UNIVERSITI PUTRA MALAYSIA

MICROSATELLITE CHARACTERIZATION AND PHYSIOLOGICAL CHANGES OF CRYOPRESERVED SPERMATOZOA SUPPLEMENTED WITH ANTIOXIDANTS IN MALAYSIAN MAHSEER, Tor tambra (VALENCIENNES, 1842)

CHEW POH CHIANG

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By

CHEW POH CHIANG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

February 2021

DEDICATION

To my beloved parents, husband, children, and all my family members.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MICROSATELLITE CHARACTERIZATION AND PHYSIOLOGICAL CHANGES OF CRYOPRESERVED SPERMATOZOA SUPPLEMENTED WITH ANTIOXIDANTS IN MALAYSIAN MAHSEER, *Tor tambra* (VALENCIENNES, 1842)

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February 2021

Chairman : Annie Christianus, PhD
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The Malaysian mahseer that fetches the high market price and demand is now considered a prospect for aquaculture. However, a constant supply of the hatchery-produced seed and fry of *Tor* spp. is still minimal and inconsistent due to the lack of quality broodfish.

In the present study, samples of *Tor tambra* and *T. tambroides* were collected from 11 localities in Malaysia (prior to genetic assessment) for broodstock development and sperm cryo-banking. Meanwhile, supplementation of antioxidants in the cryopreservation media is hypothesized to reduce the resulting lipid peroxidation (LPO) and oxidative stress-related DNA damages of the post-thaw sperm. As such, the objectives of this study were: 1) to establish the microsatellites (SSR) profile for the *Tor* spp. from different geographical regions in Malaysia; 2) to determine the effects of ascorbic acid, α-tocopherol, Trolox and reduced glutathione (GSH) on post-thaw sperm quality; 3) to investigate the effects of cryostorage duration on post-thaw sperm quality with and without antioxidants supplementation; and, 4) to assess the oxidative stress and DNA damage caused by freeze-thawing and cryostorage procedures in *T. tambra* sperm. Genetic characterization was conducted using 22 SSR markers via fragment analysis. Post-thaw quality of the cryopreserved sperm was assessed for sperm motion characteristics, viability, morphology and membrane plasma integrity. Effect of the cryostorage duration on post-thaw sperm quality was assessed a week after cryostorage and subsequently on a three-month basis for 12 months. The level of LPO and oxidative DNA damages after sperm cryopreservation was assessed by measuring malondialdehyde (MDA) content, 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration, DNA lesion analysis, relative sperm telomere length (STL) measurement and DNA fragmentation.

In the SSR characterization, results revealed that the *Tor* spp. collection retained their genetic variation but exhibited excessive homozygosity. A low gene flow over all loci indicates little genetic variation transfer between populations. The genetic structure of all populations was successfully resolved into four main clusters following geographical regions.

With antioxidants supplementation, the concentration that produced the best post-thaw motility in *T. tambra* sperm was 0.01 mM ascorbic acid, 0.025 mM α-tocopherol, 0.025 mM Trolox and 0.5 mM GSH, respectively. Overall, the motility duration of post-thaw sperm was significantly improved ($p<0.05$, >3 min) in treatments supplemented with antioxidants in combinations. Treatment supplemented with ascorbic acid and α-tocopherol (T5) showed the best performance with significantly ($p<0.05$) improved sperm motile duration, percentages of morphology, viability, morphology, reduction in LPO, 8-OHdG and DNA lesion rates while maintaining STL. Of all treatments, only treatment T3 supplemented with Trolox significantly ($p<0.05$) improved post-thaw sperm motility and velocity. In treatments with positive responses to antioxidants supplementation, the sperm physiology characteristics were not affected by the prolonged cryostorage period. Out of seven developmental-related genes studied, *HoxC4a-2* was the most vulnerable to DNA damage.

