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Age-Dependent Expression of Humanin in the Bull (*Bos Taurus*) Testis and Its Potential Role in Regulating Hormonal Status, Oxidative Stress and MicroRNA Expression

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A B S T R A C T

Humanin (HN), a mitochondrial-derived peptide with cytoprotective functions, has not been thoroughly investigated in the bull gonad. This study characterized the developmental localization of HN in the bull testis and its relationship with hormonal status, oxidative stress, and key miRNAs. An age dependent HN expression pattern was seen by immunofluorescence examinations of testicular tissues from pre-pubertal, puberty, mature, and aged bulls. So that, pre-pubertal and aged groups showed faint HN immunoreactivity, pubertal animals showed moderate immunoreactivity, and mature adults showed the most intense immune response ($P \leq 0.0001$). In contrast to this, miR-202-5p and miR-21 had their highest expression levels during pre-pubertal, pubertal, and mature testes and a significant decrease in aged bulls ($P \leq 0.0001$). Despite the contrasting developmental trends observed at the group level, a correlation analysis involving all individual animals (n = 40) demonstrated a robust positive association between HN and miR-202-5p levels (r = 0.8196, $P < 0.001$). This finding implies a close regulatory connection between these molecules that functions throughout various developmental stages. Quantitatively, HN levels was correlated positively with serum testosterone (r = 0.5852, $P < 0.01$) and the antioxidant enzyme SOD (r = 0.8208, $P < 0.001$), and negatively with the oxidative stress marker MDA (r = - 0.7140, $P < 0.001$). The current findings show the first developmentally-specific HN expression in the bull testis, which is linked to peak reproductive maturity, hormonal balance, and redox homeostasis. The correlation with miR-202-5p suggests that there is a potential new regulatory network, and that HN is a crucial peptide for testicular function and aging. The results of this study could facilitate additional investigations into the distribution of HN throughout gonadal development, ultimately enhancing the reproductive efficiency of livestock.

Keywords: bull, histology, testes, microRNA, oxidative stress

INTRODUCTION

The principle products of testes are testosterone and spermatozoa, both of which play notable roles in the development and maintenance of various physiological functions, particularly male fertility (**1**). The health and reproductive capabilities of bulls are vital for the sustainability of the global cattle industry (**2**). Aging process adversely affects male fertility by interfering with sperm generation and also functionality through several mechanisms including DNA damage and genetic alterations (**3**). Furthermore, numerous studies across different animal species have demonstrated the age-related alterations in the testes, serum hormonal levels of growth hormone, follicle-stimulating hormones, antioxidant status, and testosterone levels (**4,5**). Recent advancements in

knowledge and analytical techniques have heightened interest in exploring issues related to male fertility and infertility during adolescence process, as well as evaluating the therapeutic potential of whey in alleviating age-related changes in the testes (**5**). Gaining an understanding of the factors contributing to male infertility and subfertility is crucial for the development of new strategies or treatments for their management (**1**).

Humanin (HN) is a signaling peptide which derived from mitochondria and is encoded by mitochondrial genes and was first identified in the early 2000s. It is involved in mediating mitochondrial function and enhancing cell survival and integrity through both intracellular processes and as a secreted factor that affects extracellular pathways. Presently, HN is recognized to be extensively distributed throughout various body tissues including the testes of both humans and laboratory rodents indicating its regulatory roles in multiple physiological signaling (**6-7**). In addition, HN has been detected in several tissue fluids such as seminal plasma, where its levels are positively associated with sperm quality (**8**). Furthermore, HN is crucial for the proper functioning of mitochondria, being encoded by the MT-RNR2 gene found within the mitochondrial genome (**9**). In addition, alterations in HN expression related to aging have been observed in the ovarian tissue of rats (**10**). Prior studies have reported evidence of exogenous HN-like peptides and the role of HN in enhancing the survival of spermatozoa, as well as improving the post-thaw quality of buffalo spermatozoa (**11,12**).

MicroRNAs (miRNAs) are short, non-coding RNA molecules that serve as key regulators of spermatogenesis in mammals. Testicular development and functionality are intricately controlled by microRNAs (miRNAs), which influence the expression of numerous protein-coding genes that play critical roles in the cell differentiation processes of the male reproductive system (**13**). MicroRNAs are crucial tiny regulators expressed in the testis, where they control the genetic programs essential for gametogenesis. Their role in facilitating cell to cell communication is vital for the proper execution of developmental processes underlying male fertility (**14**). MiR-202 acts as a crucial developmental timer in male fertility, preventing the premature differentiation of spermatogonia and meiotic initiation. The loss of miR-202 leads to a depleted stem cell pool and age-dependent infertility thereby demonstrating its essential role in maintaining the proper timing and longevity of spermatogenesis process (**13**). In addition, MiR-202-5p is a testis specific miRNA that tightly regulates various gonadal functions across multiple animal species (**15,16**). It has been recognized as a highly testis enriched gene which affirming its involvement in testicular cell functions (**17**). Evidence from zebrafish models identifies it as a fundamental component of the both male and female germ plasm as well as crucial for the specification and development of primordial germ cells (**15,18**). MiR-202-5p is a critical determinant of embryonic viability because its deficiency causes fatal developmental arrest at the middle blastula transition, triggering massive apoptosis. This demonstrates that proper miR-202-3p expression is essential for early embryogenesis, directly linking its dysfunction to severe fertility issues and embryonic lethality (**19**). Importantly, its role is critical in adult fertility so that in human testes, miR-202-5p is specifically localized to Sertoli cells and is crucial for male fertility, as its expression is dramatically reduced in the testicular tissue of infertile men with a complete absence of germ cells (**20**).

