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Humanin Preserves Sperm Quality and Natural Mating Fertility after Testicular Ischemia-Reperfusion Injury in Mice

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ABSTRACT

Testicular torsion-detorsion induces ischemia-reperfusion (I/R) injury that compromises sperm quality and fertility, yet whether mitochondrial-derived peptides can mitigate this damage has not previously been examined. This study investigated the dose-dependent protective effects of humanin, an endogenous mitochondrial-derived cytoprotective peptide, on epididymal sperm parameters and natural mating fertility following testicular I/R injury in mice. Adult male mice underwent 720° testicular torsion for two hours followed by detorsion, with or without a single intraperitoneal dose of humanin (126, 252, or 504 µg/kg) administered thirty minutes before reperfusion. Sperm concentration and motility were assessed by computer-assisted sperm analysis (CASA); viability, DNA integrity, and membrane functional competence were evaluated by eosin-nigrosin staining, acridine orange fluorescence, and the hypo-osmotic swelling test, respectively; and fertility outcomes were determined by natural mating trials with untreated females. Untreated I/R animals exhibited marked reductions in sperm concentration, motility, and DNA integrity, together with a near-complete collapse in pregnancy index (7%) and male fertility index (14%) relative to sham-operated controls. Humanin treatment produced a clear, dose-dependent restoration of all sperm-functional parameters, with the highest dose (504 µg/kg) restoring pregnancy and male fertility indices to values not statistically different from sham controls (pregnancy index 86% vs. 100%; male fertility index 100% vs. 100%). These findings provide the first evidence that humanin preserves not only sperm quality but also whole-animal fertility after testicular ischemia-reperfusion injury, identifying this peptide as a promising candidate adjunct therapy for reproductive protection in testicular torsion.

Introduction

Testicular torsion constitutes a genuine urological emergency arising from twisting of the spermatic cord, most frequently affecting neonates and adolescent males.[1] Even when the affected testis is anatomically salvaged by timely surgical detorsion, spermatogenic function is frequently compromised, and the consequences of missed or delayed diagnosis are severe enough that testicular torsion remains one of the leading causes of urological malpractice litigation.[2] Beyond the acute surgical dilemma, the condition casts a long shadow: clinical and experimental series have repeatedly documented reduced sperm counts, impaired motility, and diminished fertility potential following torsion, even when the affected testis is ultimately preserved.[3] The paradox at the heart of this condition is that restoring blood flow, the only effective treatment, does not simply reverse the ischemic insult.

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Reperfusion itself initiates a second, often more destructive, wave of tissue injury, setting the stage for the oxidative and apoptotic cascades that are increasingly recognized as the principal drivers of long-term reproductive impairment.

Interruption of testicular blood flow depletes ATP and generates a pro-oxidant intracellular milieu; upon reperfusion, the abrupt reintroduction of molecular oxygen fuels a burst of reactive oxygen species that overwhelms endogenous antioxidant defenses, including superoxide dismutase, glutathione peroxidase, and total antioxidant capacity.[4] This oxidative surge damages membrane lipids, proteins, and sperm DNA, and concurrently activates the intrinsic mitochondrial apoptotic pathway, shifting the Bax/Bcl-2 balance toward germ cell death.[3,5] A range of pharmacological and phytochemical agents, including quercetin, fisetin, melatonin, and erythropoietin, have been evaluated as pre-reperfusion adjuncts, with reported improvements in sperm concentration, motility, and histological injury scores.[3,6] Most of these agents, however, act primarily as generic free-radical scavengers rather than engaging a defined, endogenous cytoprotective signaling pathway, and comparatively few studies have extended their evaluation beyond biochemical or histological endpoints to functional, whole-animal outcomes such as natural mating fertility. This gap leaves open the more clinically relevant question of whether biochemical or histological protection actually translates into preserved reproductive capacity.