In conclusion, the findings from this study provide a significant baseline reference for the genetic status of the *Tor* spp. collection. New basic knowledge on LPO and oxidative DNA damage from cryostorage and improved extender formulations by exogenous antioxidants supplementation for better post-thaw quality in *T. tambra* sperm were generated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untk ijazah Doktor Falsafah

PENCIRIAN MIKROSATELIT DAN PERUBAHAN FISIOLOGI SPERMA KRIOAWET DENGAN PENAMBAHAN ANTIOKSIDAN DALAM IKAN KELAH MALAYSIA, *Tor tambra* (VALENCIENNES, 1842)

Oleh

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Ikan kelah dengan harga dan permintaan pasaran yang tinggi kini berpotensi dibangunkan untuk akuakultur. Walau bagaimanapun, bekalan benih dan rega *Tor* spp. yang berterusan masih amat terhad kerana kekurangan induk yang berkualiti tinggi.

Dalam kajian ini, sampel *Tor tambra* dan *T. tambroides* telah dikumpul dari 11 lokaliti di Malaysia (sebelum penilaian genetik) untuk tujuan pembangunan induk dan pembankan sperma secara krioawet. Sementara itu, dengan andaian bahawa penambahan antioksidan dalam media pengkrioawetan berupaya mengurangkan tekanan oksidatif dan kerosakan DNA yang terhasil dalam sperma pasca-nyahbeku. Justeru itu, objektif utama kajian ini adalah untuk: 1) mewujudkan profil mikrosatelit (SSR) bagi *Tor* spp. yang diperoleh dari kawasan geografi yang berbeza di Malaysia; 2) mengenalpasti kesan penambahan asid askorbik, α-tokoferol, Trolox dan glutation terturun (GSH) ke atas kualiti sperma pasca-nyahbeku, 3) mengkaji kesan tempoh masa simpanan secara krioawet ke atas kualiti sperma pasca-nyahbeku dengan dan tanpa penggunaan antioksidan, dan 4) untuk menilai tekanan oksidatif dan kerosakan DNA yang terhasil akibat proses sejukbeku/nyahbeku dan simpanan secara krioawet dalam sperma *T. tambra*. Pencirian genetik telah dijalankan mengunakan 22 penanda SSR menerusi analisis fragmen. Kualiti sperma krioawet *T. tambra* pasca-nyahbeku dinilai untuk ciri-ciri pergerakan, viabiliti, morfologi dan integriti membran plasma. Kesan tempoh masa simpanan krioawet juga dinilai selepas seminggu dikrioawet, dan seterusnya setiap tiga bulan selama 12 bulan. Tahap kejadian peroksidasi lipid (LPO) dan kerosakan DNA sperma krioawet dinilai dari segi kandungan malondialdehid (MDA), kepekatan 8-hidroksil-2'-deoksiguanozin (8-

OHdG), analisis DNA dalam bentuk lesi dan ukuran panjang relatif telomer sperma dan fragmentasi DNA.

Dalam kajian pencirian SSR, hasil kajian mendapati koleksi *Tor* berkenaan masih memelihara variasi genetik tetapi menunjukkan homozigot yang berlebihan. Aliran gen merentasi semua lokus adalah rendah menandakan pemindahan variasi genetik antara populasi adalah rendah. Struktur genetik kesemua populasi telah berjaya dikelompokkan dalam empat cabang utama mengikut kawasan geografi.

Kepekatan keempat-empat antioksidan digunakan dalam media ekstender yang menunjukkan kualiti sperma pasca-nyahbeku yang terbaik adalah asid askorbik 0.01 mM, α-tokoferol 0.025 mM, Trolox 0.025 mM dan GSH 0.5 mM. Pada keseluruhannya, jangka masa motiliti sperma pasca-nyahbeku *T. tambra* dalam rawatan yang menggunakan antioksidan dalam kombinasi telah menunjukkan peningkatan signifikan ($p<0.05$, >3 min). Rawatan T5 yang menggunakan asid askorbik dan α-tokoferol menunjukkan prestasi yang terbaik dengan memberi kesan signifikan ($p<0.05$) terhadap peningkatan jangka masa motiliti, peratusan viabiliti dan morfologi, serta penurunan kandungan LPO, 8-OHdG dan kadar kerosakan DNA dalam bentuk lesi di samping memelihara panjang telomer sperma. Antara kesemua rawatan, hanya T3 (menggunakan Trolox) yang menunjukkan kesan signifikan ($p<0.05$) dalam meningkatkan motiliti dan kelajuan sperma pasca-nyahbeku. Di dalam rawatan yang menunjukkan respon positif terhadap antioksidan, ciri-ciri sperma pasca-nyahbeku didapati tidak terjejas dengan tempoh masa simpanan sejukbeku yang berpanjangan. Daripada tujuh gen yang dikaji, kerosakan DNA didapati paling cenderung berlaku dalam *HoxC4a-2*.

