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Enhancing Sustainable Rabbit Meat Production: A Feed-Based Strategy Using Natural Antioxidants to Improve Breeding Stock Fertility and Genetic Integrity

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ABSTRACT

Oxidative stress represents a major threat to male fertility by compromising sperm quality and DNA integrity. This study investigated the effects of natural antioxidant supplementation on semen quality parameters and sperm chromatin structure in rabbit bucks under oxidative stress conditions. Forty New Zealand White rabbit bucks (aged 7-9 months, 3.0-3.5 kg) were randomly allocated to four groups (n=10 per group): Control (C), Oxidative Stress (OS), OS + Natural Antioxidants (OS+NA), and Natural Antioxidants alone (NA). The natural antioxidant blend included vitamin E (200 mg/kg), organic selenium (0.3 mg/kg), vitamin C (500 mg/kg), and herbal extracts (150 mg/kg) for 90 days. Parameters assessed included sperm concentration, total and progressive motility, viability, morphological abnormalities, DNA fragmentation index (DFI), acrosome integrity, seminal plasma antioxidants (TAC, SOD, GPx, GSH, vitamins E and C, selenium), oxidative markers (MDA, ROS, 8-OHdG), and liver enzyme activities. Results showed oxidative stress significantly impaired progressive motility (47% reduction, $P<0.001$), viability (38% decrease, $P<0.001$), and dramatically increased DNA fragmentation (165% elevation, $P<0.001$). Natural antioxidants effectively restored progressive motility to 89% of control values ($P<0.01$), reduced DNA fragmentation by 58% versus OS group ($P<0.001$), and improved acrosome integrity by 43% ($P<0.01$). Seminal TAC increased 127% ($P<0.001$) while MDA decreased 62% ($P<0.001$) with antioxidant treatment. However, sperm concentration showed non-significant changes ($P=0.186$), and liver enzymes remained unaffected ($P>0.05$). Strong negative correlations were observed between seminal ROS and progressive motility ($r=-0.81$, $P<0.001$) and between DNA fragmentation and fertility markers ($r=-0.76$, $P<0.001$). This study demonstrates natural antioxidants effectively protect sperm DNA integrity and functional parameters through enhanced antioxidant defense, with DNA preservation representing the most dramatic protective effect.

1.Introduction

Within the framework of sustainable animal production, the efficiency of breeding stock is a cornerstone of economic viability and food security [1]. In rabbit meat production, the reproductive performance of buck is a critical determinant of herd expansion and genetic progress [2]. However, modern intensive rearing systems often expose animals to various environmental, nutritional, and metabolic challenges that can induce oxidative stress—a state characterized by an imbalance between the production of reactive oxygen species (ROS) and the biological system's ability to detoxify them [3]. In male reproduction, this imbalance poses a significant threat, as spermatozoa are particularly vulnerable to oxidative damage due to their high polyunsaturated fatty acid content and limited antioxidant defenses [4].

The repercussions of oxidative stress on semen quality are profound, leading to impaired sperm motility, reduced membrane integrity, and, most critically, fragmentation of nuclear DNA [5]. This degradation of sperm DNA integrity not only compromises fertilization rates but also affects embryo development and the long-term health of the offspring, thereby undermining the genetic potential and productivity of the herd [6]. Consequently, developing nutritional strategies to mitigate oxidative damage is a key objective in advanced animal nutrition, which aligns with the industry's goals of enhancing productivity, sustainability, and animal welfare [7].

The use of synthetic antioxidants in animal feed has been common, but growing consumer demand for natural and clean-label products has shifted research focus toward natural alternatives [8]. This trend presents an opportunity for the food industry to valorize plant-derived extracts and food-grade antioxidant compounds (such as vitamin E, vitamin C, and selenium) that can be repurposed from the human food chain or its co-products [9]. Incorporating these bioactive compounds into animal feed represents a synergistic approach to improving animal health and product quality while adhering to market-driven preferences [10-12].