Humanin is a 24-amino-acid peptide encoded within an open reading frame of the mitochondrial 16S ribosomal RNA gene, first isolated from surviving neurons of an Alzheimer's disease brain and subsequently recognized as the founding member of the mitochondrial-derived peptide family.[7] Endogenously secreted, and also amenable to synthetic production, humanin and its potent analog S14G-humanin (HNG) engage extracellular receptor complexes and modulate intracellular signaling to suppress mitochondrial complex I-linked oxidative stress and inhibit intrinsic apoptosis through direct interaction with pro-apoptotic effectors such as Bax.[7] These properties have translated into demonstrated cytoprotection in murine myocardial ischemia-reperfusion,[8] cardiac ischemia-reperfusion models,[9] and, more recently, in a rodent model of intestinal ischemia-reperfusion injury, where a single pre-reperfusion dose attenuated apoptosis and preserved organ motility and function.[10] Despite this expanding evidence base, and despite an emerging suggestion that humanin exerts a specific, reproductively relevant anti-apoptotic role within testicular tissue,[7] no study to date has evaluated whether humanin administration prior to detorsion can preserve sperm quality and, critically, whole-animal fertility following testicular torsion-detorsion injury. The present study was designed to evaluate the dose-dependent protective effects of humanin on epididymal sperm parameters and natural mating fertility outcomes in a mouse model of testicular ischemia-reperfusion injury induced by 720° torsion-detorsion.

Materials and Methods

Animals, Experimental Design, and Testicular Ischemia-Reperfusion Model

Adult male mice (25-30 g body weight, 8-10 weeks of age) were acclimatized for seven days under standard housing conditions before random allocation into five groups (n = 7 per group): a sham-operated group, an untreated ischemia-reperfusion (I/R) group, and three I/R groups pretreated with humanin at 126, 252, or 504 µg/kg. Testicular ischemia was induced under ketamine-xylazine anesthesia by exposing the left testis through a lower midline incision and rotating it 720° clockwise, fixed in place with 4-0 nylon suture for two hours. Humanin (catalog no. AABH93DE47F1; AA Blocks, Inc.; purity ≥ 95%, sequence confirmed by mass spectrometry; endotoxin level < 1 EU/mg) was dissolved in 0.9% saline and administered as a single intraperitoneal dose thirty minutes before detorsion, a pretreatment window consistent with that reported for humanin in a rodent intestinal ischemia-reperfusion model. The three doses (126, 252, and 504 µg/kg) were selected to span a half-log range around this reference dose, based on extrapolation from the reference dose of 252 µg/kg reported in a rodent intestinal ischemia-reperfusion model, in order to characterize the dose-response relationship rather than to test a single fixed dose.[10] Following detorsion and wound closure, animals were allowed to recover for 30 days, corresponding to one complete murine spermatogenic cycle, before tissue and gamete collection.

All procedures involving animals were reviewed and approved by the Animal Ethics Committee of Islamic Azad University (protocol number IR.IAU.URMIA.REC.1403.092), and were performed in accordance with relevant institutional and national guidelines and regulations. The study adhered to the ARRIVE guidelines (Animals in Research: Reporting *In Vivo* Experiments); group sizes were determined a priori as detailed above, animals were allocated to groups by a computer-generated random sequence, and outcome assessors were blinded to group allocation during computer-assisted sperm analysis (CASA), staining, and mating assessments.

Ketamine-xylazine anesthesia was administered at 80 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally, with anesthetic depth monitored during the two-hour

torsion period by pedal withdrawal reflex and respiratory rate. Post-operative analgesia was provided as buprenorphine 0.05 mg/kg subcutaneously. Pre-defined humane endpoints, including severe lethargy, inability to eat/drink, or signs of distress, were monitored throughout the 30-day recovery period, and no animals required early euthanasia.

Epididymal Sperm Collection and Computer-Assisted Sperm Analysis

The cauda epididymis of the left testis was minced in pre-warmed saline and incubated at 37 °C to allow sperm to disperse into suspension, which was subsequently diluted in human tubal fluid medium. Sperm concentration was determined using a Neubauer hemocytometer under phase-contrast microscopy. Kinematic parameters (Table 1), including total and progressive motility, average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF), were quantified using a CASA system (Test Semen 3.2, Videotest, St. Petersburg, Russia) configured with a 60 Hz frame rate, 30 acquired frames per field (0.5 s of capture per assessment), a VAP cutoff of 10.0 µm/s, and a minimum of 500 cells evaluated across at least five fields per sample. Sperm exhibiting a curvilinear velocity greater than 180 µm/s, together with an amplitude of lateral head displacement exceeding 9.5 µm and a linearity below 38%, were additionally classified as hyperactivated, consistent with established CASA sub-population criteria for rodent semen analysis.