Numerous miRNAs such as miR-21 play a vital protective role in male fertility by functioning as anti-apoptotic regulators within testicular cells thereby averting the death of Sertoli and Leydig cells. The dysregulation of these miRNAs represents a key mechanism by which different stressors and toxins can contribute to male infertility and positioning them as a promising target for future therapeutic interventions (**21,22**). In addition, miR-21-5p is directly implicated in male fertility issues by negatively regulating Leydig cell function as well as it can promotes apoptosis and inhibits proliferation in these cells. Since Leydig cell health is vital for spermatogenesis; therefore, dysregulation of miR-21-5p can impair androgen production and lead to infertility (**23**). The critical gap is the unknown developmental profile of HN in the bull testis and its unexplored relationship with key microRNAs. This study therefore aims to examine HN's age dependent expression and its correlations to genital hormones, oxidative stress, and special testes localized miRNAs to understand its role in testicular integrity and thereby bull fertility.

MATERIALS AND METHODS

Ethical Approval

Experimental protocols complied with ARRIVE guidelines and received ethical approval from the Animal Care and Ethics Committee of Ilam University, Iran (Approval ID: IR.ILAM.REC.1403.048).

Study Design

Testicular tissue from clinically healthy Holstein bulls (*Bos Taurus*) raised in Ilam Province, Iran was used in this study. The samples were transported on ice and processed within 30 minutes of collection.

Animals were obtained from local abattoirs during regular slaughter operations with veterinary assessments verifying the absence of reproductive disorders as well as systemic illnesses in all subjects. All bulls underwent a routine pre-slaughter veterinary examination to confirm they were free from evident systemic diseases. To specifically mitigate potential confounding factors associated with fertility and overall health, the selection for this study was further refined based on abattoir documentation and visual assessment of the reproductive tract. For this study the animals were categorized into four age groups (n = 10 biological replicates/group): pre-pubertal bulls (6 months old with average weight: 220 ± 15 kg), pubertal bulls (11-15 months old with average weight: 380 ± 25 kg), sexually mature adults (36-72 months old with average weight: 750 ± 50 kg), and aged bulls (≥ 96 months old with average weight: 800 ± 45 kg) (**24**). The

categorization of bulls into different age groups was a result of standard herd management and slaughter schedules for beef production rather than individual health or fertility status; thereby facilitating the sampling of a population-representative, healthy cohort across the developmental stages. All animals were raised following standard commercial husbandry practices, which included a diet comprising both concentrate and forage, along with unrestricted access to water. Although detailed individual fertility records and semen analyses were not available for these animals, the reproductive capabilities of all animals except for the pre-puberty group were corroborated through historical data provided by the farmer. Moreover, the selection of animals for this study was carefully refined to exclude bulls with a documented history of chronic illness or those that displayed any visible lesions including orchitis during the postmortem examination of the reproductive system.

Immunofluorescence (IF) Labeling

For IF studies, the right gonads were swiftly excised immediately following slaughter. Testicular tissue samples were then transported in ice-cold phosphate-buffered saline (PBS) and processed within thirty minutes of collection. The tissues were fixed in 4% formaldehyde in 0.1 M PBS for a duration of 24 h at room temperature, subsequently undergoing dehydration through a graded series of ethanol and paraffin embedding. Sections of five microns were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol. Antigen retrieval was conducted in preheated Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 95°C for thirty min, followed by cooling and washes with PBS. To prevent non-specific binding, a blocking solution of 5% normal donkey serum and 0.3% Triton ×-100 in PBS was applied for two hours at room temperature.

