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Oviductal Sperm Storage Activates the NRF2 Antioxidant Pathway in *Mauremys reevesii*

El Almacenamiento de Esperma en el Oviducto Activa la Vía Antioxidante NRF2 *en Mauremys reevesii*

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SUMMARY: Most reptiles can store sperm for subsequent use. After mating, sperm stored in the female reproductive tract experience oxidative stress due to limited cytoplasmic volume, which restricts antioxidant enzyme storage, and sperm are sensitive to reactive oxygen species (ROS), potentially activating the nuclear factor erythrocyte 2 related factor 2 (NRF2) pathway in response. This study aimed to investigate the role of the NRF2 pathway during oxidative stress in the oviduct of *Mauremys reevesii*. We evaluated the expression of NRF2-related genes (*NRF2, HO1, NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX3, GPX4*), the activities of antioxidant enzymes Catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD), and the localization of the NRF2 protein by fluorescence quantification, enzyme assays, and immunofluorescence. Hematoxylin-eosin staining revealed an amount of sperm in the uterus and isthmus, and only a little sperm in the vagina. qRT-PCR indicated that after fertilization ($P<0.05$), *NRF2* and its downstream genes showed reduced expression except for *SOD3* and *GPX3* on day one, with increased expression on day seven, and after one month. Enzyme activity assays indicated that after fertilization ($P<0.05$), one day the activities of SOD, CAT, and GPX were markedly reduced; seven days the activities of CAT and GPX were significantly reduced; were inconsistent with mRNA; one month all of the enzyme activities were increased. Immunofluorescence revealed increased NRF2 protein intensity in fertilized turtles. These results suggest that activation of the NRF2 pathway enhances the expression of NRF2-related genes in the oviduct, resulting in a protective effect on sperm storage in *M. reevessii.*

KEY WORDS: *Mauremys reevesii*; Oviduct; Sperm storage; Antioxidant pathway; Nuclear factor-erythroid 2-related factor 2.

INTRODUCTION

The storage of sperm in the female reproductive tract can contribute to post-mating sperm quality selection and the co-evolution of male and female reproductive systems in animals that undergo asynchronous mating and ovulation (Holt, 2011; Orr & Brennan, 2015; Wen *et al*., 2020; Degueldre & Aron, 2022). The storage of sperm in the female oviduct as a breeding strategy has been demonstrated in many species other than reptiles, including mammals, birds, and insects (Han *et al*., 2019; Shankar *et al*., 2022). However, the duration of sperm storage varies between animals, and some species of turtles and snakes have been reported to store sperm for four or even seven years (Brillard, 1993; Holt, 2011). Some studies have suggested that the microenvironment of the female reproductive tract may influence how long sperm can be stored and reported that the microenvironment of the female tract has a protective effect on sperm during storage by reducing the harmful effects of ROS on sperm, reducing the risk of attack from the female immune system and microbial infection, and inhibiting the expression of apoptotic genes (Cai *et al*., 2021). The addition of antioxidants, such as antioxidant enzymes and vitamin C, to prolong sperm preservation is common when preserving sperm *in vitro* (Keogh *et al*., 2017; Kumar *et al*., 2019; Hakim Abed Bresm & Mohammed Hassan Habeeb, 2023).

The nuclear factor erythrocyte 2 related factor 2 (NRF2) pathway is the most important pathway in terms of providing resistance to oxidative stress when cells are attacked by reactive oxygen species (ROS) (Zhang, 2020). The positive effects of the NRF2 signaling pathway on the stress caused by ROS has been validated in *NRF2* knockout

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mice (Chen *et al*., 2012; Cheung *et al*., 2012). In reptiles, studies have found that CAT, a gene that acts downstream of NRF2, was expressed at high levels in vessels of various oviduct segments in the turtle *Pelodiscus sinensis*, especially in sperm storage tubules (SSTs) in the uterus (Liu *et al*., 2016). The molecular mechanisms responsible for why turtle sperm can be stored in the oviduct for tens of days or even years, and what mechanisms are in place in the oviduct to protect sperm from or minimize ROS attack on sperm, are not yet clear. Although the NRF2 pathway plays an important role in inducing the antioxidant response, it is still unclear how the turtle oviduct regulates antioxidant levels to protect sperm from oxidative stress. Therefore, it is important that we investigate major antioxidants in the oviducts of turtles.

In the present study, we investigated the expression of NRF2 pathway-related genes in *M. reevesii* by fluorescence quantitative PCR and detected changes in the activity of major antioxidant enzymes (CAT, SOD, GPX) after various durations of sperm storage. The localization patterns of NRF2 protein was assessed by immunofluorescence. Our aim was to investigate the regulatory process of antioxidant levels in the oviduct during sperm storage in *M. reevesii* and to determine the association between the NRF2 pathway and sperm storage.