Kesimpulannnya, hasil kajian ini boleh dijadikan satu panduan rujukan tentang status genetik koleksi *Tor* spp. Pengetahuan asas yang baharu tentang LPO dan kerosakan DNA secara oksidatif akibat simpanan sperma secara krioawet dan formulasi bagi ekstender yang berpotensi untuk meningkatkan kualiti sperma *T. tambra* pasca-nyahbeku dengan penambahan antioksidan eksogenus telah berjaya dibangunkan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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A_r	Allelic richness
A_e	Effective number of alleles
A_p	Number of private alleles
AE%	Amplification efficiency
α-tocopherol	Vitamin E
AGHR	AgroHarvest, Raub, Pahang
ALH	Amplitude of lateral sperm head displacement
BCF	Beat cross-frequency
BHT	Butylated hydroxytoluene
bp	Base pairs
°C	Degree Celcius
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CASA	Computer-assisted sperm analysis
cm	Centimeter
DNA	Deoxyribonucleic acid
% DNAt	Tail DNA %
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EMS	Empurau, Sarawak
EXT	Sperm samples after dilution
f	Inbreeding coefficient
FFRC	Freshwater Fisheries Research Centre, Batu Berendam, Melaka (currently FRIGL)
FS	Fresh sperm samples before dilution
FST	Fixation index

g	Gram
g/L	Gram per liter
GPRK	Grik, Perak
GSH	Reduced glutathione
He	Gene diversity
Ho	Heterozygosity
HLKW	Kelah World, Hulu Langat, Selangor
HLS	Hulu Langat, Selangor
HOS	Hypo-osmotic swelling test
HWE	Hardy Weinberg Equilibrium
8-OHdG	8-hydroxy-2'-deoxyguanosine
IAM	Infinite allele model
kb	Kilobase pair
KCl	Potassium chloride
KENS	Kg Esok, Jelebu, Negeri Sembilan
kg	Kilogram
LIN	Linearity
LMP	Low melting point agarose
LPO	Lipid peroxidation
m	Meter
M	Molar
MAF	Major allele frequency
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate
µg	Microgram

µg/mL	Micro gram per mililiter
µL	Microliter
µM	Micromolar
mg/kg	Milligram per kilogram body weight (dosage)
mOsmol/kg	Milliosmoles per kilogram
MiliQ water	Ultrapure deionized water
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MSJ	Mersing, Johor
NaCl	Sodium chloride
Na2-EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
NaOH	Sodium hydroxide
N_A	Number of alleles per locus
Ne	Effective population size
N_G	Number of genotypes
ng	Nanogram
ng/µL	Nanogram per microliter
NGS-PBS	Normal goat serum in PBS
nm	Nanometer
Nm	Gene flow
NMPA	Normal melting point agarose
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PHG	Raub, Pahang
PI	Propidium iodide
PIC	Polymorphism information content
pmol	Picomole
pmol/mL	Pico mol per mililiter
PPAP	Aquaculture Extension Center, Perlok, Jerantut, Pahang
qPCR	Real-time PCR, quantitative Polymerase Chain Reaction
rpm	Centrifuge rotor speed
ROS	Reactive oxygen species
RNA	Ribonucleic acid
s	Second
SD	Standard deviation
SEM	Standard error mean
SFMM	Seminal fluid mineral medium
SMM	Stepwise mutational model
spp.	Species
SSRs	Microsatellites
STL	Sperm telomere length
STR	Straightness
TAE buffer	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TGN	Terengganu
Tm	Annealing temperature
TPM	Two-phase model
USD	US Dollar