A polyclonal rabbit anti-HN antibody (1:200 dilution; MBS9460407, MyBioSource, Inc., San Diego, USA) was used. A bovine-specific HN antibody is not commercially available. The use of this antibody, raised against the human HN peptide, was justified by the 100% amino acid sequence identity of the mature canonical HN peptide between humans and cattle. The specificity of the immunostaining in our bovine tissue was confirmed by the following internal controls: (1) Negative control experiments where the primary antibody was omitted, which resulted in a complete absence of specific immunoreactive signal; and (2) A secondary antibody control, which also showed no signal. The specific, developmentally regulated staining pattern observed provides further validation of the antibody's applicability in this context. Following the washing procedure, the sections were incubated for 2 h with goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor® 488 (1:500; A-21244, Thermo Fisher Scientific) in a blocking solution, shielded from light. To test the specificity of secondary antibody, it was run a secondary control with normal serum from the bovine species, instead of primary antibody. No stained tissue signal was detected after removing the primary antibody. Slides were washed, counterstained with DAPI (1 µg/mL; D1306, Thermo Fisher Scientific) for 5 min, and mounted with ProLong™ Diamond Antifade Mountant (Thermo Fisher, P36970).

Imaging was performed using a fluorescence microscope. For each animal (n = 5 biological replicates per group), five random fields of view were captured at 20× magnification under identical settings. Semi-quantitative analysis of HN immunoreactivity was performed using ImageJ (NIH v1.53t). The analysis was designed to determine the percentage area of the seminiferous tubules that was immunopositive for HN. The data were then expressed as the percentage of immunopositive area per tubule, calculated using the formula: Area of immunopositive pixels / total tubule area) × 100 (**25,26**).

Real-Time PCR

For the molecular analysis, each section of the left testis was rapidly taken and stored at -80°C. The expression levels of miR-202-5p and miR-21 in the tissue were assessed using real-time PCR. Total RNA was extracted from the testicular tissue samples according to the manufacturer's instructions using Invitrogen TRIzol® reagent. RNA purity was evaluated using a Thermo Scientific NanoDrop 2000 spectrophotometer, and A260/A280 ratios between 1.8 and 2.0 were considered acceptable. First-strand cDNA was synthesized from 500 ng of total RNA using the miRcute miRNA First Strand cDNA Synthesis Kit (TianGen, China) under the following conditions: Poly-A tailing at 37°C for 60 min, reverse transcription at 42°C for 30 min, and enzyme inactivation at 85°C for 5 min.

qPCR was performed in a total reaction volume of 25 µL using the miRcute miRNA qPCR Detection Kit (SYBR Green) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Each reaction contained 2× miRcute miRNA Premix (10 µL), cDNA template (2 µL), forward primer (10 µM; 0.4 µL), reverse primer (10 µM; 0.4 µL), ROX Reference Dye (0.4 µL), and RNase-free water (11.8 µL). Amplification was carried out under the following cycling conditions: initial denaturation for one cycle at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The primer sequences are listed in **Table 1**.

Table 1. The list of primers used in this study

Target Name	Type	Sequence (5' to 3')	Note / Source	Accession No
miR-21	Forward Primer	AGCTTATCAGACTGATGTTG	Custom designed, sequence from miRBase	MI0004742
miR-202-5p	Forward Primer	UAGCUUAUCAGACUGAUGUUGACU	Custom designed, sequence from miRBase	MIMAT0009259
Universal Reverse	Reverse Primer	GAACATGTCTGCGTATCTC	Custom designed	-
U6 snRNA	Forward Primer	GTGCTCGCTTCGGCAGCA	NCBI Reference Sequence	NR_004394
U6 snRNA	Reverse Primer	TTTGCGTGTCATCCTTGCG	NCBI Reference Sequence	NR_003027

All reactions were performed in triplicate, and specificity of the reactions was confirmed through melt curve analysis. Two distinct control reactions including lacking cDNA and containing RNA were conducted simultaneously. The Ct values for miRNA were normalized against U6 snRNA. The comparative expression levels of the microRNAs were determined using StepOne™ software version 2.3, which was in accordance with the $2^{-\Delta\Delta CT}$ methodology.

Oxidative Stress Analysis

Superoxide dismutase (SOD) was measured spectrophotometrically following the methodology established by previous researcher (**27**). The levels of malondialdehyde (MDA) were assessed utilizing the thiobarbituric acid reactive substances (TBARS) assay. In summary, 100 µL of testicular homogenate supernatant was mixed with 500 µL of TBARS reagent to avert any artifactual oxidation during the processing phase. This mixture was incubated at 95°C for 60 min in a water bath shielded from light, and subsequently cooled rapidly on ice for 10 min to halt the reaction. After centrifugation at 10,000 × g for 10 min at 4°C to eliminate precipitates, the absorbance of the supernatant was recorded at 532 nm using a microplate spectrophotometer.