Therefore, this study investigates the efficacy of a tailored dietary regimen comprising natural, food-industry-relevant antioxidants—including vitamin E, organic selenium, vitamin C, and standardized herbal extracts—on the seminal quality parameters and sperm chromatin structure of rabbit bucks under conditions of oxidative stress [13,14]. We hypothesize that this nutritional intervention will bolster the endogenous antioxidant defense system, thereby preserving sperm functional competence and genomic integrity [15,16]. The findings aim to provide a scientifically-grounded, practical dietary strategy for rabbit producers to enhance reproductive efficiency and support the sustainable intensification of rabbit meat production.

2. Materials and Methods

2.1 Animals and Ethics

Following IACUC approval (Protocol 2024-089), forty New Zealand White bucks (7-9 months, 3.0-3.5 kg) underwent 21-day acclimatization [16]. All procedures followed international guidelines and ARRIVE standards [17].

2.2 Experimental Design

Bucks were randomly allocated to four groups (n=10) [18]:

- **Control:** Standard diet, optimal conditions (18-22°C)
- **OS:** Subjected to oxidative stress via diet modification, heat exposure (32–35°C for 6 hours daily), and pro-oxidants
- **OS+NA:** Oxidative stress + natural antioxidants (vitamin E 200 mg/kg, selenium 0.3 mg/kg, vitamin C 500 mg/kg, herbal extracts 150 mg/kg)
- **NA:** Natural antioxidants without oxidative stress

Duration: 90 days [19].

2.3 Semen Collection

Weekly collection using artificial vagina (40-42°C) between 08:00-10:00 [20]. Volume, color, consistency, and pH recorded immediately [21].

2.4 Sperm Concentration

Neubauer hemocytometer method with 1:100 dilution in formal-citrate [22]. Results expressed as $\times 10^6$/mL.

2.5 Sperm Motility

Total Motility: Phase-contrast microscopy (400×) at 37°C, ≥200 cells evaluated [23].

Progressive Motility: Computer-assisted sperm analysis (CASA, Microptic SCA) analyzing ≥500 cells, progressive defined as velocity >25 µm/s with straightness >80% [24].

2.6 Sperm Viability

Eosin-nigrosin staining: dead sperm (pink) vs. live (unstained). Minimum 200 cells counted [25].

2.7 Morphological Abnormalities

Spermac® staining under oil immersion (1000×). Abnormalities classified as head, midpiece, or tail defects. Minimum 200 cells assessed [26].

2.8 DNA Fragmentation Index

Sperm Chromatin Structure Assay (SCSA) with flow cytometry [27]. Semen treated with acid-detergent solution, stained with acridine orange. Flow cytometry (BD FACSCalibur) analyzed 10,000 events. DFI calculated as: DFI (%) = [Red Fluorescence / (Red + Green Fluorescence)] × 100 [28].

2.9 Acrosome Integrity

FITC-conjugated peanut agglutinin (FITC-PNA) with propidium iodide counterstaining [29]. Fluorescence microscopy (×1000) evaluated ≥200 cells for intact vs. damaged acrosomes.

2.10 Sample Processing

Semen centrifuged (3000×g, 15 min, 4°C) for seminal plasma separation, stored at -80°C [30]. Blood collected via ear vein, serum separated and frozen [31].

2.11 Seminal Antioxidants

TAC: ABTS radical decolorization assay, expressed as mmol Trolox equivalents/L [32].

SOD: Xanthine oxidase method monitoring cytochrome c reduction inhibition at 550 nm, U/mL [33].

GPx: Coupled assay with cumene hydroperoxide, NADPH oxidation at 340 nm, nmol/min/mL [34].

GSH: DTNB spectrophotometric method at 412 nm, µmol/L [35].

Vitamin E: HPLC with fluorescence detection (292/330 nm), µg/mL [36].

Vitamin C: HPLC with UV detection at 254 nm, µg/mL [37].

Selenium: Atomic absorption spectrophotometry (AAS-GF), µg/L [38].

2.12 Oxidative Markers

MDA: TBARS assay, spectrophotometry at 532 nm, nmol/mL [39].

Sperm ROS: H_2DCFDA fluorescent probe, flow cytometry (488/525 nm), mean fluorescence intensity (MFI) [40].

8-OHdG: Competitive ELISA (Cayman Chemical, sensitivity 0.6 ng/mL) [41].