Table 1. Computer-Assisted Sperm Analysis (CASA) Parameter Settings

Parameter	Setting
Frame rate	60 Hz
Number of frames	30
Duration of capture	0.5 second
Stage temperature	37 °C
Minimum cell size	4 pixels
Cell size	13 pixels
Minimum contrast	30
Cell intensity	75 pixels
Chamber type	Slide-Coverslip (22 × 22 mm)
Volume per slide	7 µl
Chamber depth	≈ 20 µm
Minimum number of fields analyzed	500 cells
Image type	Phase contrast
Average path velocity (VAP) cutoff	10.0 µm/sec
Straight line velocity (VSL) cutoff	0.0 µm/sec
Curvilinear velocity (VCL) threshold — hyperactivation classification	> 180 µm/sec
Amplitude of lateral head displacement (ALH) threshold — hyperactivation classification	> 9.5 µm
Mean linearity (LIN) threshold — hyperactivation classification	< 38%

Duration of capture (0.5 s) is derived from frame rate × number of frames (60 Hz × 30 frames = 0.5 s). Analysis was performed using Test Semen 3.2 (Videotest, St. Petersburg, Russia). Settings are consistent with validated CASA protocols for mouse epididymal sperm analysis. The last three parameters (VCL, ALH, LIN) define the threshold used to classify a sperm sub-population as hyperactivated and are not general filters applied to all counted cells. Progressive motility was additionally classified according to WHO-based criteria for rodent sperm analysis (VAP ≥ 50.0 µm/s and straightness ≥ 50%), which are independent of, and not to be confused with, the 10.0 µm/s VAP cutoff used by the instrument to distinguish motile from immotile cells.

Sperm Viability, Morphology, and DNA Integrity

Sperm viability and morphological normalcy were assessed by eosin-nigrosin staining, with 200 spermatozoa scored per sample under 400× light microscopy; viable sperm excluded the eosin stain, while non-viable sperm-stained pink. Sperm chromatin integrity was evaluated using the acridine orange (AO) fluorescence assay, a metachromatic technique originally developed to differentiate native, double-stranded DNA (green fluorescence) from denatured, single-stranded DNA associated with chromatin damage (orange-red fluorescence).[11] Acridine orange fluorescence correlates with independent measures of sperm fertilizing capacity and has been widely applied as a rapid, cost-effective alternative to flow-cytometric DNA fragmentation assays in both clinical and experimental andrology.[12,13] Two hundred spermatozoa per sample were scored under fluorescence microscopy (Olympus BX51 fluorescence microscope or equivalent) using a fluorescein isothiocyanate filter set (excitation/emission: ~488/525 nm for green, ~488/630–650 nm for red-orange emission).

Hypo-Osmotic Swelling Test

Functional integrity of the sperm plasma membrane was assessed using the hypo-osmotic swelling test (HOST), in which sperm suspensions were incubated in a hypotonic sodium citrate-fructose solution; spermatozoa with intact, functionally competent membranes develop characteristic tail coiling and swelling in response to osmotic influx, whereas membrane-compromised sperm fail to react.[14] Sperm suspensions were incubated in the hypo-osmotic solution for 30 min at 37 °C prior to assessment. Two hundred sperm were evaluated per sample in duplicate.

Natural Mating Fertility Assessment

To determine whether biochemical and kinetic improvements in sperm quality translated into functional

reproductive capacity, each of the seven males per group was subsequently housed with two untreated, reproductively naive females (14 females per group) for a cohabitation period of 7 days. Female estrous cycle stage was not controlled for (natural cycling); mating was confirmed by the presence of a vaginal plug, and pregnancy was confirmed by abdominal palpation and post-partum litter count at 14–18 days after the start of cohabitation. Female mating index, male mating index, pregnancy index, and male fertility index were calculated as the proportion of females mated, males that achieved copulation, females confirmed pregnant, and males that successfully impregnated at least one partner, respectively. The mating indices reflect mating behavior only (mounting and copulation) and are distinct from the pregnancy and male fertility indices, which reflect reproductive success; a mating index of 100% therefore indicates that copulatory behavior was preserved after injury, independent of whether fertilization was ultimately achieved. This four-index framework for whole-animal fertility assessment following testicular torsion-detorsion parallels that recently applied in a rat model of testicular I/R injury.[3]

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). The normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was verified with Levene's test. For normally distributed data satisfying variance homogeneity, group differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparisons. Where these assumptions were not met, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied. Tukey's *post hoc* test and Dunn's multiple comparisons test both inherently control the family-wise error rate across pairwise comparisons within each endpoint, mitigating, though not eliminating, the risk of false-positive findings arising from the number of endpoints assessed across a small sample size; this constraint is acknowledged in the Limitations section. A p-value ≤ 0.05 was considered statistically significant. All analyses were performed using SPSS version 27.0 (IBM Corp., Armonk, NY, USA).

