Role of Wnt signaling and zinc in common carp testicular function

A thesis submitted to University of Hyderabad for the award of the degree, Doctor of Philosophy in
Animal Sciences

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CERTIFICATE

This is to certify that the thesis entitled "***Role of Wnt signaling and Zinc in common carp testicular function***" submitted by Ms**. S. Deepa** bearing registration number **13LAPH04** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the School of Life Sciences is a bonafide work carried out by her under my supervision and guidance. This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. <u>Publications:</u>

1. **Deepa, S.,** Murugananthkumar, R., Gupta, Y.R., Gowda K.S.M., Senthilkumaran, B. **2019.** Effects of zinc oxide nanoparticles and zinc sulfate on the testis of common carp, *Cyprinus carpio*. **Nanotoxicology 13:1-18.** (ISSN: 17435390, Taylor & Francis Group); (***see Chapter 2***)
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Further, the student has passed the following courses towards the fulfillment of course work required for the award of Ph.D

Course Code	Name	Credits	Pass/Fail
AS 801	Seminar 1	1	Pass
AS 802	Research Ethics & Management	2	Pass
AS 803	Biostatistics	2	Pass
AS 804	Analytical Techniques	3	Pass
AS 805	Lab Work	4	Pass

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DECLARATION

I, **S. Deepa**, hereby declare that this thesis entitled ***"Role of Wnt signaling and Zinc in common carp testicular function"*** submitted by me under the guidance and supervision of ***Prof. B. Senthilkumaran*** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

Date:

Name: S. Deepa

Signature of the Student
Regd. No.: 13LAPH04

Dedicated to my Ph.D supervisor and my loving parents

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Abbreviations

11-KT – 11-ketotestosterone

ad4bp/sf-1 – Adrenal 4 binding protein/steroidogenic factor 1

AP – Alsterpaullone

Cyp19a1a - Ovarian aromatase

DAB – 3,3′-diaminobenzidine

DAPI – 4',6-diamidino-2-phenylindole

DCFH-DA – 2′, 7′-dichlorodihydrofluorescein diacetate oxidation

DMEM – Dulbecco's Modified Eagle's Medium

Dmrt1 – Doublesex and mab-3 related transcription factor 1

dph – Days post hatch

EDS – Energy Dispersive X-Ray Spectroscopy

FBS – Fetal bovine serum

FE-SEM – Field emission scanning electron microscopy

FITC – Fluorescein isothiocyanate

FSH – Follicle stimulating hormone

FSH-R – Follicle stimulating hormone receptor

FTIR – Fourier-transform infrared spectroscopy

h11b – 11β-hydroxylase

hsd11b – Hydroxysteroid (11- β) dehydrogenase 1

hsd20b – Hydroxysteroid (20- β) dehydrogenase

ICP-OES – Inductive coupled plasma resonance spectroscopy

IF – Immunofluorescence

IHC – Immunohistochemistry

Iono – calcium ionophore A23187

kDa – Kilo Dalton

L-15 – Leibovitz -15

LH – Luteinizing hormone

LH-R – Luteinizing hormone receptor

Mt – Metallothione

PBS – Phosphate buffered saline

PCP – Planar cell polarity

qPCR	–	Quantitative real-time PCR
SEM	–	Scanning electron microscope
Sox9	–	SRY-related high mobility group box 9a
T	–	Testosterone
TEM	–	Transmission Electron Microscopy
TPEN	–	N,N,N,Ntetraki(2pyridylmethyl)ethylenediamine
Wnt	–	Wingless-type MMTV (Mouse mammary tumor virus)
Wt1	–	Wilms' tumor suppressor 1
XRD	–	X-ray powder diffraction
Zn	–	Zinc
Zn-NPs	–	Zinc oxide nanoparticles
$ZnSO_4$	–	Zinc sulphate
Znt1	–	Zinc transporters