MATERIAL AND METHOD

Specimen and tissue collection

In this study, six mate adult female turtles, weighing 700 - 900 g each, were purchased from Xiaolaohai Farm in Wuhu City, Anhui Province, China. All six turtles were healthy and free of disease. The turtles were euthanized by the inhalation of 100 % CO_2. Oviduct tissues (vagina, uterus, isthmus) were collected, fixed in 4 % paraformaldehyde at 4°C overnight, paraffin-embedded, sectioned, and stained with H&E. The storage of spermatozoa in the three sites was observed by light microscopy. Unmated female and male *M. reevesii* were obtained from the Wuhu Turtle Farm in Anhui Province, China. All turtles were healthy, sexually mature, and of a similar size and weight. There were six male turtles; following euthanasia, we collected the epididymis and testes from male turtles; 2 mL of semen was extracted and diluted with 1 mL of PBS. The female turtles were divided into two groups (experimental and controls): 12 turtles in each of the experimental and control groups of unmated turtles, and 4 turtles as a replicated group. The experimental turtles received 1mL diluted sperm in the cloacal cavity, while controls received 1mL of PBS. Both groups were observed on day one, day seven, and one month post-injection, with tissue samples taken from the vagina, uterus, and isthmus. Tissues from experimental and control groups were used for immunofluorescence, RNA extraction with qPCR analysis, and enzyme activity tests. Total RNA was also extracted and stored at -80°C. The detailed experimental groups are shown in Table I. All procedures involving animals and animal care were approved by the Animal Care and Use Committee of Anhui Normal University (approval Number: AHNU-2022035).

Light microscopy. Fresh tissues were fixed with 4 % paraformaldehyde (Boster), dehydrated in a gradient series of alcohol concentrations, embedded in paraffin, and serially sectioned (5mm-4mm). Sections were then dewaxed with xylene, hydrated with a descending series of alcohol concentrations, stained with hematoxylin and eosin (HE), and observed under a light microscope (Olympus BX61). Images were captured by a camera (Olympus DP71).

Table I. Breakdown of experimental materials grouping.

Groups \ Stage	One day		Seven days		One month		Main application
	Sample	Amount	Sample	Amount	Sample	Amount	
Unmated female turtles Experimental	Vagina	4	Vagina	4	Vagina	4	Used for Total RNA extraction, q-RTPCR, Enzyme viability assays, NRF2 Immunofluorescence
	Uterus	4	Uterus	4	Uterus	4	
	Isthmus	4	Isthmus	4	Isthmus	4	
Unmated female turtles Control groups	Vagina	4	Vagina	4	Vagina	4	
	Uterus	4	Uterus	4	Uterus	4	
	Isthmus	4	Isthmus	4	Isthmus	4	
	Sample	Amount	Main application				
Mixed adult female groups	Vagina Uterus Isthmus	6	Collection of vagina, uterus, and isthmus for HE staining				
Adult male groups	2 mL of semen	6	Collection of 2 mL of semen from the epididymis and testes for injection into the unmated experimental group of turtles.				

RNA extraction and qRT-PCR. Total RNA was extracted from the vagina, uterine, and isthmus tissues of animals in the experimental and control groups. Total RNA was extracted with a TRIzol kit (Thermo Fisher Scientific, USA) and cDNA was synthesized with a Reverse Transcription Kit (Thermo Fisher Scientific, USA). Primers were then designed (Oligo version 7 software) (Rychlik, 2007) and synthesized for NRF2 and genes located downstream (HO1, *NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX3, GPX3,* and *GPX4*). The primer sequences are shown in Table II; β-actin was used as the reference gene. For qRT-PCR, we used a Roche LightCycler 480 real-time PCR cycler (Bio-Rad, Hercules, CA, USA) and MonAmpTM ChemoHS Qpcr Mix (SYBR Green) (Monad, Suzhou, China) to amplify the *NRF2, HO1, NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX2, GPX3* and *GPX4* genes. The total volume for each PCR reaction was 20 mL (containing 10 μL of MonAmpTM ChemoHS Qpcr Mix, 1 μL of cDNA, 1 μL of each upstream and downstream primer, and 7 μL of RNA-free H_2O). Parameters for the qPCR reaction were as follows: pre-denaturation at 95 °C for 10 min; denaturation at 95 °C for 10 s; annealing at 56 °C for 30 s; extension at 72 °C for 30 s; 40 cycles; and a final 65 °C-95 °C melting curve to evaluate normal amplification. Three biological replicates were performed for each individual sample. The specific mRNA expression levels of the 11 genes were calculated by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Table II. NRF2 pathway Oligonucleotide PCR primers.

Gene	Primer sequence (5'-3')	Orientation
NRF2	GCTCCAACTTTTCGGAAGAC	Forward
	CAGAATGTTCAGGTGATGCAG	Reverse
HO1	CGGCCTATATCCCCGTTTAC	Forward
	GGAGAGCTTTCTGGGCAATC	Reverse
NQO1	ACTGGTAAGCCAAAAGACCC	Forward
	TCTTGGTAAGGGAGTAGGCA	Reverse
CAT	GGCAAAGCCATTTACTGCAA	Forward
	AGGATTTGTGAGGCCAAACC	Reverse
SOD1	AGTAACGCTCTCAGGAAGGAT	Forward
	GCGGTCCCGTAAGAGAAATTA	Reverse
SOD2	ACCACACCATCTTTTGGACA	Forward
	TGCTCCCACACATCAATTCC	Reverse
SOD3	TGAATGACCTGTGGCAGAAT	Forward
	CTCCGAGCTTATGGATGTGA	Reverse
GPX1	TCCTTAAAGCATGTCCGCC	Forward
	GAGATGTCATTCCTGCACACT	Reverse
GPX2	TCCAGAAATGCCAGGTGAAC	Forward
	GATGGCAATGGTCTGGAACT	Reverse
GPX3	ACAATCTACAGGTATGGGGC	Forward
	AAAAGCTGGAAATTCGGGAC	Reverse
GPX4	GGACAAGTACAGGGGGTATG	Forward
	GTTGTTCCCGTTGACCTCTA	Reverse
β-actin	GAAGATCCTGACAGAGAGAG	Forward
	GATTCCATACCCAGGAAAGA	Reverse

Enzyme activity assays. Tissues were homogenized at a ratio of 1:10 tissue mass (g) to extract volume (mL) and then centrifuged at 10,000g for 10 min at 4 °C. Next, we removed the supernatant and placed the sample on ice to await analysis. The activities of Catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) were then measured with a Catalase Assay Kit (Biosharp), Glutathione Peroxidase Assay Kit (Biosharp), and Superoxide Dismutase Assay Kit with NBT (COMIN), respectively.

