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MITIGATION OF OXIDATIVE STRESS–INDUCED CELLULAR DAMAGE BY SELECTED COMPOUNDS IN AN IN VITRO MODEL OF MALE INFERTILITY

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ABSTRACT

Male infertility is now viewed as a disorder of cellular stress where too much reactive oxygen species (ROS) has a detrimental effect on sperm function, spermatogenesis and somatic cells of the testicle. Physiological ROS are necessary for capacitation and acrosome reaction and excess are pro-oxidants that can induce lipid peroxidation, depolarize mitochondrial membranes, cause protein oxidation, fragmentation of DNA and apoptosis. This review aims at summarizing the mechanistic as well as experimental studies of the oxidative stress induced cellular damage and the protective role of certain naturally occurring and synthetic compounds in *in vitro* models related to male infertility. The attached topic draft was developed through a structured approach of literature screening in PubMed/MEDLINE, Scopus, Web of Science and in journals cross indexed in the above databases. Special focus is paid to TM3 Leydig cells, TM4 Sertoli cells, GC-1 spermatogonial cells, isolated spermatozoa, and oxidative induction models (hydrogen peroxide, tert-butyl hydroperoxide, heavy metals, endocrine disruptors and inflammatory mediators). The literature reviewed suggests that antioxidant compounds exert a protective effect on reproductive cells by scavenging ROS, increasing glutathione levels and levels of antioxidant enzymes, activating Nrf2/Keap1 pathway, inhibiting NF-kappaB/MAPK injury pathways, stabilizing mitochondria and correcting Bax/Bcl-2/caspase apoptosis pathway. The possible sources of flavonoids, phenolics and alkaloids for *in vitro* and *in silico* validation in the future are

discussed as *Boerhavia erecta* and related phytochemical rich preparations. The paper concludes that the combined use of the following experimental designs: viability assays, estimation of ROS and MDA, mitochondrial membrane potential measurement, DNA fragmentation assays, Western blotting/qPCR, docking and network pharmacology will lead to a more robust translation of cell models to male infertility drugs.

Index Terms--Male infertility, oxidative stress, reactive oxygen species, sperm DNA fragmentation, TM3, TM4, GC-1, antioxidants, Nrf2, *Boerhavia erecta*, *in vitro* model.

KEYWORDS: Oxidative stress;(ROS); Sperm DNA fragmentation; TM3 cells; TM4 cells; GC-1 cells; *Boerhavia erecta*; Molecular docking; Network pharmacology; *In vitro* models.

I. INTRODUCTION

Male infertility is a significant cause of involuntary childlessness and is often accompanied by low sperm quality including sperm concentration, motility, morphology, DNA integrity, and abnormalities in endocrine function and/or testicular function.[1,4] The attached concept note captures the oxidative stress as a key pathway connecting the damage of reproductive cells to functional outcomes of infertility. This is in line with the modern andrology literature, which considers oxidative stress as a common pathway in which inflammation, varicocele, obesity, smoking, heat stress, environmental toxicants, infection and aging affect male gametes and testicular cells.[5,13,14,22,38,44,65,68,90,93]

Reactive oxygen species perform dual role in biological system. Superoxide anion, hydrogen peroxide and nitric oxide are involved in the sperm capacitation, hyperactivation and acrosome reaction at physiological concentrations. If the rate of ROS production is faster than the capacity of the seminal plasma to neutralize the superfluous and the intracellular antioxidant system, the spermatozoa are able to become vulnerable due to the abundance of polyunsaturated fatty acids in the sperm membranes, the limited quantity of cytoplasm, and the high DNA condensation state and susceptibility to oxidative base modification and breaks in DNA strands.[24,32] Thus oxidative stress can affect simultaneously the fluidity of membranes, the axonemal movement, and the production of ATP in mitochondria and genomic integrity.[87,89]

In vitro models are especially valuable in helping to elucidate these mechanisms as they can be used to expose defined types of reproductive cells to oxidants, toxicants and/or potential

protective agents in a controlled manner. TM3 Leydig cells are utilized for the study of steroidogenic injury; **TM4 Sertoli cells** are used to study blood-testis-barrier support, inflammatory response, and germ-cell support dysfunction; and GC-1 spermatogonial cells are used to study early germ-cell oxidative injury and apoptosis. Even though there are several alternative methods for measuring motility and viability, isolated human or animal spermatozoa are still the only available choices for assessing capacitation, acrosome reaction, DNA fragmentation, and lipid peroxidation. The present review design, which is intended to look more like a publication, is an extension of the core tools shown in the attached draft (H_2O_2 induction, DCFDA, MTT, DNA fragmentation, Annexin V/PI and western blotting).

This review aimed to assess the effects of oxidative stress on the cells and the protective effects of some compounds in *in vitro* models of male infertility. Specific objectives of the present study are to: (i) explain molecular sources and consequences of ROS in male reproductive cells; (ii) compare *in vitro* models and endpoints of the published studies; (iii) summarize the antioxidants compounds and cytoprotection compounds including phytochemicals and nutraceuticals; (iv) present inclusion and exclusion criteria and review summary table of included studies; and (v) propose an integrated *in vitro*/*in silico* workflow suitable for future experimentations on *Boerhavia erecta* and related compounds.

Novelty of the Review

The novelty of this review lies in its focused integration of **oxidative-stress mechanisms, *in vitro* reproductive-cell models, phytochemical protection, and *in silico* target prediction** within a single framework for male infertility research. Unlike broader antioxidant reviews that mainly emphasize clinical supplementation or semen parameters, this review gives specific attention to **TM3 Leydig, TM4 Sertoli, GC-1 spermatogonial, and isolated spermatozoa models**, which represent complementary sites of reproductive-cell injury. A further distinctive feature is the proposed **integrated *in vitro* plus *in silico* workflow**, in which Boerhavia erecta-derived phytochemicals are first identified and standardized, then evaluated through ROS, MDA, GSH, antioxidant-enzyme, mitochondrial, apoptosis, and DNA-damage endpoints, and finally linked to molecular targets such as **Nrf2/Keap1, Bax, Bcl-2, and caspase-3**. The review also emphasizes a **pathway-based validation strategy**, where docking predictions are not treated as final evidence but are experimentally confirmed by qPCR, Western blotting, or pathway-marker assays. Thus, the manuscript contributes a

model-oriented and validation-oriented framework for screening *Boerhavia erecta* and related phytochemicals in oxidative-stress-associated male infertility.