Results

Sperm Concentration and Motility

Testicular ischemia-reperfusion markedly reduced epididymal sperm concentration and both total and progressive motility relative to sham-operated animals (Table 2). Humanin pretreatment produced a clear, dose-dependent attenuation of this decline: the 504 µg/kg dose restored all three parameters to values approaching those of the sham group, while the 126 µg/kg and 252 µg/kg doses conferred intermediate, incremental protection.

Sperm Kinematic Parameters

Average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) were all substantially decreased in the I/R group compared with sham controls (Table 3). Linearity (LIN) and straightness (STR) showed a numerically smaller

Table 2. Effects of Humanin on Sperm Concentration and Motility.

Parameter	Sham	I/R	I/R+HN 126	I/R+HN 252	I/R+HN 504
Concentration (× 10^6/ml)	32.4 ± 2.3^a	15.2 ± 2.9^e	23.1 ± 2.5^d	28.7 ± 2.1^c	31.2 ± 1.8^b
Total motility (%)	81.2 ± 2.1^a	43.5 ± 3.4^e	64.8 ± 2.9^d	75.6 ± 2.3^c	79.4 ± 2.0^b
Progressive motility (%)	42.1 ± 1.9^a	8.1 ± 1.7^e	28.4 ± 2.2^d	36.8 ± 1.6^c	40.5 ± 1.4^b

Sham = sham-operated group; I/R = ischemia-reperfusion (torsion-detorsion) group without treatment; I/R+HN 126/252/504 = I/R groups treated with Humanin at 126, 252, or 504 µg/kg, respectively. Different superscripts within the same row indicate significant differences ($p \leq 0.05$, one-way ANOVA with Tukey's *post-hoc* test). Data are presented as mean ± SD (n = 7/group).

Table 3. Effects of Humanin on Sperm Kinematic Parameters.

Parameter	Sham	I/R	I/R+HN 126	I/R+HN 252	I/R+HN 504
VAP (µm/s)	29.81 ± 1.4^a	13.94 ± 2.1^e	19.25 ± 1.7^d	25.42 ± 1.5^c	28.78 ± 1.3^b
VCL (µm/s)	85.48 ± 2.3^a	41.29 ± 2.8^e	67.51 ± 2.4^d	79.16 ± 2.1^c	83.20 ± 1.9^b
VSL (µm/s)	24.15 ± 1.3^a	10.80 ± 1.9^e	15.93 ± 1.6^d	21.34 ± 1.4^c	23.44 ± 1.2^b
LIN (%)	24.36 ± 0.02^a	21.74 ± 0.03^a	22.46 ± 0.02^a	23.81 ± 0.02^a	24.30 ± 0.02^a
STR (%)	46.20 ± 0.02^a	42.67 ± 0.03^a	43.67 ± 0.02^a	44.09 ± 0.02^a	45.17 ± 0.02^a
ALH (µm)	2.75 ± 0.11^a	1.12 ± 0.14^e	1.95 ± 0.12^d	2.52 ± 0.10^c	2.68 ± 0.09^b
BCF (Hz)	12.8 ± 1.1^a	6.4 ± 1.3^e	8.9 ± 1.2^d	11.5 ± 1.0^c	12.4 ± 0.9^b

VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; LIN = linearity; STR = straightness; ALH = amplitude of lateral head displacement; BCF = beat cross frequency. Sham = sham-operated group; I/R = ischemia-reperfusion (torsion-detorsion) group without treatment; I/R+HN 126/252/504 = I/R groups treated with Humanin at 126, 252, or 504 µg/kg, respectively. Different superscripts within the same row indicate significant differences ($p \leq 0.05$, one-way ANOVA with Tukey's *post-hoc* test). Data are presented as mean ± SD (n = 7/group).

decline that did not reach statistical significance between any of the five groups. Humanin treatment reversed the significant kinematic changes in a dose-dependent manner, with the highest dose (504 µg/kg) restoring VAP, VCL, VSL, ALH, and BCF to values approaching those of the sham group.