NRF2 immunofluorescence. Paraffin sections are baked at 60 °C for 2 to 3 hours in an oven; then dewaxed and cleared with xylene and graded alcohol dehydration. Next, these were washed three times in PBS three times (5 min per wash) and preheated for antigen retrieval solution in a microwave (100 °C for 5 min). The sections were placed in antigen retrieval solution and microwaved (50 °C for 5 min; 30 °C for 8 min) and then allow to cool naturally. Next, the sections were permeabilized with 0.1 % PBST (1mL PBS + 1μL Triton-100) for 13 to 15 min and then washed three times in PBS (3 min per wash). For routine serum blocking, we applied 5 % BSA and incubated the sections at 37 °C for 30 min. Then, excess liquid was absorbed with filter paper. Primary antibody was applied at a dilution ratio of 1:500 and sections were placed in a humid chamber, and incubated overnight at 4 °C (NRF2 Antibody purchased from Affinity Biosciences, product number: AF0639). The next morning, the sections were placed at room temperature for 30 min and then washed three times with 0.05 % PBST (5 min per wash) to remove the primary antibody. Next, a fluorescent secondary antibody was added to the sections and incubated in a humid chamber. (37 °C for 60 min) Then, the slides were washed three times with PBST (5 min per wash and excess liquid was absorbed with filter paper. From the addition of the fluorescent secondary antibody onwards, all subsequent operations were carried out in the dark. After absorbing excess liquid with absorbent paper, we applied an anti-fluorescence quenching agent containing DAPI and the slides were sealed. Finally, the slides were observed and images were captured by an inverted fluorescence microscope.

Data analysis. We used IBM Statistics version 19 (https://www.ibm.com/support/pages/spss-statistics-190) to perform independent samples t-tests and one-way analysis of variance (ANOVA) and determine whether the expression levels of key genes varied significantly between samples (*NRF2, HO1, NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX2, GPX3, GPX4*) (*$P < 0.05$; **$P < 0.01$; *** $P < 0.001$) and graphs were prepared using GraphPadPrism8 (https://www.graphpad.com/guides/prism/8/user-guide/index.htm).

RESULTS

Artificial insemination. The presence of sperm in the lumen of the vagina, uterus, and isthmus from *M. reevesii* was observed by the HE staining of fixed tissues. When adult female turtles of both sexes were raised together, few sperm were detected in the vagina, although a large number of sperm were present in the uterus and isthmus; some of the sperm in the uterus had their heads embedded in uterine cilia (Fig. 1).

