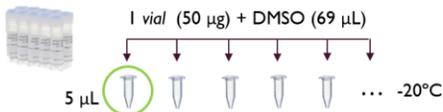


PERCENTAGE OF SPERM WITH ACTIVE MITOCHONDRIA

❖ PRIMARY STOCK SOLUTION (1 mM)



MitoTracker™ Red FM
(ThermoFisher Scientific)



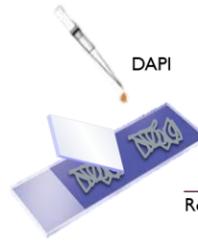
❖ SECONDARY STOCK SOLUTION (10 µM)

+ PBS (pH 7.2) (495 µL)



Incubation: 10×10^6 SPZ/mL + Secondary stock solution [300 nM]
(~3,09 µL of the secondary stock solution for 100 µL of the sample)

-20 min, 37°C, in the dark-



10 min
Room Temperature



Figure S1: representative scheme of the protocol applied to assess the percentage of spermatozoa with active mitochondria, using the MitoTracker™ Red FM (MTRFM) dye.

ALKALINE COMET ASSAY

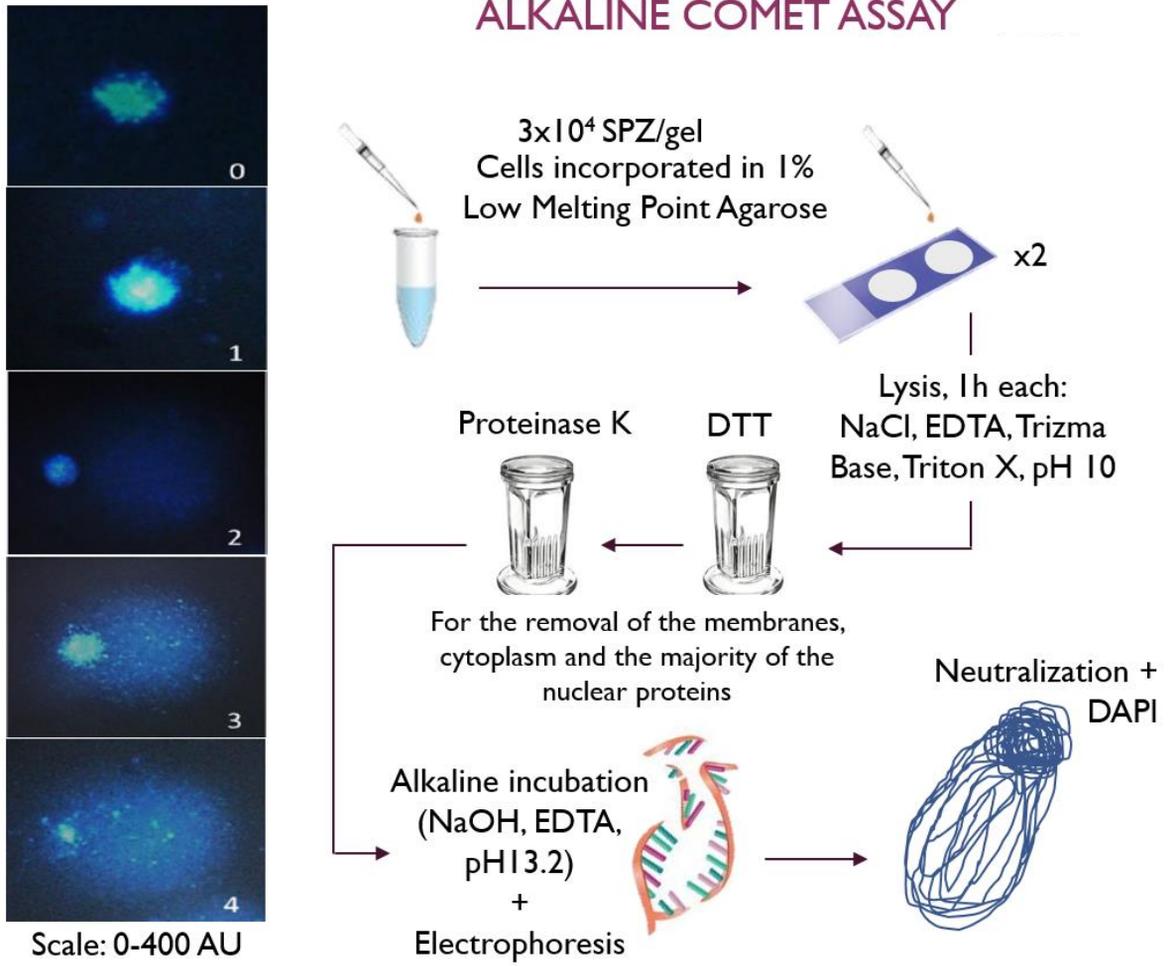


Figure S2: representative scheme of the protocol applied to assess the sperm DNA damage by alkaline comet assay and of the visual score, on a scale of 0-400 arbitrary units (AU).

TUNEL ASSAY



In Situ Cell Death Detection Kit
(Roche Diagnostics)

3 washes 1x PBS → 4% Paraformaldehyde → -20°C

↓ 500 x g, 10 min

Permeabilizing
Solution

← 2 washes 1x PBS + 1% BSA

2 washes 1x PBS + 1% BSA →

Incubation, 37°C, 1h,
in the dark:

TUNEL solution
(with enzyme TdT)

TUNEL solution
(without enzyme TdT)

Recombinant
DNase I (37°C, 20 min)
+ TUNEL solution
(with enzyme TdT)

Sample

- Control

+ Control

2 washes + resuspension in 1x PBS



SPZ with
DNA fragmentation?