V	Volt
VAP	Average path velocity
VCL	Curvilinear
VSL	Straight linear velocity

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malaysian mahseer comprised of *Tor tambra* (Valenciennes, 1842) and *T. tambroides* (Bleeker, 1854) of the family Cyprinidae, are indigenous large riverine species that occur only in Southeast Asia (Walton et al., 2017; Ambak et al., 2012, Ng, 2004). These two *Tor* spp. are the popular freshwater fish for food, ornamental and recreational. However, their wild populations are declining due to over-exploitation, natural habitat degradation, and water pollution (Dudgeon, 2011; Ng, 2004). These ecological threatened species are currently classified as data deficient in the IUCN Red List (Froese & Pauly, 2018; Kottelat et al., 2018a,b). The taxonomy of these indigenous mahseers remained uncertain, and revisions on their taxon names had been made occasionally since the 1990s (Kottelat et al., 2018a,b; Walton et al., 2017; Kottelat, 2013; Roberts, 1999; Roberts, 1993). Various conservation measures such as sperm cryo-banking, the establishment of mahseer sanctuary, restocking programmes, restricted fishing zones, and community-based management were part of the efforts to safeguard the genetic resources of this indigenous *Tor* spp. (Ansley et al., 2017; Chew et al., 2010a; Wong et al., 2009; Ambak et al., 2007).

Malaysian mahseer always receives high market demand and fetches high value (Kamarudin et al., 2014). However, mahseer price varies depending on the source (cultured or wild-caught), grade, and the fish as food or ornamental. Fish price is usually much higher for the wild-caught mahseer. Breeding and growing *Tor* spp. has lately gained much attention and popularity among local farmers in Malaysia and Indonesia (M-Zudaidy et al., 2021; Muchlisin, 2013).

By far, previous studies on Malaysian mahseer were mainly on the aspects of species identification through morphology (Haryono & Tjakrawidjaja, 2006), molecular systematics, and population genetics using both mitochondrial and nuclear DNA markers (Adzhar & Hassan, 2015; Norfatimah et al., 2014; Esa & Abdul-Rahim, 2013; Esa et al., 2008a, b, 2011; Keong et al., 2008; Nguyen et al., 2006, 2007, 2008; Siraj et al., 2007). Past studies also focused on endocrinology and reproductive biology (Ismail et al., 2011), breeding, nursing, and grow-out (Kunlapapuk & Kulabtong, 2011; Ingram et al., 2005, 2007a, 2007b), fish health (Koh et al., 2019; Asaduzzaman et al., 2018) and feeding regime and nutrition requirements (Asaduzzaman et al., 2016; Muchlisin et al., 2015; Entri, 2013; Ramezani-Fard et al., 2011, 2014; Misieng et al., 2011; Ng & Andin, 2011; Ng et al., 2008). Besides that, some sperm cryopreservation work has also been initiated on this *Tor* spp., with significantly lower egg fertilization rates using the frozen-thawed sperm (35%) compared to fresh sperm (70%)

(Chew & Zulkafli, 2012; Chew et al., 2010a). In addition, successful fry production through artificial spawning using the commercially available spawning inducer has also been established for Malaysian mahseer (M-Zudaidy et al., 2021).

1.2 Problem statements

The main constraints in sustainable aquaculture are the continued supply of egg and larval (Migaud et al., 2013). Like other commercially cultured species, the seed supply of Malaysian mahseer depends on imported or wild-collected seed and fry. A constant supply of the hatchery-produced seed and fry of *Tor* spp. is still very limited and inconsistent, although artificial spawning was recently established for Malaysian mahseer. This constraint is due to difficulties in obtaining sufficient numbers of excellent performance broodstocks such as female broodfish with high fecundity and male broodfish that produce high-quality sperm (Mylonas et al., 2015; Migaud et al., 2013; Mylonas et al., 2010). The problem can be solved by proper broodstock development and the establishment of sperm cryobank from superior broodstocks with known genetic makeup. To achieve this, the basic genetic assessment of the broodstock collection is thus essential for effective management and utilization.