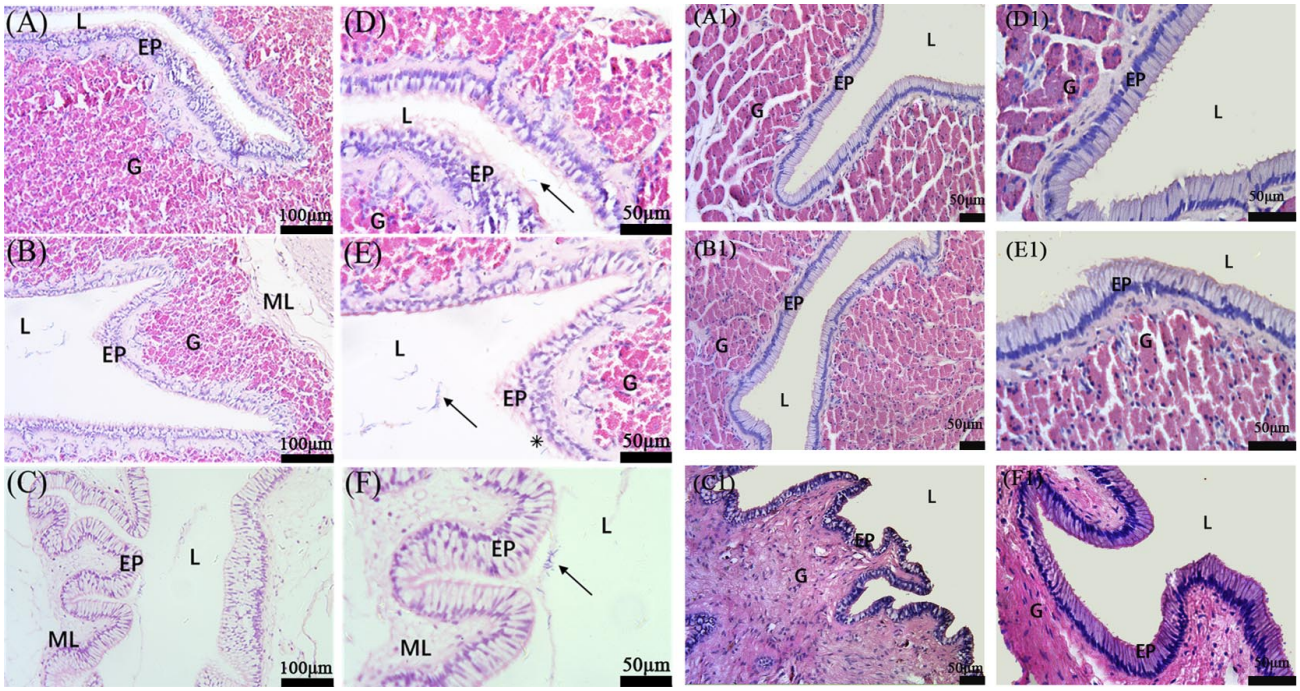

Fig. 1. Hematoxylin-eosin staining staining of oviduct tissue from a turtle. (A), (B), and (C) represent sections of vaginal, uterine, and isthmus tissues at low magnification; (D), (E), and (F); represent sections of the vaginal, uterine, and isthmus tissues at high magnification: (A1), (B1), and (C1); represent sections of vaginal, uterine, and isthmus tissues at low magnification; (D1), (E1), and (F1). Lumen (L), Epithelial cell layer (EP), Glandular cell layer (G), Muscle layer (ML), Sperm (Sperm, Æ). á represents sperm embedded in cilia. Scale bar: 50 μm and 100 μm.

The expression of NRF2-pathway-related genes in the oviducts at various periods. The analysis of oviducts revealed that the expression levels of *NRF2, HO1, NQO1, CAT, SOD1, SOD2, GPX1, GPX2,* and *GPX4* were lesser than those of unfertilized turtles. In contrast, the expression levels of SOD3 and GPX3 were increased in fertilized turtles than in unfertilized turtles (Fig. 2A, B, C) ($P < 0.05$). However, analysis of oviducts seven days and one month after fertilization showed that the expression levels of NRF2 and downstream genes *HO1, NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX2, GPX3,* and *GPX4* were greater than those of unfertilized turtles (Figs. 2D, E, and F) ($P < 0.05$).

CAT, GPX, and SOD activity in the oviduct of turtles after different durations. To further investigate enzyme activity in the oviducts of turtles, we determined the enzyme activity of major antioxidant enzymes (GPX, SOD, and CAT) and found that the results were consistent with those of mRNA expression in the oviducts of turtles, as shown in Figure 3. When considering the oviducts of turtles one day after fertilization, we found that the vagina (Fig. 3A), uterus (Fig. 3B), and isthmus (Fig. 3C) all exhibited significantly lower activities of SOD, CAT, and GPX than unfertilized turtles ($P < 0.05$), thus concurring with mRNA expression findings. Seven days after fertilization (Fig. 3D), the enzyme activities of CAT and GPX in the uterus (Fig. 3E) and isthmus (Fig. 3F) were lesser than those of unfertilized turtles; however, SOD activity was lesser than in unfertilized turtles ($P < 0.05$). One month after fertilization, the vagina (Fig. 3G), uterus (Fig. 3H), and isthmus (Fig. 3I) all showed greater activities of CAT, GPX, and SOD than unfertilized turtles ($P < 0.05$); these findings concurred with mRNA levels.

Immunofluorescence localization of NRF2 protein. In the experimental group at 1 month, we tested the expression and localization of NRF2 protein in the oviducts by