Sperm Membrane Functionality, Viability, DNA Damage, and Morphology

Sperm plasma membrane functionality, assessed by the hypo-osmotic swelling test (Figure 1), and sperm viability (Figure 2) were both substantially decreased in the I/R group compared with sham controls, whereas DNA damage (Figure 3) and abnormal morphology rates exhibited a corresponding and marked increase (Table 4). Humanin treatment reversed these changes in a dose-dependent manner: the highest dose (504 µg/kg) reduced DNA damage to a level statistically comparable to the sham group, while membrane functionality, viability, and morphology likewise showed near-complete recovery at this dose. Intermediate doses again produced graded, partial protection.

Natural Mating Fertility Outcomes

Female and male mating indices remained at 100% across all experimental groups, indicating that testicular torsion-detorsion did not impair mating behavior itself (Table 5). In contrast, the pregnancy index and male fertility index demonstrated a pronounced and dose-dependent decline following I/R injury: the untreated I/R group exhibited a pregnancy index of only 7% and a male fertility index of 14%, compared with 100% in the sham group. Humanin treatment substantially and dose-dependently restored fertility outcomes, with the 504 µg/kg dose yielding a pregnancy index of 86% and a male fertility index of 100%; the pregnancy index at this dose was not statistically different from the sham group, although the numerical value (86%) remained below the sham mean, indicating near-complete rather than complete restoration (Table 5).

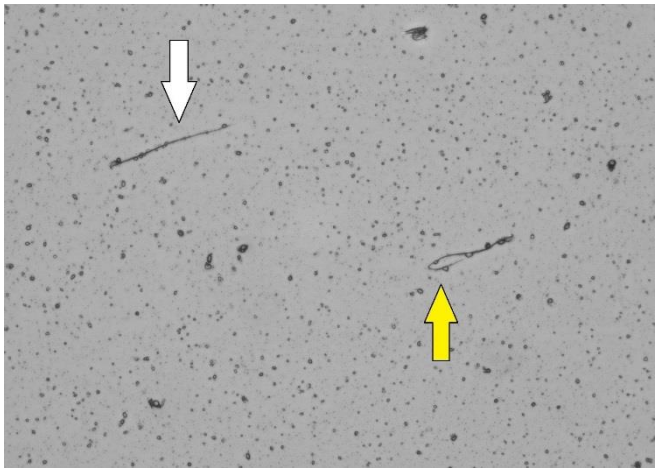

Figure 1. Sperm membrane functional integrity assessed by the hypo-osmotic swelling test (HOST). Representative photomicrograph (light microscopy, 400×) of mouse epididymal spermatozoa incubated in a hypo-osmotic sodium citrate-fructose solution. Yellow arrow: spermatozoon with an intact, functionally competent plasma membrane, exhibiting characteristic tail coiling and swelling in response to osmotic influx; white arrow: membrane-compromised spermatozoon showing no tail curling and remaining straight. Scale bar = 20 µm.

Discussion

The principal finding of this study is that humanin, administered as a single pre-reperfusion dose, confers

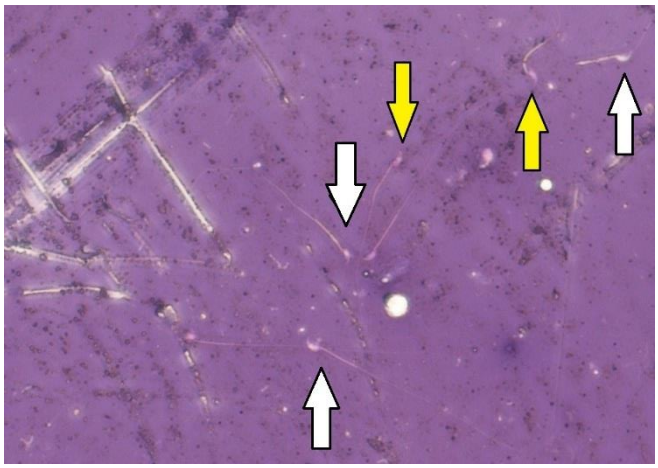

Figure 2. Sperm viability assessed by eosin-nigrosin staining. Representative photomicrograph (bright-field light microscopy, 400×) of mouse epididymal spermatozoa stained with eosin-nigrosin. White arrows indicate viable spermatozoa with unstained (white) heads, which excluded the eosin dye due to intact plasma membranes; yellow arrows indicate non-viable spermatozoa with stained heads, indicating membrane damage and dye uptake. Scale bar = 20 µm.

