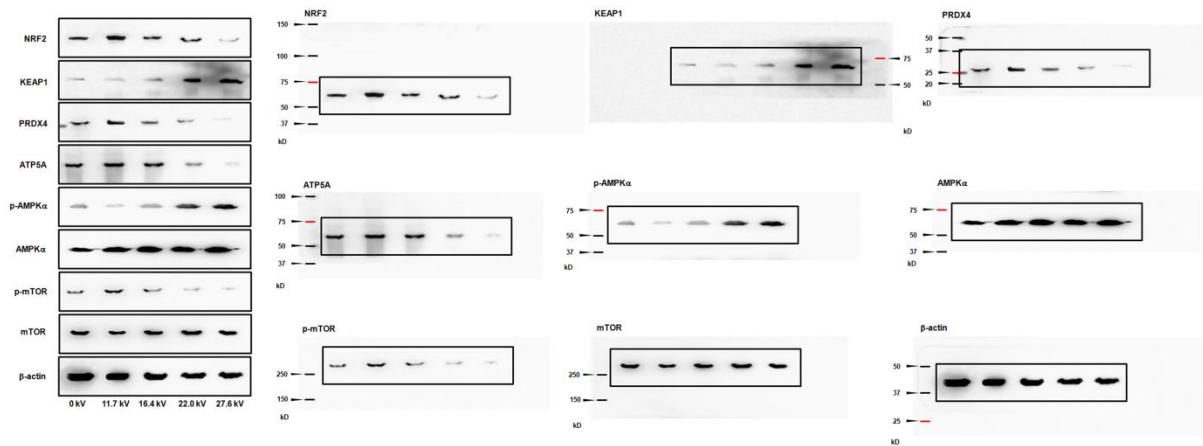
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**Supplementary Figure S1.** Western blot analysis of the protein levels of NRF2, KEAP1, PRDX4, ATP5A, p-AMPK $\alpha$ , AMPK $\alpha$ , p-mTOR, and mTOR in chicken spermatozoa exposed to different potentials for 20 s.

Uncropped immunoblot scans for Figure 3f. The grouping of gels/blots cropped from different gels.

All blots were visualized with 5 min exposure time.

**Supplementary Table S1.** Effect of plasma exposure for different durations on ROS and antioxidant enzyme in chicken spermatozoa

Exposure duration (s)	ROS (nmole DCF/10 <sup>9</sup> spz)	MDA (nmole/10 <sup>9</sup> spz)	SOD (U/10 <sup>9</sup> spz)	CAT (U/10 <sup>9</sup> spz)	GPx (mU/10 <sup>9</sup> spz)
0	6.47±0.18	3.04±0.18	42.53±0.91	2.16±0.11	4.34±0.10
10	6.07±0.04*	2.66±0.17	67.48±2.61**	2.85±0.17**	5.86±0.16**
20	5.79±0.18*	2.04±0.17**	72.64±3.17**	3.45±0.23**	6.15±0.31**
40	6.20±0.10	2.76±0.18	58.42±2.35**	2.64±0.14**	5.44±0.25**
60	7.61±0.19**	3.35±0.27	40.37±2.29	1.85±0.07	4.13±0.10
80	8.65±0.08**	3.85±0.18**	33.58±0.80**	1.65±0.10**	3.54±0.17**

Plasma exposure intensity was 11.7 kV. Values are expressed as the mean ± standard error (n=10) of three replicates; n represents an individual cock. Within a column: \*,  $p < 0.05$  versus control; \*\*,  $p < 0.01$  versus control, according to one-way ANOVA and LSD test.

**Supplementary Table S2.** Effect of plasma exposure for different durations on ATP and activity of mitochondria respiratory enzyme in chicken spermatozoa

Exposure duration (s)	ATP (nmole/10 <sup>9</sup> spz)	NADH (U/10 <sup>9</sup> spz)	Cytochrome c oxidase (mU/10 <sup>9</sup> spz)	ATP synthase (mU/10 <sup>9</sup> spz)
0	3.62±0.15	0.57±0.02	34.55±0.93	21.53±0.93
10	4.85±0.10**	0.68±0.04**	46.74±1.66**	27.47±1.80**
20	5.84±0.40**	0.79±0.02**	56.40±3.16**	31.30±2.38**
40	4.64±0.16**	0.71±0.02**	49.46±2.44**	28.46±1.75**
60	3.26±0.10	0.50±0.05*	30.40±1.52	19.32±0.80
80	2.74±0.10**	0.42±0.01**	24.48±1.69**	16.37±1.80**

Plasma exposure intensity was 11.7 kV. Values are expressed as the mean ± standard error (n=10) of three replicates; n represents an individual cock. Within a column: \*,  $p < 0.05$  versus control; \*\*,  $p < 0.01$  versus control, according to one-way ANOVA and LSD test.