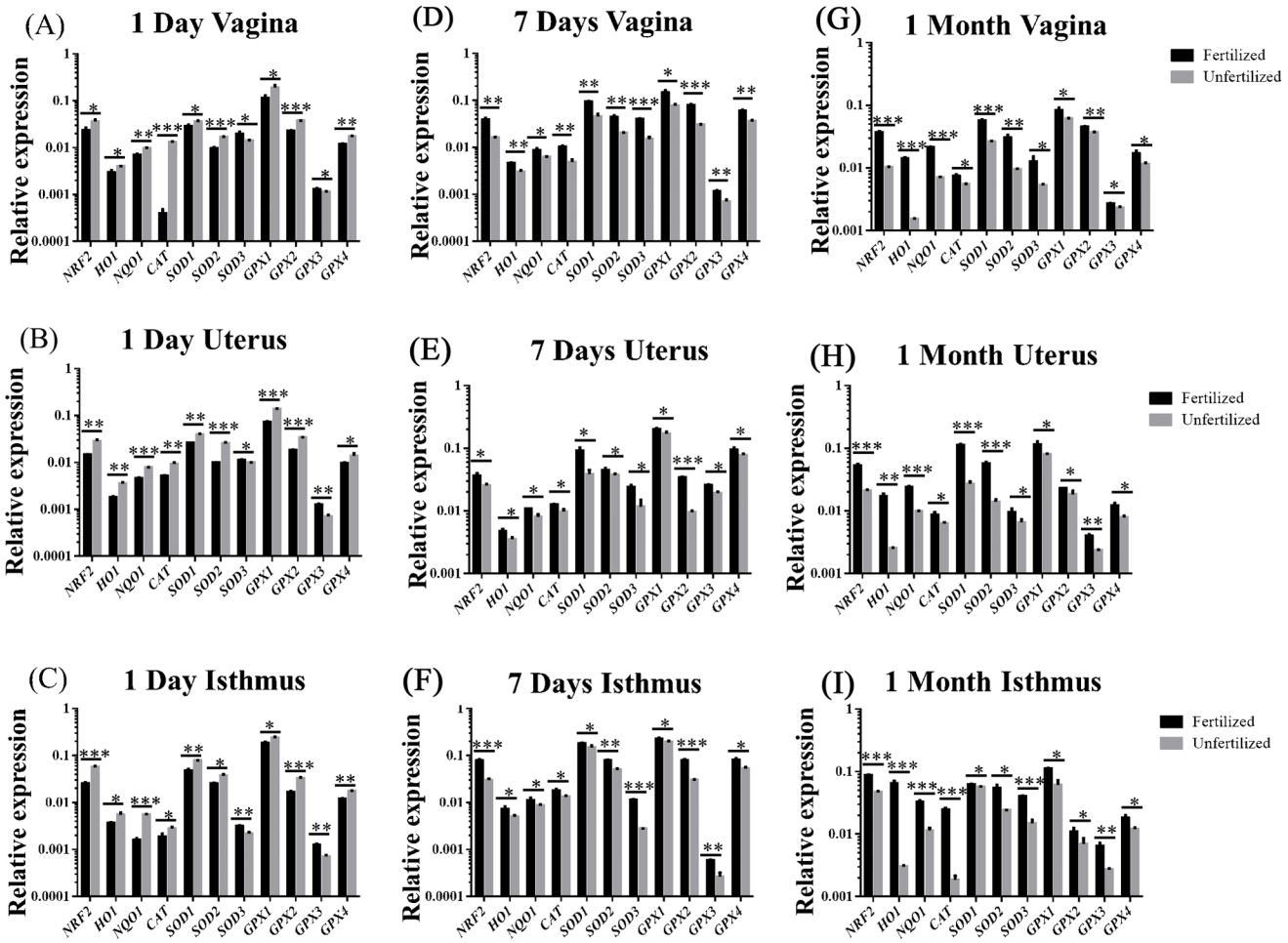

Fig. 2. Gene expression levels of genes in the NRF2 pathway within the oviduct of female turtles after one day, seven days and one month of fertilization when compared to unfertilized animals. Significant differences are identified as *, $P < 0.05$; **, $P < 0.01$. ***$P < 0.001$, significant.

immunofluorescence with an NRF2 antibody. One month after fertilization, NRF2 protein was mainly distributed in the epithelial and glandular tissues of the oviducts of fertilized turtles; lesser levels of protein were detected in muscle tissues (Fig. 4A1, A2). In oviductal epithelial tissues, NRF2 protein was expressed in the cilia, on the surface of ciliated cells, and in the glandular tissue; NRF2 was also expressed in glandular cells (Fig. 4 A1, A2), the uterus (Fig. 4 C1, C2), and the isthmus (Fig. 4 E1, E2). The immunofluorescence intensity of NRF2 in the vagina, uterus, and isthmus was increased in fertilized turtles relative to the unfertilized turtles (Fig. 4 A1, A2, 4 C1, C2,4 E1, E2). In the oviducts of unfertilized turtles, NRF2 protein was detected in the epithelial tissue and secretory glands, but not in muscle tissue. Compared to fertilized turtles, the immunofluorescence intensity of NRF2 was lesser in the ciliated and secretory cells of unfertilized turtles (Fig. 4B1, B2, 4 D1, D2, and 4 F1, F2)

DISCUSSION

For animals that undergo *in vivo* fertilization, one of the most important mechanisms for successful fertilization is the simultaneous presence of male and female gametes at the site of fertilization at the right time; this is unlikely to happen by chance *in vivo* because the sperm must travel a long distance through the female reproductive tract and must arrive at the site of fertilization at the right time. Thus, to increase the chance of game fusion, some females have developed the ability to store sperm in the reproductive tract (Mahé *et al*., 2021). Sperm are terminally differentiated cells that usually lack a distinct cytoplasm and have limited antioxidant defense capability compared to the egg cell. Sperm are unable to restrict or repair molecular damage to DNA, proteins, and membrane lipids following insult by oxidative stress (González-Marín & Roy, 2012; Aitken *et al*., 2014). In a previous study, Reinhardt & Ribou (2013),