Hormonal Profiling Via Radioimmunoassay

Blood samples were collected via jugular venipuncture using serum separating tubes and allowed to clot at room temperature for 30 min prior to centrifugation at 3,000 × g for 15 min at 4°C. Testosterone quantification was performed using the assay genie bovine testosterone enzyme-linked immunosorbent assay ELISA Kit (Assay Genie, Ireland; BOEB1196) validated for bovine species. The assay employs competitive binding between endogenous testosterone and ^{125}I-labeled testosterone to polyclonal rabbit anti-testosterone antibodies immobilized on polypropylene tubes. After 3 h incubation at 37°C, unbound components were decanted, and tube-bound radioactivity was measured for 1 min/tube using a gamma counter.

Serum levels of inhibin B were measured utilizing a commercial ELISA kit (Bovine Inhibin B ELISA Kit, Assay Genie, Dublin, Ireland; BOEB0861). Each assay was conducted in duplicate following the manufacturer's instructions. The optical density was assessed at 450 nm with the aid of a microplate reader.

Luteinizing hormone (LH) concentration was determined using species-specific enzyme immunoassays (Bovine LH (Luteinizing Hormone) ELISA Kit (Assay Genie, Ireland, BOFI00094) according to manufacturer's protocols. In summary, 25 µL of serum samples were incubated in wells coated with antibodies alongside enzyme conjugates for a duration of 60 min and at a temperature of 25°C. This was followed by the development of tetramethylbenzidine substrate for 15 min and an acid stop. The absorbance was recorded at 450 nm utilizing a microplate reader with hormone concentrations derived from sigmoidal standard curves.

Statistical Analysis

The results of this study were evaluated by descriptive statistics to determine measures of central tendency and assess the assumptions of normality. Subsequently, the data of different experimental groups were analyzed using one-way ANOVA test followed by comparison Tukey's post hoc test to identify differences among the means. All variables including hormone levels, oxidative stress amounts, and miRNA expression levels were scrutinized to investigate their relationship with HN quantity through the Pearson or Spearman correlation tests. A *P*-value of less than 0.05 was deemed to indicate significant differences between the groups.

RESULTS

Based on their position, size, and shape; different cell types and associated tissues from the testis organ were identified. Also, the normal and developmentally appropriate architecture of the testicular tissue in the pre-pubertal, pubertal, and mature adult groups were confirmed using H&E prepared sections. These sections showed normal seminiferous tubules with distinct germinal epithelium and a clearly organized interstitial area. In contrast, testicular sections from the aged group exhibited structural alterations including degeneration of various germ cells as well as disruption of seminiferous tubule morphology which implied testicular aging (**Figure 1A-D**).

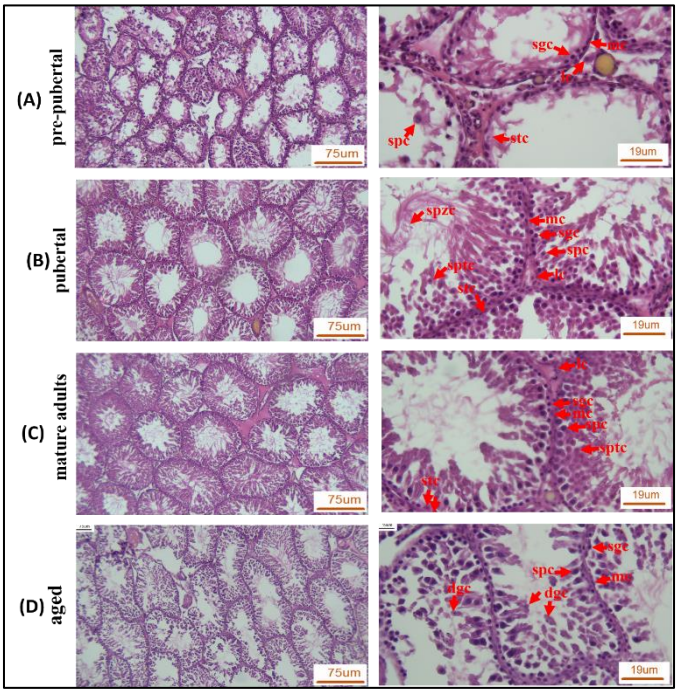

Figure 1. Representative H&E-stained sections of bull testis at different stages. Pre-pubertal **(A)**, pubertal **(B)**, mature adult **(C)**, and aged bulls **(D)**. The malformation of the seminiferous tubules and the number of degenerated cells (dgc) in the seminiferous tubules of the elderly group increased. Magnification: Left sides: 100×; Right sides: 400×. mc: myoid cells, sgc: spermatogonia lc: Leydig cells, spc: spermatocytes , sptc: spermatids , stc: Sertoli cells, spzc: spermatozoa cells

To examine the localization of HN in the bull testis; IF assay was performed on the tissues obtained from different animal groups. Then, the H&E stained serial sections were compared with IF images for aid appropriate identification of testicular cell types based on morphological criteria (**Figure 2**). The results show faint HN immunoreactivity in the pre-pubertal and aged bulls. In this groups, the HN immunoreactivity signals were observed in cells located within the seminiferous tubules morphologically consistent with various germ cell populations and as well as in the interstitial testicular compartments (**Figure 2B and E**).