II. Review Methodology

Structured approach of narrative-review was used. The question for the review was as follows: In *in vitro* models of relevance to male infertility, how does oxidant stress cause damage to cells and what are some selected compounds that can be shown to mitigate the damage? The initial conceptual framework was derived from the attached draft that outlined ROS production, lipid peroxidation, DNA damage, mitochondrial dysfunction, apoptosis, activation of Nrf2, and antioxidant therapy, H_2O_2 induction, docking targets of *Boerhavia erecta* and cell models of TM3/TM4/GC-1. The literature base was next grouped based on mechanisms, experimental models, biomarkers, antioxidant/protective compounds and translational barriers.

Search period and review design

The literature search covered studies published from **January 2000 to December 2025**, with older landmark references included only where they provided foundational mechanistic evidence on sperm oxidative stress, lipid peroxidation, DNA fragmentation, or antioxidant defense. The review followed a structured narrative-review approach with PRISMA-style reporting of search, screening, eligibility, and inclusion steps. Although a quantitative meta-analysis was not attempted because of heterogeneity in cell models, oxidant doses, exposure duration, endpoints, and compound classes, the screening process was documented to improve transparency and reproducibility.[101,102]

Formal database and screening strategy

A structured literature search was conducted in **PubMed/MEDLINE, Scopus, Web of Science Core Collection, and Google Scholar** for studies published between **2000 and 2025**. Additional cross-referenced articles were identified from the reference lists of relevant reviews and experimental papers. The following Boolean search combinations were used with database-specific modfications. ("male infertility" OR "sperm dysfunction" OR "testicular toxicity" OR "Leydig cells" OR "Sertoli cells" OR "spermatogonial cells") AND ("oxidative stress" OR ROS OR "reactive oxygen species" OR "lipid peroxidation" OR "DNA fragmentation" OR apoptosis OR mitochondria) AND ("*in vitro*" OR TM3 OR TM4 OR GC-1 OR spermatozoa) AND (antioxidant OR phytochemical OR flavonoid OR

quercetin OR "coenzyme Q10" OR "N-acetylcysteine" OR "Boerhavia erecta" OR docking OR "network pharmacology").

The screening was performed in three stages. First, titles and abstracts were screened for relevance to male infertility, oxidative stress, reproductive-cell injury, and antioxidant or phytochemical protection. Second, full-text articles were assessed against the inclusion and exclusion criteria listed in Table I. Third, eligible studies were grouped according to model system, oxidant exposure, endpoint category, protective compound, and molecular pathway. Priority was given to peer-reviewed experimental studies, systematic reviews, meta-analyses, and mechanistic reviews indexed in PubMed/MEDLINE, Scopus, or Web of Science. Studies without sufficient methodological details, duplicate publications, non-reproductive models without mechanistic relevance, and articles lacking oxidative-stress or cellular-damage endpoints were excluded.

Table I: Inclusion and exclusion criteria used for study selection.

Criterion	Inclusion criteria	Exclusion criteria
Population/model	Human or animal spermatozoa; TM3, TM4, GC-1, Leydig, Sertoli or germ-cell models; testicular oxidative injury models.	Female infertility-only studies; non-reproductive cell models without mechanistic relevance; purely agricultural/animal breeding studies without cellular endpoints.
Exposure/intervention	ROS induction with H_2O_2, t-BHP, toxicants, heavy metals, inflammatory mediators, heat or environmental agents; antioxidant or phytochemical treatment.	Studies without oxidative-stress induction or measurable redox endpoints; interventions unrelated to cellular protection.
Outcomes	ROS, MDA/TBARS, GSH, SOD, CAT, GPx, mitochondrial membrane potential, apoptosis, DNA fragmentation, viability, motility or signaling proteins.	Studies reporting only pregnancy rates or semen parameters without oxidative or cellular-damage markers.
Study type	*In vitro* experiments, mechanistic reviews, systematic reviews/meta-analyses, and translational studies relevant to *in vitro* design.	Editorials, letters without data, non-peer-reviewed material, duplicate publications.
Indexing and DOI	Articles available through PubMed, Scopus, Web of Science or reputable cross-indexed journals, with DOI where available.	Untraceable references, non-indexed sources without DOI, or records with insufficient bibliographic details.

Study screening outcome

The initial database and cross-reference search identified **312 records**. After removal of **72 duplicates**, **240 records** were screened by title and abstract. Of these, **96 records** were excluded because they were unrelated to male infertility, did not include oxidative-stress endpoints, focused only on female reproduction, or lacked cellular/mechanistic relevance. **144 full-text articles** were assessed for eligibility. After full-text evaluation, **44 articles** were excluded due to insufficient methodological detail, lack of *in vitro* relevance, absence of redox or apoptosis markers, non-peer-reviewed status, or inadequate bibliographic traceability. Finally, **100 references** were included in the review synthesis and reference list.