**Supplementary Table S3.** Primer sequences for the RT-PCR

Gene	Sequence number	Sequence position	Product Annealing		
			length (bp)	Temperature (°C)	Sequence (5'to3')
<i>β-actin</i>	NM_205518.1	625-818	194	57	F: GTGCGTGACATCAAGGAGAAGC R: CCACAGGACTCCATACCCAAGA
<i>NOX4</i>	NM_001101829.1	28-157	130	57	F: CGAGGATCTCAGAAGGTTGC R: GAGCATTACCAGATGAGCA
<i>NRF2</i>	NM_205117.1	484-619	136	57	F: AAAACGCTGAACCACCAATC R: GCTGGAGAAGCCTCATTGTC
<i>KEAP1</i>	KU321503.1	1227-1485	259	57	F: GTATCACAGCAGCGTGGAGA R: GCGTAGATGCAGTTGTTGA
<i>SOD</i>	NM_205064.1	106-278	173	55	F: ATTACCGGCTTGTCTGATGG R: CCTCCCTTGCAGTCACATT
<i>CAT</i>	NM_001031215.2	1067-1276	210	55	F: CTCATTCCAGTGGGCAAGAT R: GTAGGGGCAATTCACAGGAA
<i>GPx</i>	NM_001277853.2	353-474	122	55	F: ATGTTTCGAGAAGTGCAGGT R: ATGATGTACTGCGGGTTGGT
<i>PRDX1</i>	NM_001271932.1	358-545	188	56	F: ACAAGGTGGTTTGGGCACTA R: TCTCATCAACAGAACGGCCA
<i>PRDX3</i>	XM_426543.5	414-551	138	56	F: TTTCACCTTTGTGTGCCCA R: TTGCGCGGGGTATTTATCCA
<i>PRDX4</i>	XM_001233999.3	595-733	139	56	F: TGCACTTAGGGGCTTTTCA R: TTCTCCATGCTTGTCCGTGT
<i>PRDX6</i>	NM_001039329.2	189-340	152	58	F: TGAGTTCAGCAAACGCAACG R: GCTCTCGGTCCCTATCAGCG
<i>ATP5A1</i>	NM_204286.1	1207-1364	158	57	F: GGTATCCGTCCAGCCATCAA R: GCATCCAAATCAGACCCAAACT
<i>ATP5B</i>	NM_001031391.2	482-637	156	57	F: GCCCATCACAACGAAACAG R: CGCCTCCAAACAAACCAATC
<i>ATP5C1</i>	NM_001278096.1	272-411	140	57	F: ATTAAGGCACCCGAGGACAA R: ACTTCCTTCCCTGCATTGGA
<i>ATP5F1</i>	XM_417993.4	437-644	208	57	F: CATTGGAGACTGCCATTGAGG R: TGATCTTGCTCTTTCTGACGCTT
<i>ATP5G1</i>	XM_001233602.3	287-536	250	57	F: CAGGAGCAGGTATTGGGACA R: TTGTCAGTCTGGAACGCTCT
<i>ATP5G3</i>	NM_001277855.1	141-288	148	57	F: CAAAACGCTGTCTCCCAAC R: ACCGAAGACCGTTCCAATACC
<i>ATP5H</i>	XM_001232598.3	332-551	220	57	F: CTGAAGGTCCCTGAACCAGT R: ACTTCTCCCTGTCCAGTCTG
<i>ATP5I</i>	NM_001097534.2	74-240	167	57	F: TCTCGCCCCTCATCAAGTTC R: TGCCAGTTCCTTTGCAATCC

<i>ATP5J</i>	XM_004938370.1	58-197	140	58	F: CACTTGCGGAGAAACATCGGT R: CCTACATCAACAGGTCCTCCAGC
<i>ATP5J2</i>	NM_001257200.1	170-263	94	57	F: GCCTCGGTGGTATCAGTATGGT R: TACTTCCTGCGGCGGTCAT
<i>ATP5L</i>	XM_015298211	250-377	128	57	F: CCATGGTCAGGAGCTTTCAG R: GCCTCGTTTGCCTATGATCTC
<i>ATP5S</i>	NM_001277562.1	46-279	234	57	F: TCCCCTTCCCCTTTCTTTCC R: CATAGCCTTGATAGCGCACC
<i>AMPK<math>\alpha</math>2</i>	NM_001039605.1	726-943	218	57	F: GGAGGCGTGTTTTACATCCC R: AACTTCTCACAGACCTCCCG
<i>AMPK<math>\beta</math>2</i>	NM_001044662.1	435-661	227	57	F: CCAGTGTTTTTCAGCTCCCAC R: GAGGTCCAGGATAGCGACAA
<i>AMPK<math>\gamma</math>3</i>	NM_001031258.2	183-320	138	57	F: GCTGGAACCCGACAACAATT R: GCCTTCTTGATCTCCAGGGT
<i>mTOR</i>	XM_417614.4	119-309	191	57	F: TGAAGGGGTCAAGGCAATCC R: GGCGAGCAGTGGTTGTGGAT

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**Supplementary Table S4.** Primer sequences for bisulfite-sequencing PCR

Gene	Sequence position	Product length (bp)	Annealing		Expected No. of CpGs
			Temperature (°C)	Sequence (5'to3')	
<i>NRF2</i>	407-562	156	55	F: TTTGTTGGTTTAGTTGGTGTAGAT R: CAATCATCAAAAAACAAAACCTCTT	4
<i>KEAP1</i>	583-849	267	55	F: AATTTGTTTTATTGTTAGTTGGTTA R: CAAAATAAAATCCTAAAAAATCTAC	30
<i>PRDX4</i>	70-343	274	56	F: GTTGTTGTTGGTTTTGTTGTTGTA R: AAAAACAAAATATTTTCCTTCATAATCTAT	24
<i>ATP5A1</i>	1082-1292	211	55	F: GAGGTTTTTTGATTGTTTTGTTTGT R: CCTACCACCTATTCATTACCCTAAT	4
<i>AMPK<math>\alpha</math>2</i>	233-463	231	52	F: AGAAATTTAAAATTTGAAATTTTTT R: AAATCCCTATAAACAACCATATATC	10
<i>mTOR</i>	1690-1890	201	52	F: ATATTTAGGATGGGTTGTTGAAAAT R: AAATCAAAAAATACCCTTCAAACCTC	4