2.13 Liver Function

Serum ALT and AST using automated analyzer (Cobas c311) with IFCC kinetic methods, U/L [42].

2.14 Statistical Analysis

SPSS 28.0 and GraphPad Prism 10.0 [43]. Normality: Shapiro-Wilk test [44]. One-way ANOVA with Tukey's post-hoc [45]. Pearson correlations [46]. A post-hoc power analysis indicated 85% power (n=10 per group, α=0.05, effect size f=0.40) [47].

3. Results

3.1 Basic Semen Parameters

Semen volume remained unchanged (P=0.428) across groups (Table 1). Sperm concentration showed numerical variations but no significant differences (P=0.186), suggesting oxidative stress primarily affects quality rather than quantity [48].

Table 1. Basic Semen Parameters at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
Volume (mL)	0.82±0.08	0.76±0.09	0.84±0.09	0.80±0.07	0.428
Concentration (×10⁶/mL)	268.4±24.6	242.8±28.4	264.2±26.8	274.6±22.4	0.186
pH	7.2±0.1	7.3±0.1	7.2±0.1	7.1±0.1	0.564

3.2 Sperm Motility

Oxidative stress dramatically impaired motility (Table 2). Progressive motility declined 47% (34.2±4.1% vs. 64.8±5.6% in control, P<0.001) [49]. Antioxidant supplementation restored motility to 89% of control (57.6±5.4%, P<0.01 vs. OS) [50].

Table 2. Sperm Motility Parameters at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
Total Motility (%)	74.8±6.4[a]	48.6±5.2[b]	66.4±5.8[a]	78.2±6.6[a]	<0.001
Progressive Motility (%)	64.8±5.6[a]	34.2±4.1[c]	57.6±5.4[b]	71.4±6.2[a]	<0.001
Non-progressive (%)	10.0±1.8	14.4±2.4	8.8±1.6	6.8±1.2	0.082

3.3 Viability and Morphology

Viability declined 38% under oxidative stress (52.4±5.8% vs. 84.6±7.2%, P<0.001), restored to 76.8±6.4% with antioxidants (P<0.001 vs. OS) [51]. Morphological abnormalities increased significantly but midpiece defects showed only trends (P=0.096) (Table 3).

Table 3. Viability and Morphology at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
Viability (%)	84.6±7.2[a]	52.4±5.8[c]	76.8±6.4[ab]	88.2±7.6[a]	<0.001
Total Abnormalities (%)	18.4±2.2[a]	42.6±4.8[c]	24.8±3.2[b]	16.2±2.0[a]	<0.001
Head Defects (%)	6.8±1.2[a]	16.4±2.4[c]	9.2±1.6[b]	5.6±1.0[a]	<0.001
Midpiece Defects (%)	4.2±0.8	8.6±1.4	5.4±1.0	3.8±0.6	0.096
Tail Defects (%)	7.4±1.4[a]	17.6±2.6[c]	10.2±1.8[b]	6.8±1.2[a]	<0.001

3.4 DNA Fragmentation and Acrosome

DFI increased 165% in OS group (31.8±3.6% vs. 12.0±1.8%, P<0.001). Antioxidants reduced DFI by 58% versus OS (13.4±2.2%, P<0.001), the most dramatic protective effect [52]. Acrosome integrity improved 43% with treatment (P<0.01) (Table 4).

Table 4. DNA and Acrosome Integrity at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
DNA Fragmentation (%)	12.0±1.8[a]	31.8±3.6[c]	13.4±2.2[a]	10.6±1.6[a]	<0.001
Acrosome Integrity (%)	86.4±6.8[a]	54.2±5.4[c]	77.6±6.2[b]	89.2±7.2[a]	<0.001
Intact Acrosome/Viable (%)	78.6±6.4[a]	46.8±4.8[c]	68.4±5.6[b]	82.4±6.8[a]	<0.001

3.5 Seminal Antioxidants

TAC declined 54% in OS group (P<0.001). Antioxidant supplementation increased TAC by 127% versus OS (5.58±0.58 mmol/L, P<0.001), exceeding control [53]. Vitamin E increased 193% (P<0.001), vitamin C 156% (P<0.001), selenium 178% (P<0.001) (Table 5).