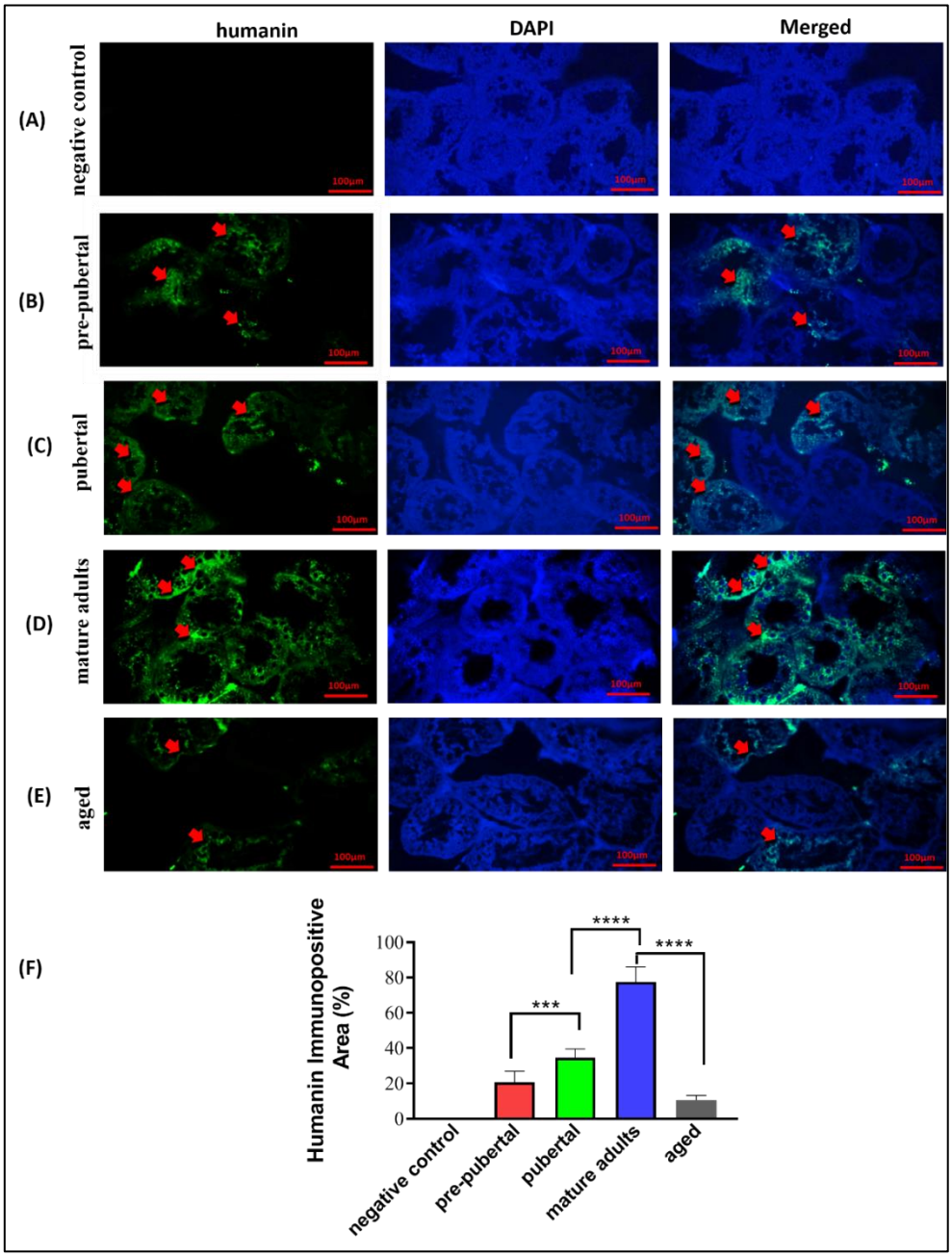

Figure 2. Immunofluorescence localization of humanin in the testes of negative control **(A)**, pre-pubertal **(B)**, pubertal **(C)**, mature adult **(D)**, and aged bulls **(E)**. The arrows indicate humanin immunoreactivity. Scale bar: **(A-E)** = 100 µm. Magnification: 100×. **(F)**, Quantification of humanin immunoreactivity in different components and cellular population in both intra-tubular germ cells and interstitial parts of the bull testes, presented as the mean percentage of the immunopositive area per field of view (±SEM). $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ indicates significant differences between the designated groups