Contents

Testicular function in fish plays a crucial role in male reproduction and it is regulated by steroidogenesis and spermatogenesis. In teleosts, sex steroids play a key role in regulating gametogenesis and gonadal physiology. In all vertebrates including teleosts spermatogenesis starts with diploid spermatogonial stem cells to develop further to haploid genome to produce spermatozoa. Teleosts are evolutionarily diverse and the process of spermatogenesis as well as steroidogenesis are variable from species to species. There are several factors like germ cell marker (*vasa* and *nanos*), steroidogenic enzyme (*h11b, hsd11b*, and *hsd20b*) and transcription factor (*ad4bp/sf-1*, *wt1*, *dmrt1* and *sox9*) genes are known to be involved in testicular function in teleosts as in other vertebrates (Borg, 1994; Raghuveer and Senthilkumaran, 2009). Regulatory mechanisms of testicular function including growth and development are under the control of ligands and co-factors (Hughes, 2001). Progression of studies associated to testicular development under gene regulatory mechanisms are limited in teleosts due to fewer reports on this line.

Spermatogenesis

Spermatogenesis is a complex biological process containing mitotic and meiotic cell divisions in all vertebrates including teleosts. The process is regulated by both intrinsic (Sertoli and germ cells) and extrinsic (androgens) factors/molecules. Sertoli cells are the key regulator of testis development induced by follicle stimulating hormone (FSH) and luteinizing hormone (LH) for the progression of spermatogenesis. Testosterone (T) and 11-ketotestosterone (11-KT) are the potent androgens for the initiation of spermatogenesis in fishes, while in mammals T and 5α-dihydrotestosterone are the counterparts (Kretser et al., 1998).

In vertebrates, the process of spermatogenesis occurs during the production of sperm from primordial germ cells (PGCs). In male mouse embryo, the PGCs migrates to genital ridge to form sex cords after maturation, the sex cords hollow out to form seminiferous tubule, where the tubules segregates in to Sertoli cells (Zhao et al., 1996). The Sertoli cells contain N-cadherin molecules and carbohydrate receptor, the galactosyltransferase molecules of spermatogonial germ cells bound to Sertoli cells for the development and nourishment of sperm (Newton et al., 1993; Pratt et al., 1993). Further, PGCs arise at the gonad to start multiple divisions to initiate spermatogenesis. Initially, it divides to type-A1 spermatogonial cells which are found adjacent to the outer basement membrane of the sex cords that is capable of regenerative in nature. The size of type-A1 spermatogonial cells is smaller than PGCs with ovoid nucleus along with chromatin associated nuclear membrane. A1 spermatogonia further divides to A2 spermatogonia which are the paler type of cell that divides to produce A3 spermatogonia which then produce type-A4 spermatogonia. Therefore, each of type-A spermatogonia stem cells are capable of self-renewal. The type-A4 spermatogonia undergo three divisions. It forms another A4 spermatogonium which is also self-renewal in nature, or it can further undergo apoptosis, or it can distinguish into the first dedicated stem cell type, the intermediate spermatogonium. The intermediate spermatogonium divides to type-B spermatogonia to become spermatozoa. The type-B spermatogonia divides once to generate the primary spermatocytes, then the cells enter meiosis (Dym, 1994). Figure 1 depicts testis architecture of vertebrates to distinguish the types.

