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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 case of rabbit meat production, the reproductive performance of buck is one of the most critical determinants of herd expansion and genetic progress [2]. However, modern intensive rearing systems frequently subject animals to a variety of environmental, nutritional and metabolic challenges which can lead to oxidative stress - a state of imbalance between the generation of reactive oxygen species (ROS) and the capacity of the biological system to detoxify them [3]. In the case of male reproduction, this imbalance is especially dangerous, because spermatozoa are especially susceptible to oxidative damage because of their high polyunsaturated fatty acid content and relatively low antioxidant defenses [4].

The consequences of oxidative stress to semen quality are far-reaching, resulting in impaired sperm motility, decreased membrane integrity and most importantly, nuclear DNA fragmentation [5]. This degradation of sperm DNA integrity not only affects fertilization rates but also has an impact on embryo development and subsequent long-term health of the resulting offspring and affects the genetic potential and productivity of the herd [6]. Consequently, development of nutritional strategies to reduce oxidative damage is an important goal in advanced animal nutrition which is in line with the industry's objectives for improved productivity, sustainability and animal welfare [7].

The use of synthetic antioxidants in animal feed has been common, but increasing consumer demand for natural and clean-label products has changed research interest to natural alternatives [8]. This trend is an opportunity for the food industry to valorize plant-derived extracts and food grade antioxidant compounds (e.g. vitamin E, vitamin C and selenium) that can be re-used from the human food chain or its co-products [9]. Incorporation of these bioactive compounds into animal feed is part of a synergistic approach to enhanced animal health and product quality and meets the market-driven preferences [10-12].

Therefore, the present study examines the effectiveness of a specific dietary regime containing natural food industry relevant antioxidants such as vitamin E, organic selenium, vitamin C and standardized herbal extracts to assess the effect on seminal quality parameters and the sperm chromatin structure of the bucks of rabbit (Oryctolagus cuniculus), under

conditions of oxidative stress [13,14]. We hypothesize that this nutritional intervention will enhance the endogenous antioxidant defense system that will maintain sperm functional competence and genomic integrity [15,16]. The overall findings are aimed at giving rabbit producers a scientific based practical approach to diet to improve reproductive efficiency and contribute to sustainable intensification of rabbit meat production.

2. Materials and Methods

2.1 Animals and Ethics

After IACUC approval (Protocol 2024-089), 21-day acclimatization of 50 New Zealand White bucks (7-9 months, 3.0-3.5 kg) was done [16]. All the procedures were based on 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

Artificial vagina (40-42degC) Weekly collection between 08.00-10.00 [20]. Immediately, volume, color, consistency, and pH were determined [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

There was no difference in volume of the semen (P=0.428) among groups (Table 1). The concentration of sperm had numerical deviations but no significant differences (P=0.186), therefore oxidative stress mainly influences the quality and not the amount [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 (×10^{6}/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 highest safeguarding action was the stunning maintenance of sperm DNA integrity.

4.1. Elucidating Oxidative Damage Mechanisms

The drastic alteration of sperm motility and viability during oxidative stress conditions can be compared with the susceptibility of spermatozoa to reactive oxygen species known [49]. The 47 percent reduction in the progressive motility is probable to be due to the oxidative harm to the sperm mitochondria, resulting in ATP depletion, and to the axonemal structures, and impairment of movement. The fact that the dramatic rise in the level of DFI of 165 percent took the level to bypass the critical level of DFI that is known to affect fertility is a confirmation that the sperm nucleus is one of the main targets of oxidative attack [52]. The direct confirmation that the DNA fragmentation was mainly mediated by oxidative pathways is the very high correlation (r=0.84) between 8-OHdG, a specific marker of oxidative damage of the DNA, and DFI [58].

4.2. Superior Efficacy in DNA Protection

The most notable result of this study is the strong protective action of the natural antioxidant blend on sperm chromatin whereby a reduction of 58 per cent was obtained in the DFI [59]. The improved DNA protection is explained by the synergistic effect of the supplemented compounds [11]. The chain-breaking antioxidant (vitamin E) inserts into sperm membranes to prevent the formation of lipid peroxidation cascades. Selenium which is a vital cofactor of glutathione peroxidase (GPx) facilitates the process of enzymatic detoxification of hydrogen peroxide in the cell [12]. Vitamin C acts to restore the oxidized vitamin E and the polyphenolic compounds which are in place. possibly play a role in the herbal extracts, through metal chelation, to inhibit the catalysis of highly reactive hydroxyl radicals by Fenton reactions [60]. This multi-mechanistic technique seems to be the unique method of the protection of the shrinked sperm DNA [13].

4.3. Enhancement of the Seminal Antioxidant Defense

The results prove that the antioxidant supplement was both being absorbed and actively concentrated into the reproductive tract as shown by the 127 percent increase in seminal TAC which was above the level of basal control [53]. The marked increases in lipid-soluble (Vitamin E) and water-soluble (Vitamin C, GSH) antioxidants, as well as, the key antioxidant enzymes (SOD, GPx), indicate the extensive enhancement of the seminal defensive mechanism. This formed a reducing microenvironment capable of suppressing the abnormal ROS and thus guarding spermatozoa under their post-testicular maturation and storage [61].

4.4. Central Role of ROS as a Mediator

The negative correlation between seminal ROS and progressive motility (r=-0.81) is significant to confirm that the mediator of the observed sperm dysfunction is ROS [57]. The 68 percent decrease in intracellular sperm ROS of the OS+NA group also gives a direct mechanistic account to the simultaneous recovery of the motility parameters and the decrease in DNA damage. This proves that the main way of action of the antioxidant intervention was the direct alleviation of oxidative stress at its origin [55].

4.5. Differential Impact on Spermatogenesis and Maturation

The major alterations in the concentration of sperms indicate that the spermatogenic process itself was preserved to a great extent, presumably, by the blood-testis barrier [48]. This signifies that the pernicious impacts of the systemic oxidative stress were mainly executed on post-testicular maturing spermatozoa, that also have a high level of membrane polyunsaturated fatty acids and are known to be especially vulnerable to oxidative damage because of their limited repair ability [62].

4.6. Confirmation of Safety and Practical Application

The fact that there is no remarkable change in the liver enzyme profiles is a confirmation of the security and hepatotoxicity free nature at the dosages of these natural antioxidants [56]. This is a serious fact to consider its adoption in livestock production. According to these findings, an effective supplementation schedule is suggested, which will start 6-8 weeks before the breeding season, to make sure that a complete turnover of spermatogenic and epididymal storage cycles will be achieved [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, the experiment has shown that dietary Supplementation of rabbit bucks with a mixture of natural antioxidants is a good way to protect the integrity of the sperm DNA and other functional parameters in rabbit bucks, who were exposed to oxidative stress. The most astonishing effect of the silencing was the safeguarding of the chromatin integrity, which is more than the recovery of the motility and viability. The oxidative stress is a primary reproductive toxin as the strong correlations of oxidative markers with sperm quality confirm. These results put forward a strong scientific foundation that natural antioxidant supplementation could be a good approach in ensuring male fertility is maintained and improved in animal production systems that are subjected to harsh environments.

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