The potential use of cryopreservation technology in the long-term storage of valuable genetic materials is undeniable (Yeste, 2016). However, the use of cryopreserved sperm in aquaculture is not practised due to poor performance and inconsistency in post-thaw sperm viability and fertility (Hagedorn, 2014). A standard cryopreservation protocol is always not available, and there are difficulties in the standardization of the protocols (Hagedorn, 2014; Yang et al., 2007). Reduced post-thaw sperm quality and poor fertilization performance were typical for the cryopreserved sperm (Anghel et al., 2010; Cabrita et al., 2001a). Lipid peroxidation (LPO) and DNA damage resulted from oxidative stress throughout freezing-thawing and sperm cryostorage has been suggested as the main reason for the low post-thaw quality of the cryopreserved sperm, and it is not well characterized in fish species. The discovery of oxidative stress on fish spermatozoa cryopreservation is very recent. Hence, only a limited amount of recent research on the understanding of oxidative stress and sperm viability rate (Sandoval-Vargas et al., 2021; Mostek et al., 2018). However, it is crucial to determine the various factors affecting the success of a proposed cryopreservation method. DNA damage from oxidative stress during and after cryopreservation is very much unknown in fish species in general, not to mention in Malaysian freshwater fish species.

1.3 Research justifications

In the present study, basic genetic information and the genetic background of the *Tor* spp. stocks collected for broodstock development and sperm cryo-banking and whether this *Tor* spp. from different geographical regions possess

different genetic makeup is unknown, as there was no genetic assessment on the stocks. Lack of information on the genetics of this locally important freshwater fish species, even though it is essential to maintain proper genetic records for effective management, utilization and ease of traceability (Senanan et al., 2015). Genetic assessment of the collected broodstocks in these species is thus necessary for efficient broodstock development and genebank management and later application in selective breeding and genetic improvement programmes.

Cryopreservation is seen as the potential method to store valuable fish gametes for future use. Successful cryopreservation of commercially important fish gametes would help stock improvement to meet the demand of seed supply and the conservation of endangered fish species (Zhang, 2011). Therefore, an in-depth understanding of cryopreservation science and technology is necessary for establishing a novel and improved cryopreservation technique. However, significantly lower post-thaw sperm quality and fertilization ability were typical in cryopreserved gametes in general (Anghel et al., 2010; Cabrita et al., 2001a). Similar findings were also noticed in the previous studies on this Malaysian *Tor* spp. (Chew et al., 2012; Chew et al., 2010a).

T. tambra was chosen as the model fish in this study because of its high potential and good perspective as an aquaculture species. Thus, it is in great need of research work and ready samples (fish and sperm) available for the study. Furthermore, the sperm cryopreservation protocol had been established for this species. But, the effect of LPO and oxidative DNA damage imposed on the post-thawed *T. tambra* sperm remained unknown. In this work, LPO and oxidative stress towards DNA damage during and after cryopreservation of sperm in *T. tambra* was the main investigation. Meanwhile, the usefulness of exogenous antioxidants supplementation in improving low post-thaw quality and poor fertilization performance of *T. tambra* cryopreserved sperm is yet to be explored. Therefore, a new extender formulation with improved post-thaw quality for cryopreservation of *T. tambra* sperm is targeted to be developed in the present study. Application of necessary antioxidants to reduce or prevent the adverse effects of oxidative stress is expected to contribute new knowledge to this field and enable improved fish reproduction in the aquaculture industry.

1.4 Research hypothesis

It is anticipated that microsatellite (SSR) genotyping of both cryopreserved milt specimens and live broodfish collected from various sources could facilitate the assessment of genetic variability and genetic structure of the *Tor* spp. in the collection. Information on the status of genetic variability is essential for the conservation (sperm cryo-banking) and effective management of the broodstock and future genetic improvement and selective breeding in *Tor* spp.