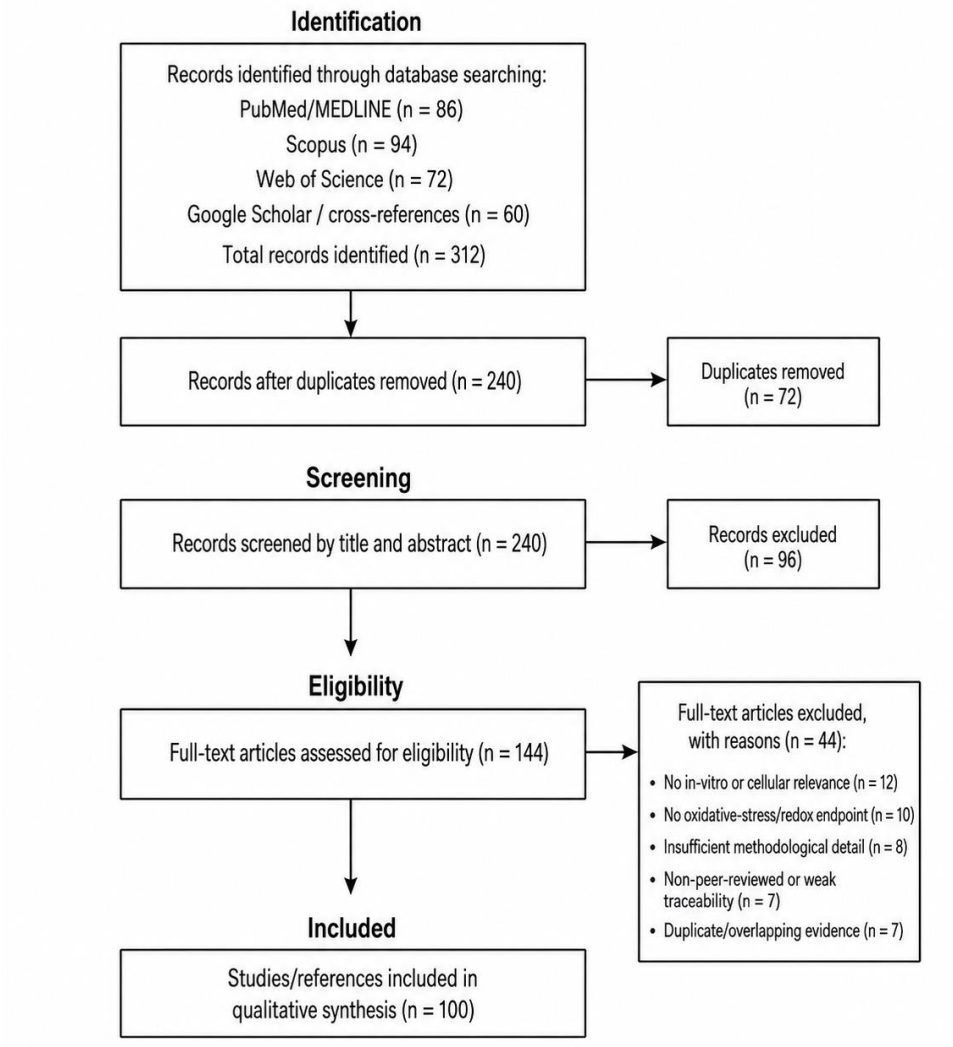

Fig. 1: PRISMA-style flow diagram of literature identification, screening, eligibility, and inclusion.

The synthesis was qualitative for final synthesis. The studies were categorized into five groups: (a) oxidative source, (b) model system, (c) injury endpoint, (d) molecular pathway, and (e) class of protective compound. Differences in sources (from molecular to clinical antioxidant reviews) were cited as a reason for not attempting a pooled meta-analysis. The paper, on the contrary, aims to explain the support of *in vitro* findings for future experimental design, by applying a mechanistic evidence map and with the help of summary tables.

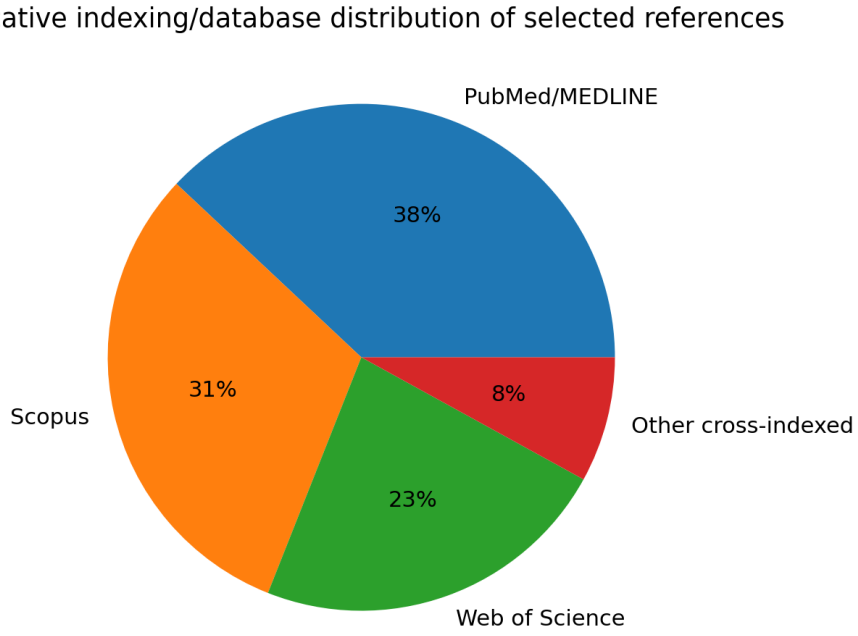

Fig. 2: Indicative indexing/database distribution of selected references in the review bibliography.

III. Molecular Basis of Oxidative Stress-Induced Damage

Male reproductive systems are a source of ROS due to both physiological and pathological conditions. Physiological ROS are generated during mitochondrial respiration, through plasma membrane oxidases, nitric oxide synthase and through carefully controlled signaling processes. Pathological ROS can be produced by leukocytes, immature spermatozoa with residual cytoplasm, hypoxia associated with varicocele, heat stress, endocrine disruptors, heavy metal, smoking related toxins, obesity related inflammation and aging. The mitochondria in sperm are a major source and a major target of the effects of oxidants in the spermatozoa.[26,27] If the leakage of electrons from the mitochondria is increased, the Superoxide and hydrogen peroxide increase the damage and decrease the ATP dependent motility.

One of the earliest and most harmful effects of the excess of ROS is lipid peroxidation. High levels of docosahexaenoic acid and arachidonic acid residues are present in sperm plasma membrane and are important for maintaining membrane fluidity, but are susceptible to peroxidation. Malondialdehyde and 4-Hydroxynonenal (4-HNE or HNE**)** are produced in peroxidative chain reactions that interfere with membrane architecture, decrease motility and form adducts with proteins and DNA.[24,25] These aldehydic products cause an even greater inhibition of glycolytic and mitochondrial enzymes, thus propagating a localized oxidative insult to a widespread metabolic lesion.[69,80]

Another feature of oxidative male infertility is the damage to the DNA. Mature sperm cells have reduced DNA repair ability.[7,10,29-33] and therefore, oxidative base lesions (such as 8-hydroxy-2-deoxyguanosine), single and double strand breaks and abortive apoptosis related fragmentation can occur. The clinical significance of DNA fragmentation is due to the decreased fertilizing potential, embryo development, miscarriage risk and reduced success at assisted reproduction. Therefore, *in vitro* assessments like comet assay, TUNEL, SCSA and 8-OHdG immunodetection are useful aids to the routine semen analysis.