Accordingly, a faint to moderate HN immunoreactivity signals were observed in the both intra tubular and interstitial compartments of pubertal animal group. In addition, specific HN immunoreactivity was not found in the negative control sections where the primary antibody was omitted. This finding confirmed the specificity of the staining protocol (**Figure 2C**). In testicular tissue from mature adult bulls, a persistent and bright HN

immunoreactivity signal was observed in the compartments of cells within the tubules and interstitium (**Figure 2D**). To semi-quantitatively evaluate these observational findings, the density of the HN immunoreactivity signal was measured across age groups. Quantitative analysis confirmed the highest HN immunoreactivity signal in mature adult testes ($P < 0.001$; **Figure 2F**). Pre-pubertal and aged bulls exhibited a significantly lower immunopositivity for HN relative to the strong signal in mature adults. Pubertal animals exhibited a moderate level of HN immunoreactivity, which was statistically greater than in the pre-pubertal group ($P \leq 0.001$) and less than in the mature adults ($P < 0.001$; **Figure 2F)**

Serum testosterone levels were found to be moderate in both pre-pubertal and pubertal groups, significantly low in aged bulls, and exhibited a dramatic increase in mature groups ($P < 0.001$; **Table 2**). Conversely, serum concentrations of inhibin B were highest in the pre-pubertal group and showed a significant decline with increasing age ($P < 0.001$). Regarding of serum LH levels; no significant differences were found between the various groups; nevertheless, there was a rise in the serum LH levels in the adult and elderly animal groups but did not reach statistical significance. Compared to other groups; the MDA levels were significantly higher in the testes of aged bulls ($P < 0.001$; **Table 2**). Also, SOD levels showed an inverse pattern with the highest concentrations observed in the pre-pubertal, pubertal, and mature groups, in contrast to aged animals ($P < 0.001$; **Table 2).** On the basis of quantitative real time PCR analysis; both miR-202-5p and miR-21 were highly expressed in pre-pubertal testes, but their levels significantly decreased with sexual maturation ($P < 0.001$; **Table 2**).

Serum testosterone level was positively associated with HN levels (r = 0.5852, $P < 0.01$). Notably, testicular HN levels exhibited a negative correlation with MDA (r = -0.7140, $P < 0.001$) and a positive correlation with SOD activity (r = 0.8208, $P < 0.001$). Interestingly, correlation analysis uncovered a positive relationship between HN and miR-202-5p (r = 0.8196, $P < 0.001$) (**Figure 3**).

Table 2. Serum reproductive hormones, testicular oxidative stress markers, and testicular miRNA expression across age groups in Holstein bulls (*Bos taurus*)

Parameters	Pre-Pubertal	Pubertal	Mature adults	Aged	*P*-value
Testosterone (nmol/L)	2.39 ± 0.11 [a]	2.35 ± 0.09 [a]	4.90 ± 0.22 [b]	1.80 ± 0.18 [c]	<0.001
Inhibin B (ng/mL)	4.06 ± 0.13 [a]	1.20 ± 0.10 [b]	1.19 ± 0.11 [b]	1.09 ± 0.05 [b]	<0.001
Luteinizing hormone (mU/L)	2.78 ± 0.17 [a]	2.86 ± 0.12 [a]	3.05 ± 0.20 [a]	3.00 ± 0.11 [a]	<0.001
Malondialdehyde (nmol/mg)	0.12 ± 0.04 [a]	0.22 ± 0.07 [a]	0.51 ± 0.12 [a]	4.02 ± 0.22 [b]	>0.05 (NS)
Superoxide dismutase (U/mg)	3.65 ± 0.19 [a]	3.75 ± 0.07 [a]	3.31 ± 0.17 [a]	1.23 ± 0.24 [b]	<0.001
miR-202-5p (fold change/U6)	3.95 ± 0.10 [a]	3.92 ± 0.14 [a]	3.77 ± 0.19 [a]	1.74 ± 0.26 [b]	<0.001
miR-21 (fold change/U6)	2.73 ± 0.16 [a]	2.72 ± 0.13 [a]	2.54 ± 0.20 [a]	0.77 ± 0.19 [b]	<0.001

Values are expressed as mean ± SEM. Within each row, means with different superscript letters ([a]–[c]) differ significantly ($P < 0.05$). NS = not significant