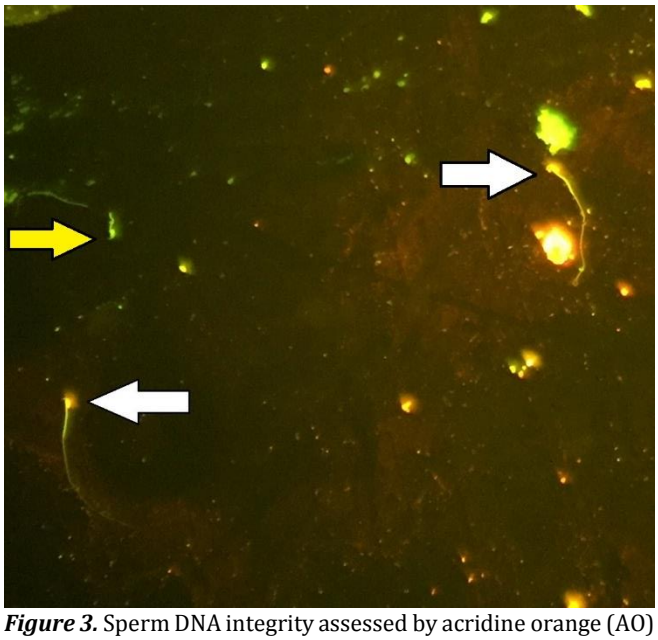

Figure 3. Sperm DNA integrity assessed by acridine orange (AO) fluorescence. Representative fluorescence photomicrograph (400×) of mouse epididymal spermatozoa stained with acridine orange. White arrows indicate spermatozoa with denatured, single-stranded DNA (orange-red/yellow fluorescence), reflecting sperm chromatin damage; yellow arrows indicate spermatozoa with native, double-stranded DNA (green fluorescence), reflecting intact chromatin. Scale bar = 20 µm.

Table 4. Effects of humanin on sperm membrane functionality, viability, dna damage, and morphology.

Parameter	Sham	I/R	I/R+HN 126	I/R+HN 252	I/R+HN 504
Sperm plasma membrane functionality (%)	84.3 ± 2.6[a]	47.8 ± 4.1[e]	65.9 ± 3.4[d]	78.4 ± 2.7[c]	83.1 ± 2.3[b]
Sperm viability (%)	87.5 ± 2.2[a]	53.4 ± 3.8[e]	70.2 ± 3.1[d]	81.9 ± 2.4[c]	85.8 ± 2.1[b]
Sperm DNA damage (%)	4.6 ± 0.5[e]	27.9 ± 1.8[a]	15.8 ± 1.3[b]	7.9 ± 0.7[c]	5.4 ± 0.6[d]
Sperm abnormal morphology (%)	6.8 ± 0.7[e]	32.4 ± 2.1[a]	17.5 ± 1.6[b]	10.2 ± 1.1[c]	7.6 ± 0.8[d]

Sperm plasma membrane functionality assessed by the hypo-osmotic swelling test (HOST). Sham = sham-operated group; I/R = ischemia-reperfusion (torsion-detorsion) group without treatment; I/R+HN 126/252/504 = I/R groups treated with Humanin at 126, 252, or 504 µg/kg, respectively. Different superscripts within the same row indicate significant differences ($p \leq 0.05$, one-way ANOVA with Tukey's *post-hoc* test). Data are presented as mean ± SD (n = 7/group).

Table 5. Fertility indices after natural mating with non-treated females.

Parameter	Sham	I/R	I/R+HN 126	I/R+HN 252	I/R+HN 504
Number of females	14	14	14	14	14
Number of females mated	14	14	14	14	14
Number of males mated	7	7	7	7	7
Number of males impregnating females	7	1	4	6	7
Number of females pregnant	14	1	5	8	12
Female mating index (%)	100[a]	100[a]	100[a]	100[a]	100[a]
Male mating index (%)	100[a]	100[a]	100[a]	100[a]	100[a]
Pregnancy index (%)	100[a]	7[d]	36[c]	57[b]	86[a]
Male fertility index (%)	100[a]	14[d]	57[c]	86[b]	100[a]