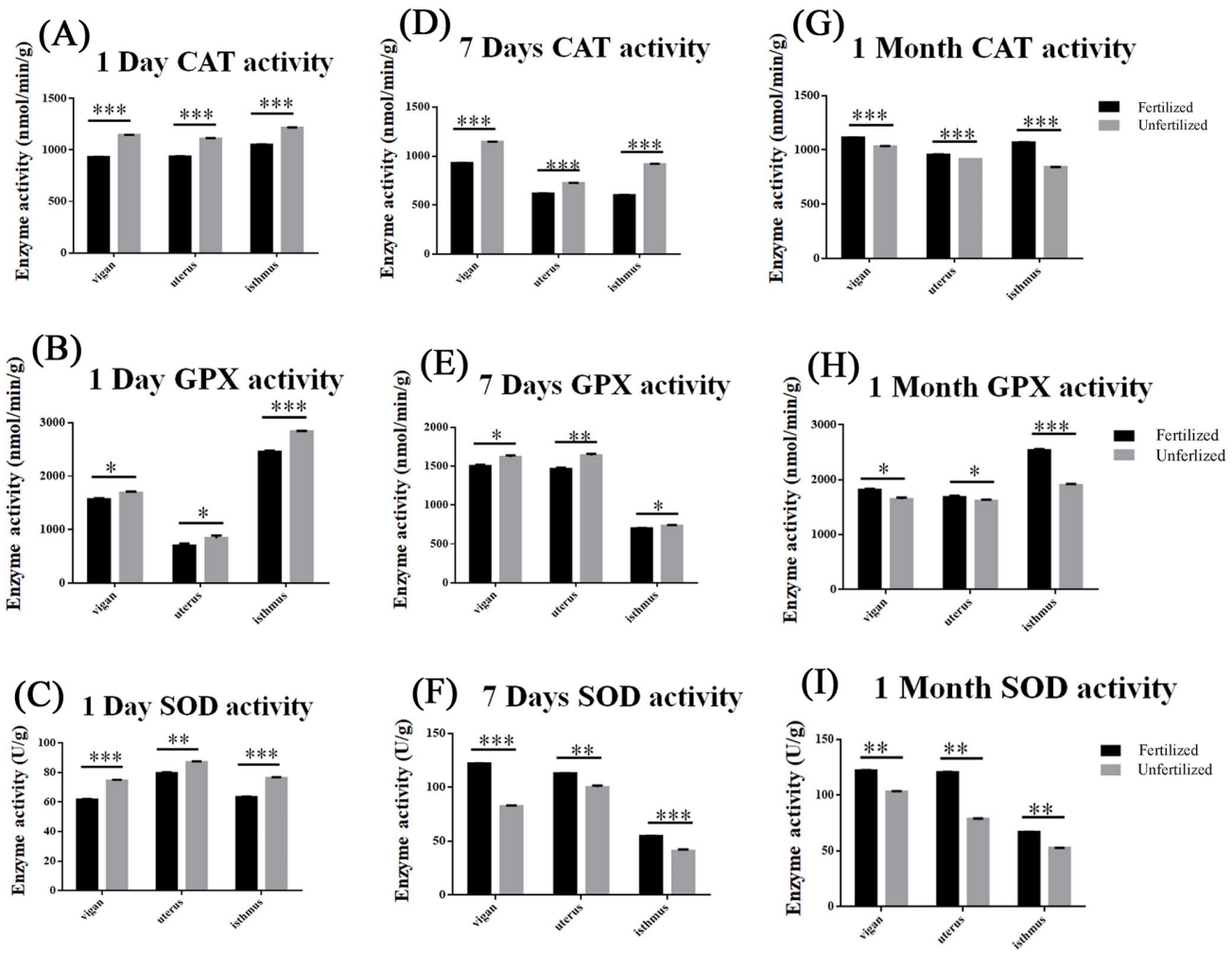

Fig. 3. Enzyme activities of CAT, GPX, and SOD in the oviduct of fertilized and unfertilized turtles after one day, seven days, and one month. *Significant difference (Significant differences are identified as *, $P < 0.05$; **, $P < 0.01$. ***$P < 0.001$).

proposed two mechanisms that can reduce oxidative damage in sperm: interfering with cellular metabolism to reduce the formation of oxygen free radicals, or the production of antioxidants that modulate the functional activity of sperm in the female reproductive tract (Baker & Aitken, 2005).

ROS is a product of physiological activity in normal sperm and somatic cells. However, in excess, ROS can be harmful; moderation appears to be the key to ROS production in sperm (Moraes & Meyers, 2018). When cells are attacked by an excess of ROS, they will defend themselves through certain mechanisms. NRF2 plays a key role in defending against oxidative damage (Zhang *et al*., 2020) and improves the expression of antioxidant genes to play a role in cell protection by combining antioxidant response elements (AREs) to increase the activity of antioxidant enzymes, and activating a series of antioxidant genes in response to oxidative stress, thus reducing the levels of ROS (Kobayashi *et al*., 2006; Chung *et al*., 2021; Egbujor *et al*., 2024). However, ROS can also exert a positive impact on cell signaling and sperm function; in mammals, the ability of sperm to penetrate and fertilize an egg is triggered by ROS. When produced at deleterious levels, ROS can be quenched by a range of endogenous antioxidant enzymes, including SOD, CAT and GPX (Monaghan *et al*., 2009). A moderate level of ROS is necessary for some sperm functions, including fertilization, motility, capacitation, the acrosome reaction, and hyperactivation (Baumber *et al*., 2003; Amaral *et al*., 2013; Gibb *et al*., 2014). In the present study, we demonstrated that one day after fertilization, the expression levels of NRF2 and downstream antioxidant-related genes *HO1, NQO1, CAT, SOD1, SOD2, GPX1, GPX2*, and *GPX4*, except for *SOD3* and *GPX3*, were down-regulated when compared to unfertilized turtles. Our previous findings identified greater expression levels of genes related to the NRF2 pathway and greater levels of enzymic activity in