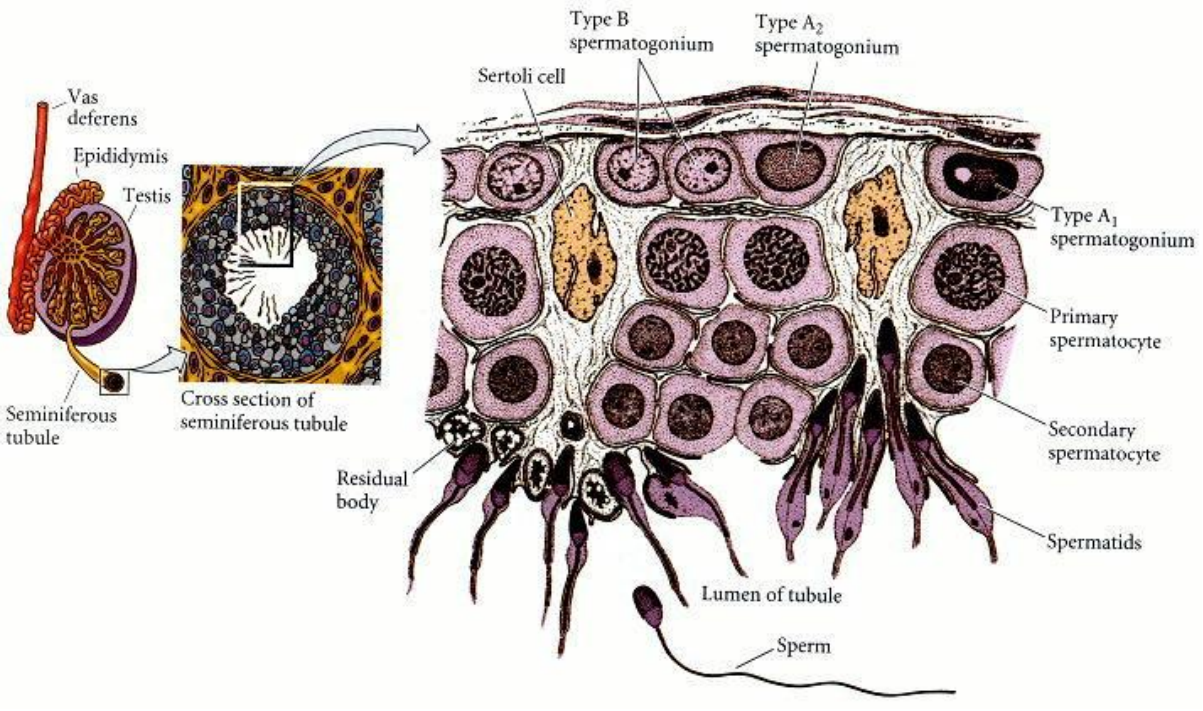

Fig. 1. Diagrammatic representation for variable structure of testis in vertebrates (cystovarian type and gymnovarian type).

Image was adopted from Dym, M. 1977.

In fishes, the process of spermatogenesis varies widely due to the species-specific overlapping cycles, the spermatogenesis process may be continuous or discontinuous (Billard, 1986). In most of the fishes, testis architecture is made up of lobule with the cyst. The cyst is surrounded by Sertoli cells for the nourishment of sperm production. The Sertoli cells contain a single or clonally developing group of spermatogenic cells. The Sertoli cells secrete various factors and hormones for the survival of germ cells. Unlike mammals, fish spermatogonial cells are not attached to basal lamina and spermatozoa lacks acrosome (Schulz et al., 2010). The lobule lumen release cysts with sperm for the process of spermiation. The Sertoli cells are suspended in fluid sperm, the milt. In natural conditions, sperm of the fish is released naturally according to their breeding cycle, whereas in laboratory conditions the stomach of the fish is gently squeezed to obtain milt.

Occasionally, it will be difficult to induce spermiation in teleosts even after induction under laboratory conditions (Sudhakumari and Senthilkumaran, 2013; Panda, 2016). Depend upon the environment and temperature the duration of spermatogenesis of fishes varies from 4-21 days (Schulz et al., 2010). In view of this, it is necessary to understand the hormone mediated regulatory mechanisms of spermatogenesis. Figure 2 represents the mammalian and fish, segments of spermatogenesis differences in cystic and non-cystic types of testis structure.