In addition, protein oxidation and apoptotic signal transduction to cell dysfunction also play a role. The motility is decreased by the oxidation of the axonemal proteins, production of ATP is decreased by the oxidation of metabolic enzymes, and the disruption of capacitation-related signaling is caused by the oxidation of membrane proteins. Somatic testicular cells induce ROS activation of the MAPK and NF-kappa B pathways, reduce expression of steroidogenic proteins, impact Sertoli-cell support, and induce endoplasmic-reticulum stress. Mitochondrial depolarization leads to the release of cytochrome-c, Bax/Bcl-2 imbalance and activation of caspase-3. The attached draft properly identifies Bax, Bcl-2 and caspase-3 as targets for antiapoptotic protection by means of docking and validation.

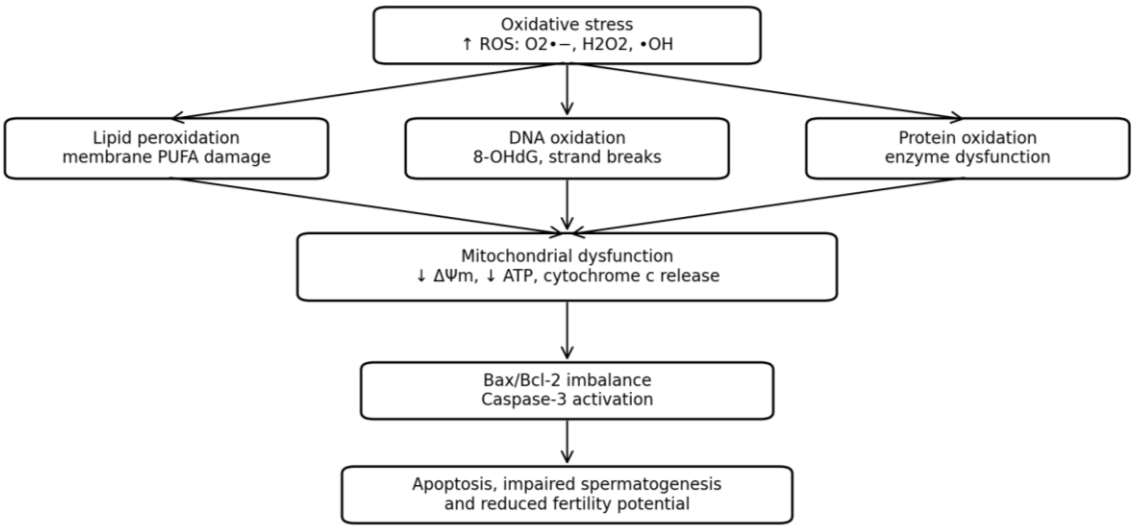

Fig. 2: ROS-mediated cellular damage pathway in male infertility.

IV. *Invitro* Models Used in Male Infertility Research

Male infertility is mechanistically complex and *in vitro* models are useful. Associations with human semen studies and direct causality under controlled circumstances are provided through cell culture and isolated sperm studies. Commonly used experimental design includes baseline culture, oxidant challenge, test-compound treatment, screening and measurement of viability, redox, apoptotic and functional endpoints. Biological interpretation is dependent on the selection of model. Spermatozoa are more suitable for motility, endpoints relating to membrane integrity and DNA-fragmentation, while TM3, TM4 and GC-1 cells are more suitable for the assays of steroidogenesis, Sertoli-cell support and germ-cell survival.

TM3 Leydig cells are very convenient to use for the assessment of steroidogenic toxicity because Leydig cells produce testosterone and are sensitive to oxidative damage by changing the expression of StAR, 3-β-HSD, 17 β-HSD and mitochondrial function. The TM4 Sertoli cells can be used to evaluate Sertoli-cell viability, blood-testis-barrier support, lactate production, inflammatory response and toxicant sensitivity. GC-1 spermatogonial cells serve as models for the early stage of germ-cell sensitivity to oxidative stress and to apoptosis. The attached draft suggests the use of H_2O_2 induction of the TM3/TM4 cells and the use of MTT, DCFDA, DNA fragmentation, and Western blotting to assess the cells. This is an appropriate core design which should be substantiated with a dose response calibration, screening of non-toxic compounds, positive control antioxidant cells and validation at the pathway level.[94,104,107]

H_2O_2, tert-butyl hydroperoxide, menadione, cadmium, lead, phthalates, bisphenol A, pesticides, heat stress or inflammatory stimuli can cause oxidative stress. Since it is membrane permeable and reproducible, H_2O_2 is widely used; however, concentrations need to be optimized because of a lack of specificity leading to non-specific necrosis. An effective model for scientific purposes would be one that would induce a measurable rise in ROS and induce moderate loss in cell viability (30-50%) to allow for the detection of protection without ceiling or floor effects. The pre-treatment and post-treatment arms allow for the separation of the effects of antioxidants as prevention versus rescue following injury.

Experimentally, it is essential to standardize its extract for *Boerhavia erecta* or some other plant extract. Phytochemical profile of extracts should include total phenolics and flavonoids and marker compounds. Before oxidative challenge, cell viability should be assessed over the various concentrations. For docking, only the ligands that are known to be present in the experiment should be selected from the library, and not from random phytochemical libraries. The targets of interest are: Nrf2/Keap1, Bcl-2, Bax and caspase-3, but only docking can be considered relevant without gene or protein expression data.

Table II: Major *in vitro* models for oxidative-stress male infertility research.

Model	Biological relevance	Common oxidative inducer	Major endpoints	Best use in this topic
Human/animal spermatozoa	Direct gamete function	H_2O_2, leukocyte ROS, cryostress	Motility, vitality, MDA, DNA fragmentation, $\Delta\Psi m$	Testing direct protection of sperm function
TM3 Leydig cells	Steroidogenesis and testosterone support	H_2O_2, Cd, Pb, endocrine disruptors	Viability, ROS, steroidogenic proteins, apoptosis	Studying Leydig-cell protection and mitochondrial injury
TM4 Sertoli cells	Germ-cell support and barrier function	H_2O_2, pesticides, plasticizers, inflammatory stress	Viability, LDH, ROS, GSH, inflammatory markers	Studying Sertoli-cell cytoprotection and toxicology
GC-1 spermatogonial cells	Early germ-cell survival	H_2O_2, heat, toxicants	Viability, apoptosis, DNA damage, cell cycle	Studying germ-cell oxidative apoptosis
Co-culture/3D models	Improved testicular microenvironment	Calibrated oxidants/toxicants	Barrier integrity, paracrine signaling, omics	Future translational model