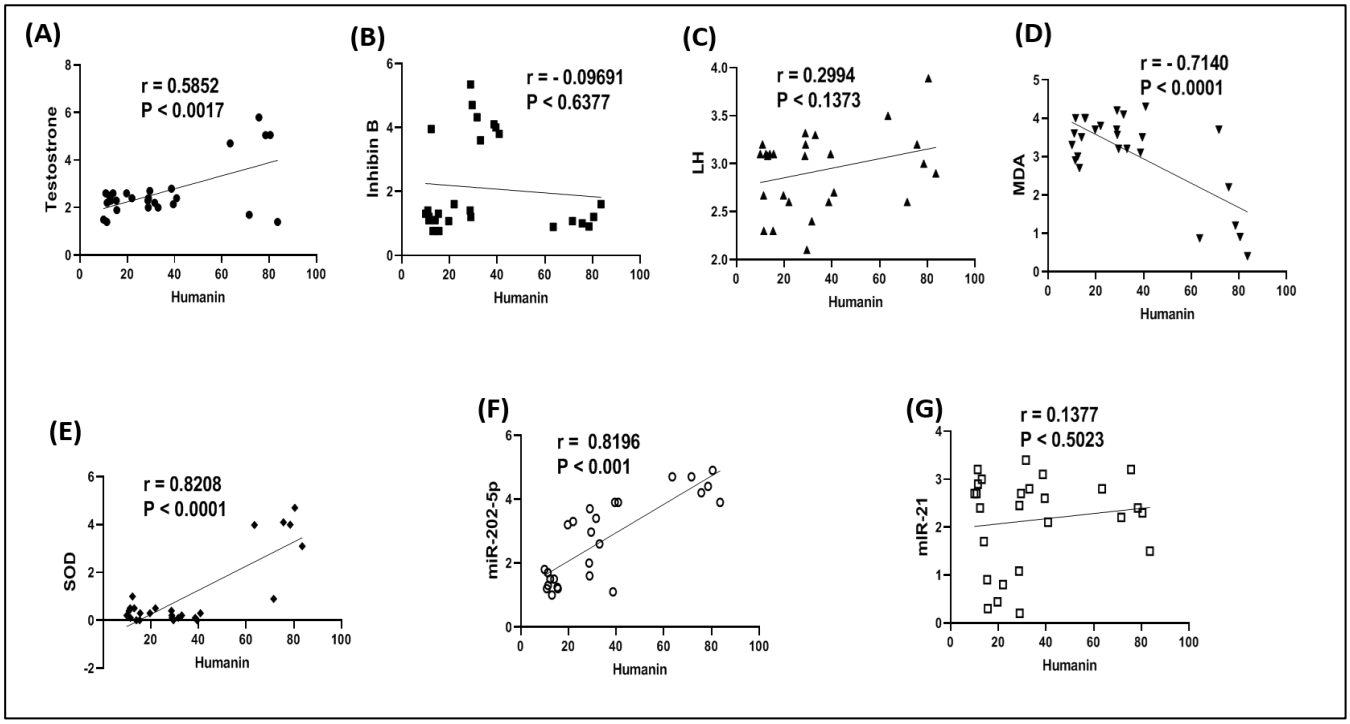

Figure 3. Scatterplots showing the Pearson's (r) correlation indices between the different parameters (Y-axis) and humanin levels (X-axis) in various aged groups of bulls. Each dot corresponds to one testicular sample

DISCUSSION

The current study provides the first tissue analysis of the HN in the bull testis and revealed a dynamic regulating expression pattern that was associated with endocrine profile, redox homeostasis, and post transcriptional signaling. The finding shows that HN expression is minimal in the pre-pubertal testis, peaks during sexual maturity, and declines significantly with advanced age. The faint HN signaling in the pre-pubertal testes was coincides with a quiescent spermatogenic state, which is consistent with previously published researches in murine models where HN expression increases alongside the initiation of spermatogenesis (**28,29**). This finding suggests that HN is not a mere constitutive housekeeping protein, but a functionally peptide involved in supporting testicular maturation and maintaining adult spermatogenic function. The IF signaling observed in the testicular cells of mature adults' animal positions HN as a key cytoprotective factor within the testis organ during the process of spermatogenesis. This result is in line with the previously established cytoprotective roles for HN, which is known to mitigate apoptosis and oxidative stress in the various tissues (**30-32**).

The testicular metabolic activity during spermatogenesis generates high amount of reactive oxygen species (**33**). In the present study it showed a high HN immunoreactivity in mature testes. Thus, it likely represents a critical endogenous defense mechanism to maintain cellular homeostasis and ensure genomic integrity during spermatogenesis process. The observed decline in the HN immunoreactivity in the aged testicular tissue is particularly intriguing and may mirror age related reductions in HN observed in other mammalian systems such as the human and rodent models (**34,35**). This profile of HN signaling could potentially contribute to the age associated increase in the testicular oxidative stress and apoptosis which leads to diminished spermatogenic efficiency (**35**). The significant positive correlation between testicular HN levels and the concentrations of testosterone suggests a potential regulatory relationship. It has been suggested that androgens are promotor of mitochondrial dynamics and Leydig cell functionality in the aging rat models (**36**).

The HN as a mitochondrially encoded peptide could protect Leydig cells against pathological insults in the mature adult's testis. This is supported by the intense HN immunostaining which was observed in the interstitial components of seminiferous tubules. Conversely, the significant decline in the inhibin B levels from pre-pubertal to aged groups aligns with the established role of inhibin B as a marker of spermatogenic activity (**37**). In the present study, the parallel decline in inhibin B and HN in the aged testes implies that HN may be a functional component of a healthy and actively spermatogenic testicular environment.