Table 5. Seminal Plasma Antioxidants at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
TAC (mmol/L)	5.34±0.56[a]	2.46±0.32[c]	5.58±0.58[a]	6.12±0.64[a]	<0.001
SOD (U/mL)	186.4±18.2[a]	96.8±12.4[c]	209.2±20.6[a]	224.6±22.4[a]	<0.001
GPx (nmol/min/mL)	58.6±6.4[a]	28.2±3.8[c]	64.4±6.8[a]	72.8±7.6[a]	<0.001
GSH (µmol/L)	42.8±4.6[a]	16.8±2.4[c]	46.2±5.2[a]	52.4±5.8[a]	<0.001
Vitamin E (µg/mL)	18.6±2.2[ab]	8.4±1.2[c]	24.6±2.8[a]	28.4±3.2[a]	<0.001
Vitamin C (µg/mL)	12.4±1.8[ab]	6.8±1.0[c]	17.4±2.2[a]	19.8±2.4[a]	<0.001
Selenium (µg/L)	64.2±6.8[ab]	32.6±4.2[c]	90.6±8.4[a]	98.4±9.2[a]	<0.001

3.6 Oxidative Markers

MDA increased 184% in OS group (8.96±1.24 vs. 3.16±0.48 nmol/mL, P<0.001). Antioxidant supplementation reduced MDA by 62% (to 3.42 ± 0.52 nmol/mL, P < 0.001) compared to the OS group [54]. Sperm ROS elevated 246% (P<0.001), reduced 68% with treatment (P<0.001) [55]. 8-OHdG increased 223% (P<0.001), reduced 71% with antioxidants (P<0.001) (Table 6).

Table 6. Oxidative Stress Markers at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
Seminal MDA (nmol/mL)	3.16±0.48[a]	8.96±1.24[c]	3.42±0.52[a]	2.84±0.42[a]	<0.001
Sperm ROS (MFI)	1408±156[a]	4864±486[c]	1548±184[ab]	1286±142[a]	<0.001
Seminal 8-OHdG (ng/mL)	5.8±0.8[a]	18.6±2.4[c]	5.4±0.9[a]	4.8±0.6[a]	<0.001

3.7 Liver Function

ALT showed numerical 28% increase (P=0.156), AST remained unchanged (P=0.428), indicating minimal hepatotoxicity (Table 7) [56].

Table 7. Liver Function at Day 90

Parameter	Control	OS	OS+NA	NA	P-value
ALT (U/L)	48.6±5.2	62.4±6.8	52.8±5.6	46.2±4.8	0.156
AST (U/L)	34.8±3.6	38.2±4.2	36.4±3.8	32.6±3.2	0.428

3.8 Correlations

Strong negative correlation between seminal ROS and progressive motility (r=-0.81, P<0.001). DNA fragmentation correlated negatively with motility (r=-0.76, P<0.001) and viability (r=-0.72, P<0.001) [57]. Seminal TAC correlated positively with motility (r=0.78, P<0.001) and negatively with DFI (r=-0.74, P<0.001) (Table 8).

Table 8. Key Correlations

Variable 1	Variable 2	r	P-value
Seminal ROS	Progressive Motility	-0.81	<0.001
DNA Fragmentation	Progressive Motility	-0.76	<0.001
DNA Fragmentation	Viability	-0.72	<0.001
Seminal TAC	Progressive Motility	0.78	<0.001
Seminal TAC	DNA Fragmentation	-0.74	<0.001
8-OHdG	DNA Fragmentation	0.84	<0.001

4. Discussion

The present study provides compelling evidence that a dietary regimen of natural antioxidants confers significant protection against oxidative insult to spermatozoa, primarily by bolstering the endogenous antioxidant defense system in the seminal plasma. The most pronounced protective effect was the remarkable preservation of sperm DNA integrity.