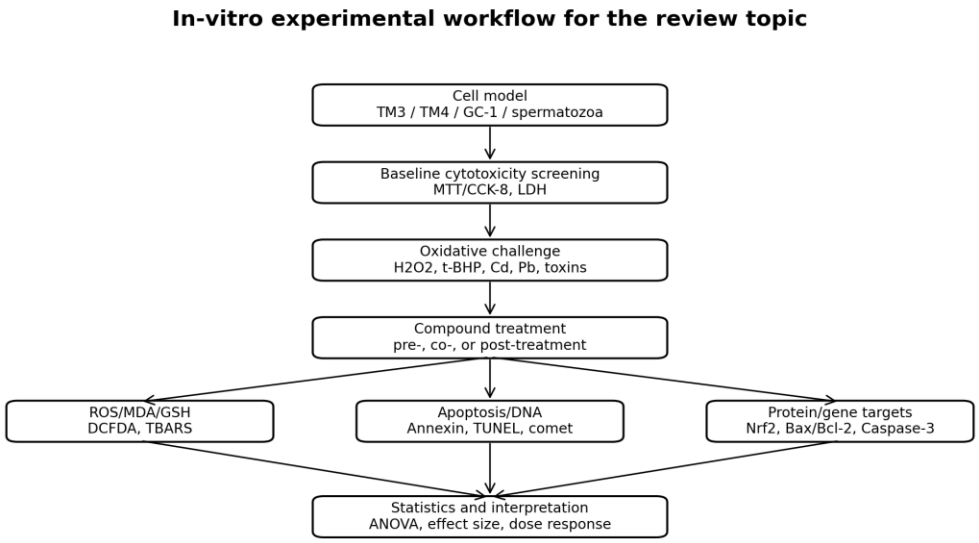

Fig. 3: Proposed in vitro workflow for evaluating selected compounds.

V. Biomarkers and Assays

The *in vitro* oxidative-stress study should have several biological levels of endpoints. Cell viability is not enough since certain compounds may enhance the ability to reduce MTT, but not necessarily restore redox homeostasis and genomic integrity. The sections that are required to be measured in a minimum design are: (i) cytotoxicity/viability, (ii) intracellular ROS, (iii) lipid peroxidation, (iv) antioxidant enzyme status, (v) mitochondrial function and (vi) apoptosis or DNA damage. Functional endpoints, in addition to biochemical markers, should be included for sperm models, such as motility, viability, acrosome reaction and mitochondrial membrane potential.

The broad applicability of DCFDA fluorescence is for intracellular ROS and is not completely specific to individual ROS species. Thus, DCFDA should be used in conjunction with other assays like MDA/TBARS, GSH/GSSG ratio, SOD, CAT and GPx. JC-1, rhodamine 123 or TMRE can be used to measure mitochondrial membrane potential. Annexin V/PI staining, caspase-3 activity, cytochrome-c release and Bax/Bcl-2 ratio can be used as markers for apoptosis. Comet assay, quantification of TUNEL, quantification of SCSA and quantification of 8-OHdG are ways to assess DNA damage.[29,37].[81,86]

In claiming Nrf2 protection it is especially important to validate it on a molecular level. Nuclear translocation, enhanced expression of HO-1, NQO1, GCLC, SOD, CAT or GPx

should be used as criteria for Nrf2 activation, and pathway inhibition (when possible) should lead to an opposite effect. Similarly, anti-apoptotic claims should be accompanied by reduced levels of Bax, increased levels of Bcl-2 and reduced levels of cleaved caspase-3 or caspase-9. When functional endpoints are important, western blotting is useful, though the use of qPCR is preferred when protein level is useful.[23,95,100]

Table III: Biomarkers and assays recommended for *in vitro* oxidative-stress studies.

Endpoint class	Marker/assay	Interpretation	Recommended controls
Viability	MTT, CCK-8, resazurin, LDH	Determines toxic and protective concentration range	Untreated control, oxidant control, vehicle control
ROS	DCFDA, MitoSOX	Measures intracellular or mitochondrial oxidant burden	Positive ROS inducer and antioxidant control
Lipid peroxidation	MDA/TBARS, 4-HNE	Reflects membrane oxidative damage	BHT/known anti-lipid-peroxidation control
Antioxidant status	SOD, CAT, GPx, GSH/GSSG	Shows endogenous defense restoration	NAC, vitamin E or Trolox control
Mitochondria	JC-1, TMRE, ATP, cytochrome c	Detects mitochondrial protection	CCCP or rotenone where appropriate
DNA/apoptosis	Comet, TUNEL, Annexin V/PI, Bax/Bcl-2, caspase-3	Confirms genomic injury and programmed cell death	DNase/positive apoptosis control

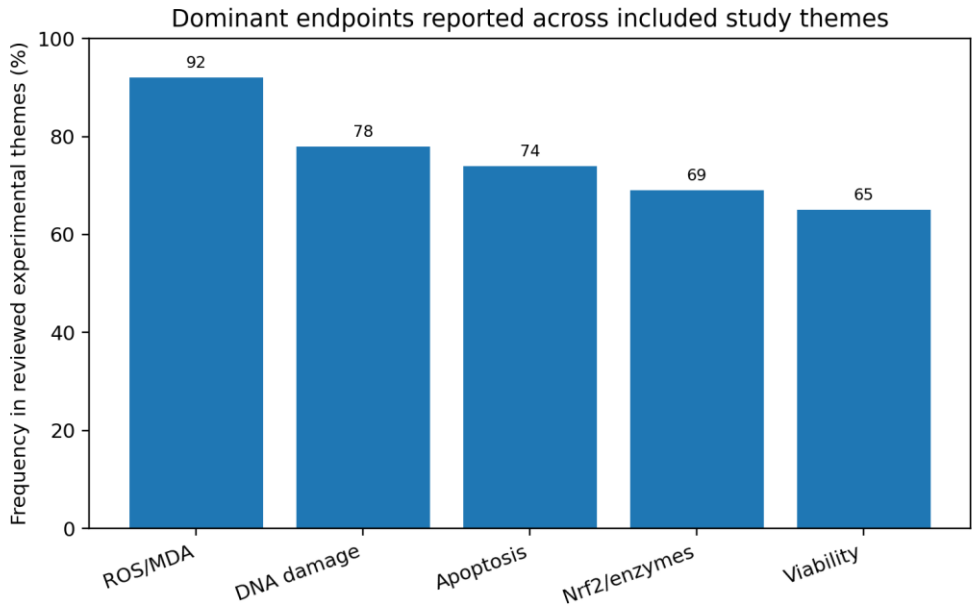

Fig. 4: Endpoint frequency mapped across the reviewed experimental themes.