The compelling evidence for HN's protective role in the testis integrity comes from the fact that by damaging to mitochondrial genomes, reactive oxygen species influence sperm mitochondrial activities and reduced energy production. This is consistent with the well-known role of HN in the other tissues where it directly interacts with stabilizes key antioxidant enzymes like SOD and as well as suppresses mitochondrial ROS production (**29,38**). The elevated MDA level, diminished SOD activity coinciding with the decreasing of HN expression in the aged testes could imply that the age-related decline in HN is a causative factor in the collapse of antioxidant defenses. This creates a permissive pathological environment for oxidative damage to cell components and ultimately promoting dysfunction of germ cells (**30,35**). Therefore, the robust expression of HN during sexual maturity which showed in the current work may acts as a critical shield against the oxidative challenge during spermatogenesis.

In the present study, a new layer of complexity was added by the examining the expression patterns of miR-202-5p and miR-21. Our results show a high level of both microRNAs in pre-pubertal testes which drops off as animal aged, this finding is in accordance with their proposed roles in early testicular differentiation and germ cell maintenance (**39,40-41**). Since miR-202-5p acts as repressors of gene expression, the positive correlation between miR-202-5p and HN protein levels seems counterintuitive. So, we propose two hypotheses to resolve this finding. First, miR-202-5p may indirectly promote HN accumulation by targeting a specific transcriptional repressor of nuclear-encoded mitochondrial factors. Second, the parallel finding may result from co activation of HN and miR-202-5p by a common upstream regulator like the hormonal shifts during puberty process which independently drive both miR-202-5p expression and the metabolic activity, leading to HN synthesis. This intriguing correlation highlights the complexity of post-transcriptional regulation in the testis and may warrants further mechanistic examinations. On the other hand, the decline of miR-21 expression in the face of significant oxidative stress in the aged testes is also notable because miR-21 is typically stress-inducible (**21**). This result may suggest a potential breakdown in this protective feedback loop with aging process which may be associated to the overall decline in testicular homeostasis.

The positive correlation between HN and the level of miR-202-5p presents an apparent paradox, given the principle role of miRNAs in the post transcriptional repression pathways. However, a direct interaction is predictable by the nature of HN as a mitochondrial derived peptide, not as a nuclear mRNA transcript. This correlation can be explained by their involvement in the co-regulated physiological process such as spermatogenesis. To explore probable indirect mechanisms, we performed a bioinformatic analysis using miRPathDB. The results revealed a significant association between miR-202-5p and key available pathways like MAPK and mTOR signaling. On the other hand, miR-21-5p was associated to FoxO and p53 signaling. Notably, these target pathways are vital to the cell survival, proliferation, and metabolic homeostasis which the later are primary targets of HN's cytoprotective action. Therefore, it is plausible that to coordinately reinforce cytoprotective state within the testis, developmental

signals driving peak testicular activity independently upregulate by both HN and miR-202-5p. Similar indirect co-regulatory relationships between miRNAs and proteins have been documented in the other biological systems (**42**). Consequently, we suggest that HN and miR-202-5p do not act within a linear pathway; instead, they work as parallel, synergistic elements within a cohesive network that is essential for preserving testicular homeostasis.

In conclusion, our findings demonstrate a comprehensive animal model in which testicular HN expression was developmentally programmed and finely tuned by reproductive hormones and oxidative stress signals. Due to state of oxidative damage in the ageing that compromises spermatogenesis; the decline of HN level in the aged animals that demonstrated in the current study appears to be a pivotal event in testicular senescence. In addition, the relationship between HN and miR-202-5p may open a new avenue into the post transcriptional control of mitochondrial peptides. While further mechanistic studies are needed to establish causality, the current data significantly establish HN as a principle player in the testicular physiology. These insights position HN as a potential biomarker for male fertility status and a promising therapeutic target for mitigating age-related testicular insufficiency.

The primary limitation of this study is its descriptive nature which cannot establish a causal relationship between HN expression and the observed physiological alterations. Moreover, since the results are derived from a singular species and a comparatively limited sample size, the applicability of the conclusions could restrict. Another limitation is that the aged bulls, while selected for the absence of overt clinical disease, may have had subclinical age-related conditions. Therefore, while the decline in HN level is associated with aging process; we cannot definitively rule out the contribution of underlying age associated health issues to this observed decrease. Finally, the precise molecular mechanisms, the receptors, and the downstream signaling pathways through which HN exerts its effects within the testicular microenvironment are still unresolved.

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N/A

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing: ARG.; Writing – Review & Editing, Investigation, Resources: ALM. All authors have read and approved the final version of the manuscript.

ARTIFICIAL INTELLIGENT DECLARATION

The authors declare that they are responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and it is not used as a co-author.

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