- No



+ Yes

Figure S3: representative scheme of the protocol applied to assess the percentage of spermatozoa with fragmented DNA with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

ANTIOXIDANT ENZYMES ACTIVITY



❖ **Sonication**
Pulse 15 secs
Amplitude 70%
Time 1 min



❖ **Centrifugation**
12,000 x g
15 min
4°C

Supernatant

SOD

Reaction mixture:

Phosphate buffer (50 mM KH_2PO_4 and 1 mM EDTA, pH 7.4) (935 μL) + 10 mM NBT (10 μL) + 10 mM hypoxanthine (10 μL) + sample supernatant (30 μL)

⌚ 25°C - 2 min

Add: Xanthine oxidase (15 μL)



Spectrophotometric reading



25°C | 3 min
560 nm

SOD activity

$\text{U min}^{-1} \text{mg protein}^{-1}$

GR

Reaction mixture:

Phosphate buffer (100 mM KH_2PO_4 and 0.5 mM EDTA, pH 7.4) (1910 μL) + sample supernatant (50 μL)

⌚ 25°C - 2 min

Add: NADPH (20 μL)

⌚ 25°C - 1 min

Add: GSSG (20 μL)



Spectrophotometric reading



25°C | 3 min
340 nm

GR activity

$\mu\text{M NADPH/NADP}^+ \text{min}^{-1} \text{mg protein}^{-1}$

Figure S4: representative scheme of the protocol applied to assess the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione reductase (GR)

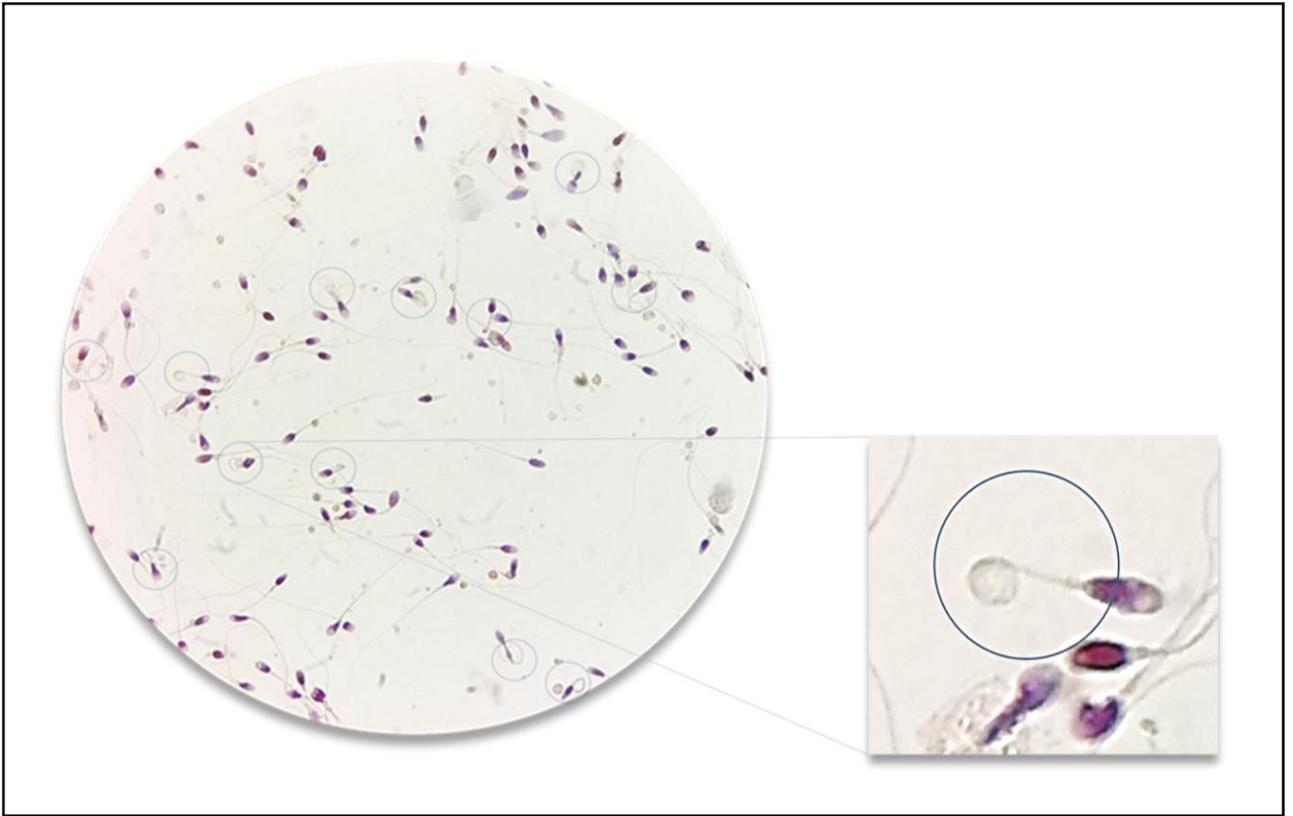


Figure S5: sperm morphology assessment after Papanicolaou–Shörr staining of a sperm sample cryo-preserved by method A (MA) (1000× magnification). A large number of spermatozoa with coiled tails (circled in blue) are evident when MA is applied.