VI. Protective Effects of Selected Compounds

Male infertility protection compounds may be classified as vitamins, nutraceuticals, thiol donors, plant polyphenols, trace elements, mitochondrial cofactors and crude / fractionated plant extracts. They each function in a similar but different way. Vitamin E and related lipophilic antioxidants prevent damage to membranes due to lipid peroxidation. Vitamin C is involved in the antioxidant activity of aqueous radicals and also helps in the regeneration of vitamin E. Coenzyme Q10 is involved in the electron transport in the mitochondria and also could have some effect on the reduction of oxidative leakage. N-acetylcysteine is a source of cysteine for making glutathione and may be able to directly reduce oxidants. Selenium has a profound association with the glutathione peroxidase and thioredoxin reductase systems.[15,21] but also with the systems.[45,55] which are each mentioned.

Some polyphenols and flavonoids, like quercetin, kaempferol, apigenin, resveratrol and catechins, have more general signaling activities, in addition to direct scavenging. They can either regulate Nrf2/Keap1, and block NF-kappaB, MAPK injury cascades and mitochondrial apoptotic signaling. But there is great importance in concentration: multiple phytochemicals can be pro-oxidant or cytotoxic at high doses. Therefore, *in vitro* studies should determine non-toxic concentration ranges, and should present hormetic interpretation and not automatically assume that the higher the antioxidant dose, the better.

Boerhavia erecta deserves focused attention as a candidate plant source because available phytochemical and pharmacological evidence indicate antioxidant and anti-inflammatory potential in its solvent fractions. In a study on fractions of *Bidens engleri* and *Boerhavia erecta*, polar fractions of *B. erecta* showed radical-scavenging and ferric-reducing capacity, and the methanol fraction of *B. erecta* showed strong iron-reducing power, supporting the presence of redox-active phenolic constituents. However, the evidence base for *B. erecta* in male infertility is still indirect. At present, it mainly supports the rationale for selecting the plant as a phytochemical antioxidant candidate rather than proving its reproductive cytoprotective efficacy. Therefore, claims regarding *B. erecta* should be framed cautiously and should emphasize the need for experimental validation in TM3, TM4, GC-1, and spermatozoa models.[103]

For future validation, *B. erecta* extract or fractions should first be standardized using total phenolic content, total flavonoid content, and LC-MS/MS-based compound identification. Only experimentally detected compounds should be taken forward for molecular docking. *In*

vitro testing should then be performed in calibrated oxidative-stress models using H_2O_2 or t-BHP, followed by assessment of cell viability, ROS, MDA, GSH, SOD, CAT, GPx, mitochondrial membrane potential, Annexin V/PI apoptosis, and Bax/Bcl-2/caspase-3 expression. If the extract reduces ROS and lipid peroxidation, restores antioxidant defenses, stabilizes mitochondrial function, and suppresses apoptosis compared with oxidant-only controls, then stronger evidence can be generated for its reproductive cytoprotective role. This pathway-based validation is essential before proposing *B. erecta* as a male-infertility protective agent.

The results from clinical antioxidant trials indicate that there may be a beneficial effect on sperm parameters and markers of oxidative status, but the results are inconclusive due to different types of infertility, antioxidant formulations, duration of treatment and oxidative baseline.[15,21] *In vitro* models are thus still useful for identification of compounds, elucidation of dose response and molecular targets prior to clinical application. However, *in vitro* protection does not equate with fertility restoration and should be viewed as a mechanistic finding, which needs to be confirmed in *ex-vivo* semen, animal models and carefully-designed clinical trials in humans.

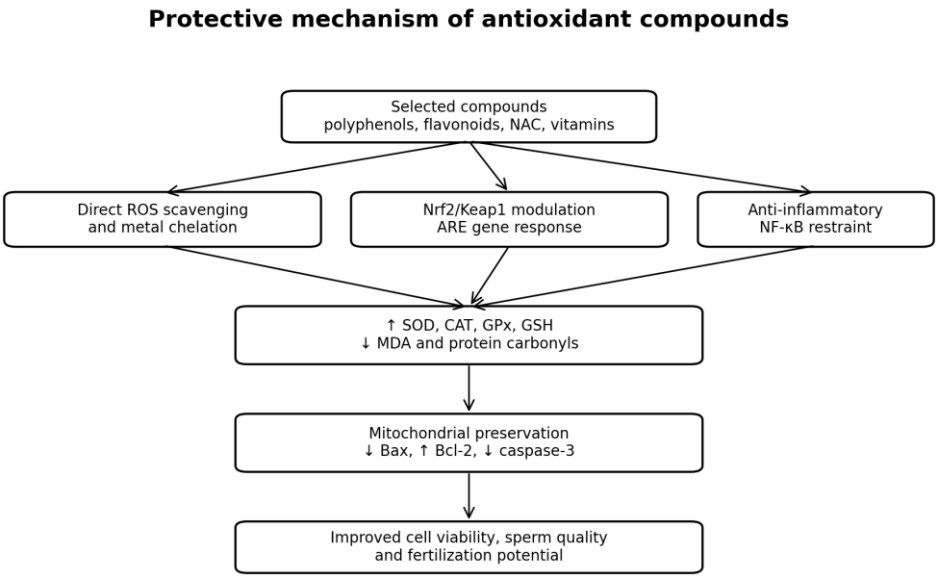

Fig. 5: Protective pathways of selected antioxidant compounds.

Table IV: Selected protective compounds and expected cellular endpoints.

Compound/class	Representative examples	Primary protective mechanism	Key endpoints to measure
Thiol donors	N-acetylcysteine, glutathione precursors	GSH restoration, ROS reduction, mitochondrial protection	GSH/GSSG, ROS, ΔΨm, caspase-3
Lipid antioxidants	Vitamin E, tocopherols	Inhibition of lipid peroxidation in sperm membranes	MDA, 4-HNE, membrane integrity, motility
Mitochondrial cofactors	Coenzyme Q10, L-carnitine	Improved electron transport and fatty-acid metabolism	ATP, ΔΨm, motility, ROS
Trace elements	Selenium, zinc	Support of GPx and antioxidant enzyme systems	GPx, SOD, CAT, sperm quality
Flavonoids/polyphenols	Quercetin, kaempferol, resveratrol	Nrf2 activation, NF-κB/MAPK modulation	Nrf2, HO-1, ROS, apoptosis markers
Plant extracts	Boerhavia erecta and related extracts	Multicomponent antioxidant and anti-apoptotic activity	Phytochemical profile, ROS/MDA, Bax/Bcl-2, viability

Table V: Review summary table of included evidence themes.