Female mating index = (number of females mated/total females) × 100; Male mating index = (number of males that achieved copulation/total males) × 100; Pregnancy index = (number of females pregnant / total females mated) × 100; Male fertility index = (number of males impregnating females/total males mated) × 100. Sham = sham-operated group; I/R = ischemia-reperfusion (torsion-detorsion) group without treatment; I/R+HN 126/252/504 = I/R groups treated with Humanin at 126, 252, or 504 µg/kg, respectively. Different superscripts within the same row indicate significant differences ($p \leq 0.05$, one-way ANOVA with Tukey's *post-hoc* test). Data are presented as mean ± SD (n = 7/group).

substantial and dose-dependent protection against the sperm-functional and whole-animal fertility consequences of testicular torsion-detorsion injury in mice. To our knowledge, this is the first report to extend evaluation of humanin, a mitochondrial-derived cytoprotective peptide with established efficacy in cardiac and intestinal ischemia-reperfusion models,[8-10] into the reproductive domain. It is also the first to demonstrate that this protection is not confined to biochemical or kinetic surrogates but extends to natural mating fertility, arguably the most clinically meaningful endpoint available in a preclinical model. This distinction matters: many previously tested antioxidant and phytochemical interventions have reported improved sperm parameters after testicular I/R injury without confirming that these improvements translate into preserved reproductive capacity.[3,6]

The marked reduction in sperm concentration, motility, and kinematic parameters observed in our untreated I/R group is consistent with the broader experimental literature on testicular torsion-detorsion, in which comparable declines in epididymal sperm concentration and progressive motility have been attributed to ischemia-driven disruption of spermatogenesis and post-testicular sperm maturation.[3] The dose-dependent recovery we observed with humanin parallels findings for other cytoprotective and antioxidant agents evaluated in similar torsion-detorsion paradigms, including quercetin and fisetin, which likewise improved sperm concentration and motility when administered prior to detorsion.[3,6] Mechanistically, this protection may be mediated through humanin's well-characterized capacity, described in other tissues, to suppress mitochondrial complex I-linked reactive oxygen species generation and to inhibit the intrinsic apoptotic cascade through direct interaction with Bax and related pro-apoptotic effectors,[7] thereby limiting the oxidative and apoptotic injury to both germinal epithelium and post-testicular sperm that is characteristic of testicular I/R injury. As oxidative stress and apoptotic markers were not directly measured in the present study, this mechanistic explanation is consistent with, rather than confirmed by, the current findings and should be interpreted with appropriate caution.[4,5]

The improvement in sperm DNA integrity and membrane functionality with humanin treatment is a particularly noteworthy finding, given that sperm DNA fragmentation is now recognized as a clinically relevant biomarker of male reproductive potential that is not always captured by conventional semen parameters such as concentration and motility alone.[12,13] The acridine orange assay used here to quantify chromatin damage reflects the susceptibility of sperm DNA to acid-induced denaturation at sites of existing strand breaks, and elevated DNA fragmentation has been consistently associated with reduced fertilization capacity in both

clinical and experimental settings.[13] Similarly, the hypo-osmotic swelling test provides a functional readout of plasma membrane integrity that is mechanistically distinct from, but complementary to, motility and viability staining.[14] The concordant, dose-dependent improvement across all of these independent assays strengthens confidence that humanin exerts a genuine protective effect on sperm quality rather than an artifact confined to a single measurement technique.

The natural mating fertility data provide the most direct evidence of biological significance in this study. Despite preserved mating behavior across all groups, reflected in unchanged female and male mating indices, the untreated I/R group demonstrated a near-complete collapse in pregnancy and male fertility indices, indicating that the sperm-functional deficits documented in Table 1 were of sufficient magnitude to compromise actual reproductive success. This finding is consistent with a recent report in a rat model of testicular torsion-detorsion, in which fertility indices calculated from natural mating trials similarly declined following untreated injury and were substantially restored by pre-reperfusion antioxidant treatment.[3] The near-complete restoration of fertility indices at the highest humanin dose in the present study, approaching values statistically indistinguishable from sham-operated animals, suggests that the degree of sperm-functional protection achieved was sufficient to translate into meaningful reproductive benefit, a translational threshold that is not always reached even when biochemical or histological improvements are documented.

From a clinical perspective, the current standard of care for testicular torsion remains prompt surgical detorsion, with no pharmacological adjunct in routine use to protect reproductive function during reperfusion. Should humanin's protective effects observed here translate to larger animal models and, eventually, to humans, it would most plausibly be positioned as a peri-operative adjunct administered alongside emergency detorsion. The principal barrier to this translation is timing: in the present study humanin was administered thirty minutes before a scheduled detorsion, whereas patients typically present after an unknown and variable period of ischemia, without a defined pre-reperfusion window in which to intervene. Establishing whether humanin retains efficacy when given at, or shortly after, detorsion, as noted below, is therefore a prerequisite for meaningful clinical translation.