As for the cryopreserved gametes, the assumption is that cryo-damage on the cryopreserved sperm of *T. tambra* can be due to oxidative stress. Oxidative

stress-related damages and high reactive oxygen species (ROS) content could cause low post-thaw quality and poor fertilization performance of the cryopreserved sperm. This cryo-damage can be ascertained by measuring the concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), LPO, analysis of DNA lesion rate of the developmental-related genes in the frozen sperm, and DNA fragmentation. An analysis of damages caused by cryopreservation in the most susceptible genes to oxidative stress could help identify the best candidate genes to be used as sentinels of DNA damage. It is also hypothesized that supplementation antioxidants in the extender media used for cryopreservation can remove oxidative stress and enhance the post-thaw quality in *T. tambra* sperm. Thus, with necessary antioxidants, adverse effects from oxidative stress can be reduced or prevented. The antioxidant properties of selected antioxidants in enhancing the post-thaw sperm quality of *T. tambra* were also explored in the study.

1.5 Objectives of the study

The overall aim of this study was to genotype the collection of Malaysian mahseer obtained for broodstock development and sperm cryo-banking using microsatellite (SSR) markers and investigate the physiological changes of the cryopreserved spermatozoa supplemented with antioxidants in *T. tambra*. This included: (i) genetic characterization of the *Tor* spp. from different sources in Malaysia, (ii) assessment of the effects of antioxidants supplementation in extender media and cryostorage duration on post-thaw quality in *T. tambra* sperm, and (iii) evaluation of the LPO and oxidative DNA damage caused by freezing-thawing and cryostorage procedures. Therefore, the specific objectives of this study were:

1) To determine the genetic diversity, population structure of this *Tor* spp. collection and their relatedness, and subsequently to establish the genetic profile for the *Tor* spp. collection by employing the SSR markers.
2) To determine the effects of exogenous supplementation of ascorbic acid, α-tocopherol, Trolox and reduced glutathione in the extender media on post-thaw sperm quality of *T. tambra* sperm.
3) To evaluate the cryostorage duration on post-thaw sperm quality of *T. tambra* sperm.
4) To determine the oxidative stress and DNA damage caused by freezing-thawing and cryostorage procedures in *T. tambra* sperm.

1.6 Scopes of work

This study covers two main research areas, i.e., molecular genetics using microsatellite (SSR) markers and spermatozoa cryopreservation. For the molecular genetics study, the main focus was on the SSR genotyping of cryopreserved milt specimens and the lived broodstock of the *Tor* spp. collected

from different geographical sources in Malaysia. As for the spermatozoa cryopreservation study, the focus was on determining the effects of antioxidants supplementation in the extender media and the cryostorage duration on post-thaw sperm quality, evaluation and quantification of oxidative stress and DNA damage caused by freezing-thawing and cryostorage procedures. Research samples (frozen milt, scale and milt samples of live broodstock) used in the study were provided by Freshwater Fisheries Research Division, FRI Glami Lemi, Titi, Jelebu, Negeri Sembilan, Malaysia. The following aspects were studied in this research project:

1) Genetic characterization and DNA profiling of *Tor* spp. sperm from cryobank and live broodstock at Fisheries Research Institute Glami Lemi using SSR markers via fragment analysis. The SSR profile generated serves as the reference for future progeny traceability purposes, essential for the effective management of broodstock and genebank of the indigenous *Tor* spp. in the region.
2) Assessment of the antioxidant properties of vitamins (ascorbic acid, α-tocopherol), Trolox (vitamin E analogue) and reduced glutathione (GSH) in enhancing post-thaw quality in *T. tambra* sperm. The use of antioxidants in cryopreservation of *T. tambra* sperm and the optimum concentration of each antioxidant with improved post-thaw quality were determined.
3) Evaluation of cryostorage duration on post-thaw sperm quality in *T. tambra*. The effects of freezing and cryostorage over time on spermatozoa quality in sperm motion characteristics, viability, morphology and plasma membrane integrity were examined.
4) Determination of the oxidative stress and DNA damage on the cryopreserved sperm of *T. tambra*. Oxidative stress and DNA damage in frozen-thawed *T. tambra* sperm were assessed through quantification of 8-OHdG content using fluorescent probes, measurement of lipid peroxidation, examination of the DNA integrity, quantification of relative sperm telomere length, and DNA damage through DNA lesion analysis in specific genes using quantitative polymerase chain reaction (qPCR).

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