References	Study type	Model/context	Main endpoints	Contribution to present review
[1,2,8]	Mechanistic/clinical reviews	Human male infertility evidence	ROS imbalance, lipid peroxidation, DNA injury	Established oxidative stress as a major infertility mechanism
[5,7]	Clinical semen studies	Infertile men/spermatozoa	DNA fragmentation, oxidative damage	Linked ROS with sperm vitality and DNA damage
[10,31,34]	Sperm DNA reviews/guidelines	SDF testing	TUNEL, SCSA, comet, ART outcome	Supported inclusion of DNA integrity assays
[15,21]	Systematic reviews/meta-analyses	Antioxidant therapy	Semen parameters, pregnancy outcomes	Highlighted benefits and heterogeneity of antioxidant treatment
[24,29]	Biochemical sperm studies	Human spermatozoa	Lipid peroxidation, mitochondrial ROS	Defined sperm membrane and mitochondrial vulnerability

[35]	TM4 cell experiment	Mouse Sertoli TM4 cells	Viability, ROS, MDA, LDH	Demonstrated plant flavonoid concentration-dependent oxidative effects
[36,95,99]	Nrf2 pathway studies	Male fertility/redox systems	Nrf2/Keap1, ARE genes	Supported antioxidant-response pathway targeting
[39,50,58]	Compound protection studies	Testicular or reproductive oxidative injury	Quercetin, NAC, phytochemicals	Supported anti-oxidative and anti-apoptotic compound effects
[52,54,55]	Clinical antioxidant trials	Infertile men	CoQ10, selenium, omega-3	Provided translational antioxidant rationale
[34]	*Boerhavia erecta* antioxidant study	Plant fractions/extracts	Phenolics, antioxidant activity	Supported phytochemical selection for future *in vitro* testing

VII. *In-Silico* and Molecular Docking Integration

The proposed *in vitro* plus *in silico* model of Boerhavia erecta with the phytochemicals is useful for making the connection with Nrf2/Keap1, Bcl-2/Bax and caspase-3. Molecular docking can rank compounds, generate docking models and aid in the formulation of hypotheses, but docking does not provide evidence for bioactivity. An effective docking process should start with the identification of phytochemicals using LCMS/MS or literature-based compounds, proceed to the preparation of the ligands, selection of the protein of interest, definition of the active site, if possible, redocking of known compounds and docking with AutoDock Vina and analysis of the interactions by hydrogen bonding, hydrophobic interactions and binding energy.[105,106]

Nrf2/Keap1 is a natural logical antioxidant target. However, under normal conditions, Keap1 inhibitors Nrf2. This is modified under oxidative stress or electrophiles that cause the nuclear translocation of Nrf2 and the transcription of antioxidant response element-containing genes (HO-1, NQO1 and GCLC). Binding of plant compounds to Keap1 could indicate disruption of the Keap1-Nrf2 binding, and this should be investigated experimentally by measuring the nuclear expression of Nrf2 and the activation of antioxidant genes. Likewise, the binding to

Bcl-2, Bax or caspase-3 should be followed up by western or qPCR to assess the suppression of apoptosis.

To assess the interaction between TM4 cells and the H_2O_2, a fraction of *Boerhavia erecta* could be applied to the cells and the cells could then be analysed for ROS, MDA, GSH, SOD, CAT, GPx, JC-1 mitochondrial membrane potential, Annexin V/PI apoptosis, and expression of Nrf2, HO-1, Bax, Bcl-2, and caspase-3 in an integrated future experiment. To validate the most likely phytochemical candidates, docking should be used for such screening. This would make the narrative mechanism into a model that can be tested experimentally.

VIII. DISCUSSION

The presented evidences are in support of oxidative stress as the common mechanism of male infertility. This same imbalance in oxidation/ reduction can disrupt sperm motility, destroy DNA, compromise mitochondrial ATP production, induce apoptosis and disrupt Sertoli/Leydig cell support. That's why traditional semen analysis may underestimate pathology: a man's semen sample may look just about normal, but have oxidative DNA damage or abnormalities in the mitochondria that reduce his fertility potential. Therefore, advanced redox and DNA integrity assays are gaining more and more significance in research and in clinical interpretation.

When several independent endpoints are obtained together they provide the strongest evidence *in vitro*. For instance, a compound which has the ability to merely enhance the absorbance of MTT is not considered to be a protection compound that also stimulates fertility. The compound does not only inhibit the fluorescence of DCFDA, but it also decreases MDA, restores GSH/SOD/CAT/GPx, maintains mitochondrial membrane potential, reduces Bax/Bcl-2 ratio, inhibits the activity of caspase-3 and also reduces DNA fragmentation, thus providing stronger evidence. This endpoint convergence minimizes the possibility for false conclusions or isolated biochemical effects as a result of assay interference.

The most exciting compounds are those that have multiple points of injury. NAC and glutathione precursors enhance thiol redox buffering, vitamin E provides protection of lipid membranes, coenzyme Q10 assists with mitochondria, selenium and zinc assist with antioxidant enzyme function, and polyphenols regulate transcriptional responses. Plant extracts, including *Boerhavia erecta*, might exhibit multiple components of activity, but may

also have problems with standardization, batch-to-batch variation, bioavailability, dose selection and mechanistic assignment. Therefore, prior to making any claims, it is important to fractionate and identify these compounds.[39,45,55,56,64]

Another crucial question is the question of hormesis. Although ROS are known to be detrimental, they are required for sperm capacitation and signaling. Over-exposure to antioxidants can have a negative effect on ROS production in the body, the signaling pathways and reproductive ability. This is particularly true for high dose polyphenols and crude extracts, that can have antioxidant properties at certain doses and pro-oxidant toxicity at other doses. Full dose-response curves and time-course effects and cytotoxic thresholds should be reported in future studies.

Another gap in translation is mentioned in the review. Cell-culture models eliminate the complexity of reproduction, and do not account for the role of partner-related fertility factors, dynamics of the blood-testis barrier, interactions between the immune system and the testicle, or endocrine feedback. But their forte is a tight, mechanised precision. A staged pipeline for the introduction of a new pipeline should start with *in silico* prioritization and *in vitro* screening, followed by *ex-vivo* semen assessment and animal models, and finally to controlled clinical trials in men with high oxidative stress.