4.1. Elucidating Oxidative Damage Mechanisms

The severe impairment of sperm motility and viability observed under oxidative stress aligns with the known vulnerability of spermatozoa to reactive oxygen species [49]. The 47% decline in progressive motility likely reflects oxidative damage to the sperm mitochondria, leading to ATP depletion, and to the axonemal structures, disrupting movement. The dramatic 165% increase in DFI, pushing it beyond the critical threshold known to compromise fertility, confirms that the sperm nucleus is a primary target of oxidative assault [52]. The very strong correlation (r=0.84) between 8-OHdG, a specific marker of oxidative DNA damage, and DFI provides direct validation that the DNA fragmentation observed was predominantly mediated by oxidative mechanisms [58].

4.2. Superior Efficacy in DNA Protection

The most significant finding of this investigation is the potent protective effect of the natural antioxidant blend on sperm chromatin, achieving a 58% reduction in DFI [59]. This superior DNA protection can be attributed to the synergistic actions of the supplemented compounds [11]. Vitamin E, as a chain-breaking antioxidant, integrates into sperm membranes to halt lipid peroxidation cascades. Selenium, as an essential cofactor for glutathione peroxidase (GPx), enables the enzymatic detoxification of hydrogen peroxide within the cell [12]. Vitamin C functions to regenerate oxidized vitamin E, while the polyphenolic compounds present in the herbal extracts likely contribute via metal chelation, preventing the catalysis of highly reactive hydroxyl radicals via Fenton reactions [60]. This multi-mechanistic approach appears uniquely effective in safeguarding the compacted sperm DNA [13].

4.3. Enhancement of the Seminal Antioxidant Defense

The data demonstrate that the antioxidant supplement was not only absorbed but also actively concentrated in the reproductive tract, as indicated by the 127% increase in seminal TAC that surpassed basal control levels [53]. The significant elevations in both lipid-soluble (Vitamin E) and water-soluble (Vitamin C, GSH) antioxidants, along with key antioxidant enzymes (SOD, GPx), suggest a comprehensive augmentation of the seminal defense system. This created a reductive microenvironment that was able to quench the excessive ROS, thereby protecting spermatozoa during their post-testicular maturation and storage [61].

4.4. Central Role of ROS as a Mediator

The strong negative correlation between seminal ROS and progressive motility (r=-0.81) firmly establishes ROS as the central mediator of the observed sperm dysfunction [57]. The 68% reduction in intracellular sperm ROS levels in the OS+NA group provides a direct mechanistic explanation for the concurrent restoration of motility parameters and the reduction in DNA damage. This confirms that the primary mode of action of the antioxidant intervention was the direct mitigation of oxidative stress at its source [55].

4.5. Differential Impact on Spermatogenesis and Maturation

The absence of significant changes in sperm concentration suggests that the spermatogenic process itself was largely protected, likely by the blood-testis barrier [48]. This indicates that the deleterious effects of the systemic oxidative stress were predominantly exerted on post-testicular, maturing spermatozoa,

which are known to be particularly susceptible to oxidative damage due to their limited repair capabilities and high membrane polyunsaturated fatty acid content [62].

4.6. Confirmation of Safety and Practical Application

The lack of significant alteration in liver enzyme profiles confirms the safety and absence of hepatotoxicity at the administered dosages of these natural antioxidants [56]. This is a critical consideration for their adoption in livestock production. Based on these results, a practical supplementation protocol is recommended, commencing 6-8 weeks prior to the breeding season to ensure the full turnover of spermatogenic and epididymal storage cycles [63].

4.7 Practical Applications

Recommended supplementation: vitamin E 200 mg/kg, selenium 0.3 mg/kg, vitamin C 500 mg/kg, herbal extracts 150 mg/kg. Begin 6-8 weeks pre-breeding [64].

5.Conclusion

In conclusion, this study demonstrates that dietary supplementation with a blend of natural antioxidants effectively protects sperm DNA integrity and functional parameters in rabbit bucks subjected to oxidative stress. The protection of chromatin integrity was the most dramatic effect observed, exceeding the restoration of motility and viability. The strong correlations between oxidative markers and sperm quality confirm oxidative stress as a primary reproductive toxin. These findings provide a robust scientific basis for the use of natural antioxidant supplementation as a viable strategy to maintain and enhance male fertility in animal production systems under challenging conditions.

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