Several limitations warrant consideration when interpreting these findings. First, the relatively small sample size per group (n=7), while consistent with comparable published rodent torsion-detorsion studies,[3] limits statistical power for detecting smaller between-group differences and constrains the precision of effect-size estimates; in addition, although Tukey's and Dunn's tests control the family-wise error rate within each endpoint, the large number of endpoints assessed across a small sample size means that a false discovery rate correction across endpoints (e.g., Benjamini-Hochberg) could be considered in future studies. Second, this study employed a single, defined torsion angle and duration (720° for two hours); the generalizability of these findings to shorter or longer ischemic intervals, or to partial torsion, remains to be established. Third, the underlying oxidative, inflammatory, and apoptotic mechanisms (e.g., oxidative stress markers, Bax/Bcl-2 expression) were not assessed alongside the functional and reproductive endpoints reported here, which limits the ability to directly link the sperm-functional and fertility findings to a specific molecular pathway. Fourth, only the torsed (left) testis was assessed, and contralateral testicular and sperm parameters were not evaluated; because unilateral testicular torsion can affect the contralateral gonad through autoimmune and inflammatory mechanisms, the relationship between this unilateral model and bilateral reproductive outcomes could not be addressed and warrants dedicated investigation. Fifth, no baseline (pre-injury) sperm parameters were obtained, so equivalence among groups prior to intervention was assumed on the basis of random allocation from a homogeneous population rather than demonstrated directly. Sixth, as with all rodent models, direct extrapolation of dosing and pharmacokinetics to human clinical application requires caution and further translational validation; in particular, humanin was administered before reperfusion in this model, whereas patients with testicular torsion typically present, and can only be treated, after the ischemic event has already occurred, and this distinction should be considered when discussing future clinical application. Finally, the natural mating fertility assessment captures cumulative reproductive outcome but does not resolve whether residual deficits in I/R and lower-dose humanin groups reflect impaired fertilization, early embryonic loss, or a combination of both mechanisms.

Future studies should extend the present findings in several directions. Long-term follow-up studies evaluating whether the fertility benefits observed at 30 days persist across subsequent spermatogenic cycles would clarify the durability of humanin's protective effect. Dose-ranging and pharmacokinetic studies, including exploration of doses beyond the highest tested here, would help define the optimal therapeutic window. Direct molecular investigation of the humanin-Bax interaction and downstream apoptotic signaling within testicular tissue, ideally performed in the same animals subjected to functional and fertility testing, would strengthen mechanistic inference. Given the clinical urgency of testicular torsion, in which detorsion timing is

often constrained by patient presentation, future work should also examine whether humanin retains efficacy when administered at, or even after, the time of surgical detorsion rather than only as a pre-reperfusion intervention, since this would substantially enhance clinical translational potential. Repeated or extended dosing schedules, combined with histopathological assessment of testicular tissue and direct measurement of oxidative stress and apoptotic biomarkers in the same experimental model, would help link the sperm-functional and fertility outcomes reported here to their underlying cellular mechanisms. The relatively modest protection conferred by the 126 µg/kg dose also warrants further evaluation to establish whether it represents a true no-effect, or minimally effective, dose that could help define the lower boundary of the therapeutic window. Future studies may also benefit from presenting fertility outcomes as Kaplan-Meier or time-to-pregnancy curves, which would provide richer information on the timing of reproductive success than simple proportions, and from including a supplemental figure depicting the full experimental timeline (acclimatization, torsion, humanin administration, detorsion, recovery, and sperm collection) to aid readers in visualizing the procedural sequence.

In Conclusion, humanin administration prior to detorsion conferred substantial, dose-dependent protection against sperm-functional impairment and, critically, natural mating infertility following testicular torsion-detorsion injury in mice, with the highest dose tested restoring fertility outcomes to levels statistically indistinguishable from uninjured controls. These findings extend the known cytoprotective repertoire of this mitochondrial-derived peptide into the reproductive domain and identify humanin as a promising candidate adjunct for preserving fertility potential in the setting of testicular ischemia-reperfusion injury, warranting further mechanistic and translational investigation.

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Conflict of Interest

The authors declare that they have no competing interests.

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