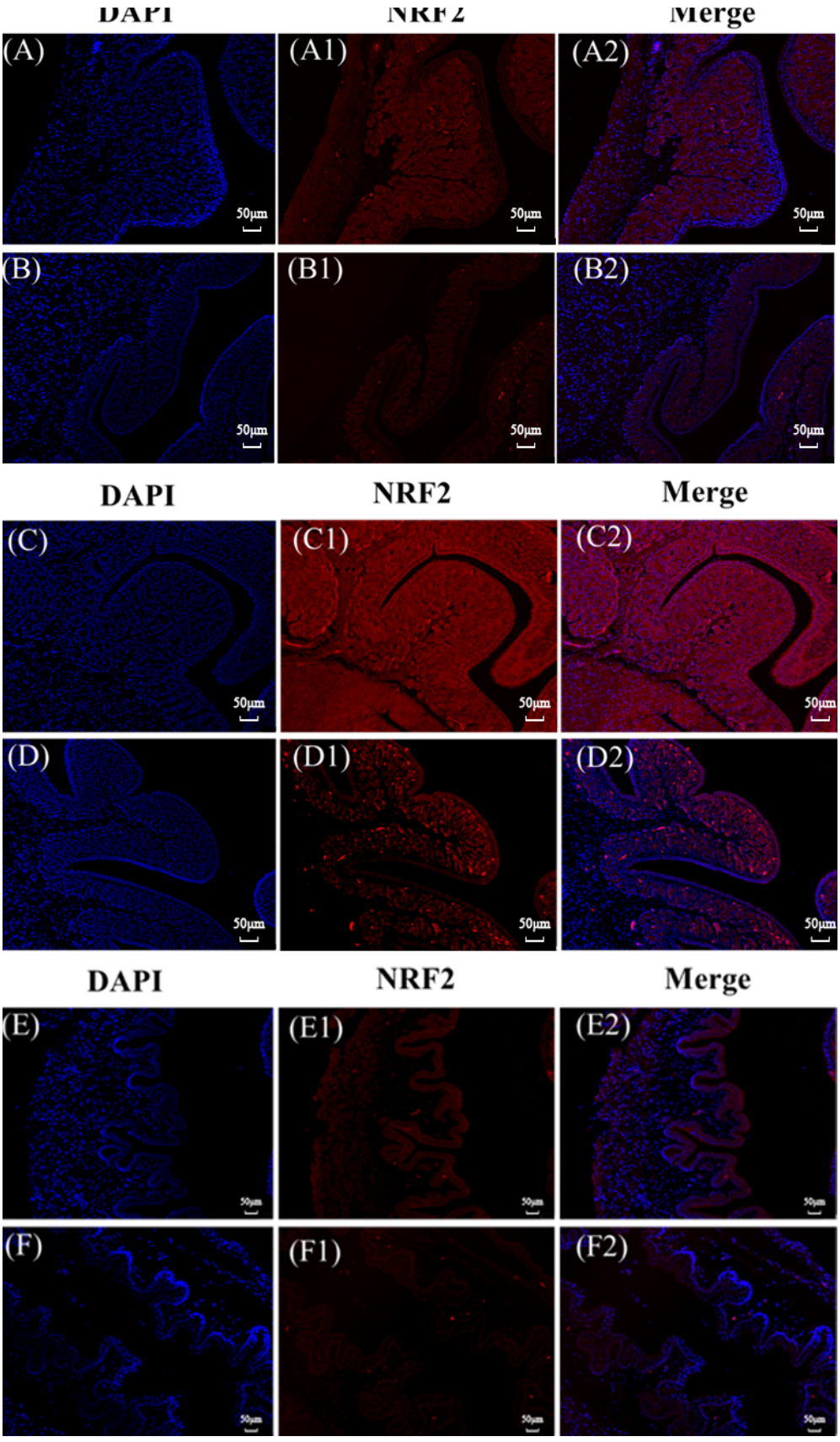

Fig. 4. Nuclear factor-erythroid 2-related factor 2 (NRF2) immunofluorescence in the vagina(A-B), uterus(C-D), isthmus (E-F) of fertilized and unfertilized turtles. Red fluorescence indicated the localization of NRF2 protein (A1, B1, C1, D1, E1, F1), while nuclei are shown in blue (A, B, C, D, E, F); (A2, B2, C2, D2, E2, F2) show merged images of the NRF2 protein and nuclei. Scale: 50mm. A, A1, A2C, C1, C2E, E1, E2: fertilized; B, B1, B2D, D1, D2F, F1, F2: unfertilized.

the oviducts of turtles one month after fertilization when compared to unfertilized turtles. These findings might suggest that there was an up-regulation of antioxidant genes and the expression of major antioxidant enzymes in the oviducts of *M. reevesii* during sperm storage. Furthermore, the levels of enzyme activity measured one day later showed a significant reduction in the activities of CAT, GPX, and SOD in the oviduct of fertilized turtles. The down-regulation of antioxidant genes in the oviduct, and the reduction in enzymic activity, may be due to the presence of antioxidant enzymes in the seminal plasma. This suggests that a moderate level of ROS is favorable for sperm to migrate to the oviduct storage site and that the ROS produced by sperm do not fully activate the antioxidant pathway in the oviduct within a short time.

In a previous study, Park *et al*. (2011), found that the NRF2/ARE signaling pathway can resist oxidative stress by upregulating the expression of HO-1 and NQO1 in rat astrocytes. In addition, in two-spotted crickets (*Gryllus bimaculatus*), sperm that had been stored in females had a 37 % lower metabolic rate and 42 % lower ROS level when compared to freshly ejaculated sperm; these changes may have helped to prolong the lifespan of sperm (Ribou & Reinhardt, 2012). In honeybees (*Apis mellifera*), sperm are protected by the up-regulation of antioxidant genes (Gonzalez *et al*., 2017); a similar phenomenon has been documented in *Anopheles gambiae mosquitoes* (Shaw *et al*., 2014). Furthermore, one month after fertilization, immunofluorescence showed that the immunofluorescence intensity of NRF2 levels in the oviducts of fertilized turtles was greater than those in unfertilized turtles. This may be due to the gradual replacement of seminal plasma by tubal fluid. These results suggest the existence of a specific antioxidant-regulating system in the oviducts of *M. reevesii* that plays an important role in sperm storage.