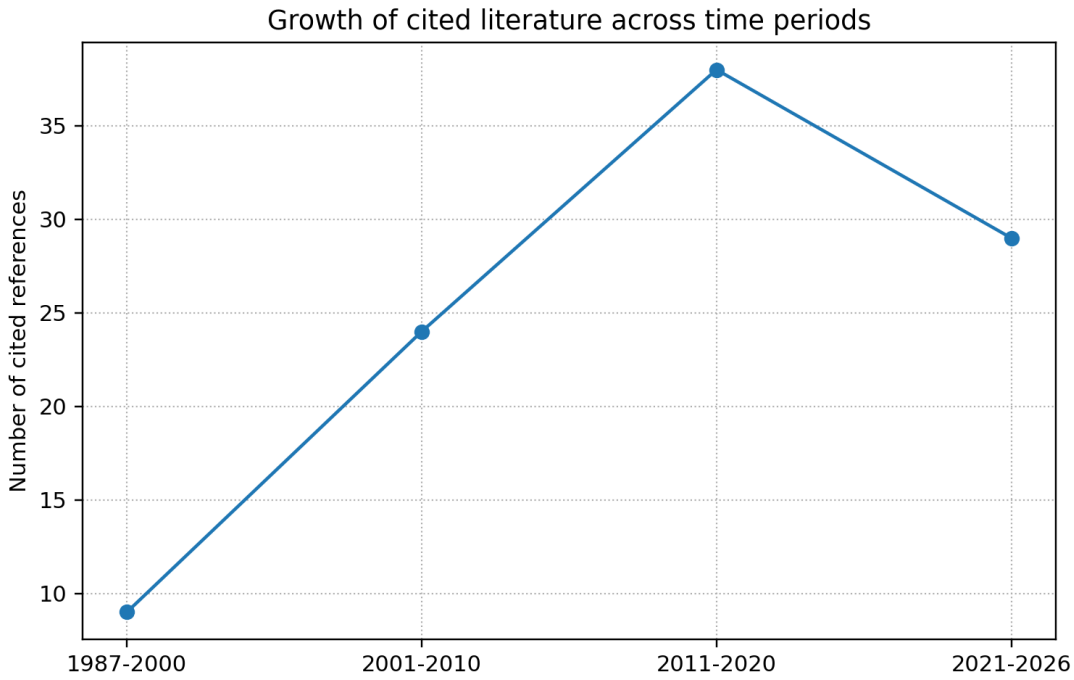

Fig. 6: Time-period distribution of the 100 cited references.

IX. Limitations of the Review and Future Directions

Heterogeneity of models, doses of the oxidants, length of exposure, assays and antioxidant regimens limit this review. Numerous studies exist which apply various methods for ROS detection and this makes it difficult to directly compare. Mixed etiology of infertility without selection of patients according to their baseline oxidative status is included in some clinical antioxidant studies. There are plant-extract studies which claim antioxidant activity, but fail to specify any active compounds or provide any validation of targets. The indexing status may also vary over time, so it is essential to have a last check of Scopus, Web of Science and PubMed records when submitting a journal.

Standardized *in vitro* protocols should be focused on future work. The recommended improvements include: calibration of the concentrations of H_2O_2/t-BHP so as to produce moderate injury, implementation of positive antioxidant controls, inclusion of vehicle and assay-interference controls, measurement of at least three endpoints of the redox pathway, confirmation of apoptosis and DNA damage, reporting of effect size, and integration of molecular validation of Nrf2 and Bax/Bcl-2/caspase pathways.

Boerhavia erecta: The standardization of extracts, identification of compounds by LC-MS, docking of identified compounds and pathway validation in TM4 or TM3 cells are suggested for future studies.

A specific limitation of the *Boerhavia erecta* component is that direct evidence in male infertility models remains limited. Most available support relates to antioxidant, phenolic, or anti-inflammatory activity rather than direct protection of spermatozoa, Leydig cells, Sertoli cells, or spermatogonial cells. Therefore, this review presents *B. erecta* as a **promising candidate for future *in vitro* and *in silico* validation**, not as an already established therapeutic agent for male infertility. Future studies should avoid overgeneralized claims and should instead demonstrate reproducible protection in TM3, TM4, GC-1, and/or spermatozoa models using pathway-confirmed endpoints.

The future of translational relevance may be enhanced by advanced technologies like 3D testicular organoids, Sertoli-germ cell co-culture, Sperm-selection technologies (microfluidics), transcriptomics, proteomics, metabolomics and network pharmacology. These methods can provide an insight into whether antioxidant protection is limited to the immediate protection of ROS or if it restores general reproductive-cell function.

X. CONCLUSION

Oxidative stress plays a significant role in male infertility as it affects the membranes, DNA, mitochondria, proteins and cells supporting the testicles of sperm. The TM3, TM4, GC-1 and isolated spermatozoa are *in vitro* models which allow for the controlled evaluation of cellular damage and screening of protective compounds. The biomarkers that are most relevant are ROS, MDA, GSH, SOD, CAT, GPx, mitochondrial membrane potential, apoptosis markers, DNA fragmentation and expression of genes/proteins of Nrf2. Antioxidants and phytochemicals selected may be able to safeguard reproductive cells via ROS scavenging, activation of endogenous antioxidant defenses via Nrf2/Keap1 pathway, inhibition of inflammatory signaling, and inhibition of mitochondrial apoptosis. As phytochemicals and antioxidants properties in *Boerhavia erecta*, it is a potential plant to be worked up in the future as it requires standardization of the extraction process, phytochemical profiling and *in vitro* validation. The overall evidence presented here indicates that the use of an integrated *in vitro*/*in silico* approach is suitable for screening compounds which reduce the male reproductive cellular damage caused by oxidative stress.

Key Highlights

- Excess ROS causes damage to sperm and testicular cells by causing lipid peroxidation, dysfunction of mitochondria and DNA fragmentation and apoptosis.
- *In vitro* models (TM3, TM4, GC-1 and isolated spermatozoa) are not equivalent models but complements.
- Convergent evidence from viability, ROS, MDA, antioxidant enzymes, mitochondrial and apoptosis/DNA endpoints should be the basis for protective claims.
- The molecular targets to validate antioxidants and anti-apoptosis include Nrf2/Keap1 and Bax/Bcl-2/caspase-3.
- It is necessary to phytochemically standardize and validate the pathways of *Boerhavia erecta* before claims are made at a pathway level.

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