It is well known that $\bullet O_2^-$ can react with NO produced by nitric oxide synthase (NOS) to produce a strong oxidant, peroxynitrite ($ONOO^-$) (Lapointe *et al*., 2006; Roy & Krishna, 2011). Our present analysis indicated an increased level of SOD activity in the oviduct just seven days after fertilization; mRNA analysis indicated high expression levels of SOD1, an enzyme that is hypothesized to prevent the formation of the toxic substance ONOO- in the oviduct. The enzymic activities of CAT, GPX, and SOD were significantly higher in the oviduct one month after fertilization. GPX enzyme activity was the highest in the isthmus and SOD enzyme activity was the lowest; this indicates that GPX plays a major role in balancing the redox state in the oviduct. Our results suggest that CAT, GPX, and SOD enzymes act together to regulate the redox state in the oviducts of turtles, thus creating a favorable environment for sperm to survive under long periods of storage. These results suggest that antioxidant systems are uniquely adapted and conserved between species and that differences in sperm survival time across organisms are associated with antioxidant enzymes that reduce the levels of ROS. Sperm in the reptilian oviduct are known to survive for several years; consequently, there must be a specific mechanism in these species that scavenges ROS (Holt, 2011). In this study, we found that as the duration of sperm storage increased in the reproductive tract, the expression levels of downstream antioxidant genes were regulated by NRF2, and the activity of antioxidant enzymes was increased. This regulatory activity, in turn, modulated the redox status in the oviduct, rendering it conducive for the long-term storage of sperm.

CONCLUSION

In summary, one day after fertilization, there was a reduction in the expression of *NRF2* and its downstream genes, except for *SOD3* and *GPX3*, and enzyme activity tests revealed reduced activity of SOD, CAT, and GPX, which matched the mRNA expression levels. Collectively, these results suggest that a moderate level of ROS can exert a positive impact on cell signaling functions. Seven days after fertilization, increased *NRF2* and downstream gene expression contrasted with decreased CAT and GPX enzymic activities; this did not align with mRNA expression. One month after fertilization, the activitiesof CAT, GPX, and SOD were significantly greater than in unfertilized turtles, thus aligning with mRNA expression data. Immunofluorescence analysis showed that NRF2 protein expression was increased in fertilized turtles compared to unfertilized turtles. These results suggest that the oviduct of fertilized turtles can regulate the expression of the *NRF2* gene and its downstream genes to protect sperm from oxidative stress, thus creating a microenvironment that is conducive to sperm storage. This pattern of regulation modulates the redox level in the oviduct, making it more conducive to the long-term storage of spermatozoa. The storage of sperm is important for reproduction and the propagation of turtles. Our findings enhance our understanding of sperm storage mechanisms.

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YU, X.; LIN, X.; WANG, W.; ZHANG, G.; XIA, X.; NIE, L. y BU, X. El almacenamiento de esperma en el oviducto activa la vía antioxidante NRF2 en *Mauremys reevesii*. *Int. J. Morphol., 43(3)*:968-977, 2025.

RESUMEN: La mayoría de los reptiles pueden almacenar esperma para su uso posterior. Tras el apareamiento, el esperma almacenado en el tracto reproductivo de la hembra experimenta estrés oxidativo debido al volumen citoplasmático limitado, lo

que restringe el almacenamiento de enzimas antioxidantes. Además, los espermatozoides son sensibles a las especies reactivas de oxígeno (ROS), lo que podría activar la vía del factor nuclear 2 relacionado con el eritrocitos 2 (NRF2) en respuesta. Este estudio tuvo como objetivo investigar el papel de la vía NRF2 durante el estrés oxidativo en el oviducto de *Mauremys reevesii*. Evaluamos la expresión de genes relacionados con *NRF2 (NRF2, HO1, NQO1, CAT, SOD1, SOD2, SOD3, GPX1, GPX3, GPX4*), las actividades de las enzimas antioxidantes Catalasa (CAT), glutatión peroxidasa (GPX) y superóxido dismutasa (SOD), y la localización de la proteína NRF2 por cuantificación de fluorescencia, ensayos enzimáticos e inmunofluorescencia. La tinción con hematoxilina-eosina reveló una cantidad de espermatozoides en el útero y el istmo, y solo una escasa cantidad de espermatozoides en la vagina. qRT-PCR indicó que después de la fertilización ($P < 0,05$), NRF2 y sus genes descendentes mostraron una expresión reducida excepto SOD3 y GPX3 en el día uno, con una expresión aumentada en el día siete y después de un mes. Los ensayos de actividad enzimática indicaron que después de la fertilización ($P < 0,05$), un día las actividades de SOD, CAT y GPX se redujeron notablemente; a los siete días, las actividades de CAT y GPX se redujeron significativamente; fueron inconsistentes con el ARNm; al mes todas las actividades enzimáticas aumentaron. La inmunofluorescencia reveló un aumento en la intensidad de la proteína NRF2 en tortugas fertilizadas. Estos resultados sugieren que la activación de la vía NRF2 potencia la expresión de genes relacionados con NRF2 en el oviducto, lo que resulta en un efecto protector sobre el almacenamiento de esperma en *M. reevessii*.

PALABRAS CLAVE: *Mauremys reevesii*; Oviducto; Almacenamiento de esperma; Vía antioxidante; Factor nuclear 2 relacionado con el factor eritroide 2.

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