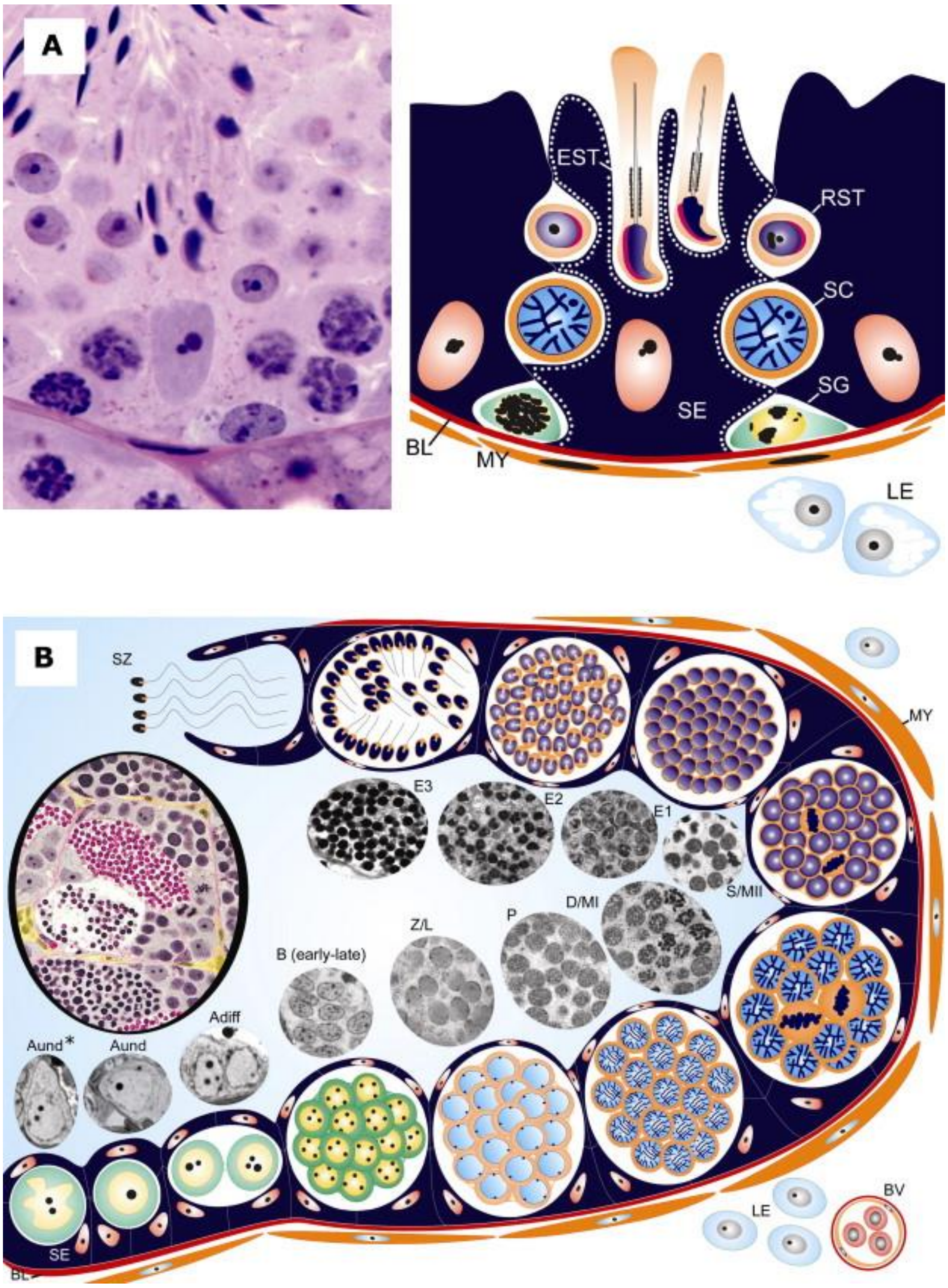

Fig. 2. Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis.

Adopted from Schulz et al., 2010

Hormonal regulation of spermatogenesis

In general, glycoprotein hormones such as FSH and luteinizing hormone (LH) are secreted by anterior pituitary that regulate somatic testicular cells for the process of spermatogenesis. These two hormones pose common α-subunit and are distinguished by variable β-subunit, which is a part of secreted growth factors of TGF β superfamily (Pierce and Parsons, 1981). In mammals, both FSH and LH bind to their specific receptors (FSH-R and LH-R) which are restricted to Sertoli and Leydig cells for testicular development (Rannikki et al., 1995). Several studies found the staining of FSH-R in Sertoli cells and LH-R in Leydig cells (Eblen et al., 2001; Lei et al., 2001). Interestingly in fish, the specificity of hormone with their particular receptor is not clearly understood. Other than hormones, few genes involved in spermatogenesis are activin and glial cell-derived neurotrophic factor, which are stimulated by FSH (Tadokoro et al., 2002). Various hormonal regulatory mechanisms are known to play an essential role in testicular development in teleosts is represented in figure 3.

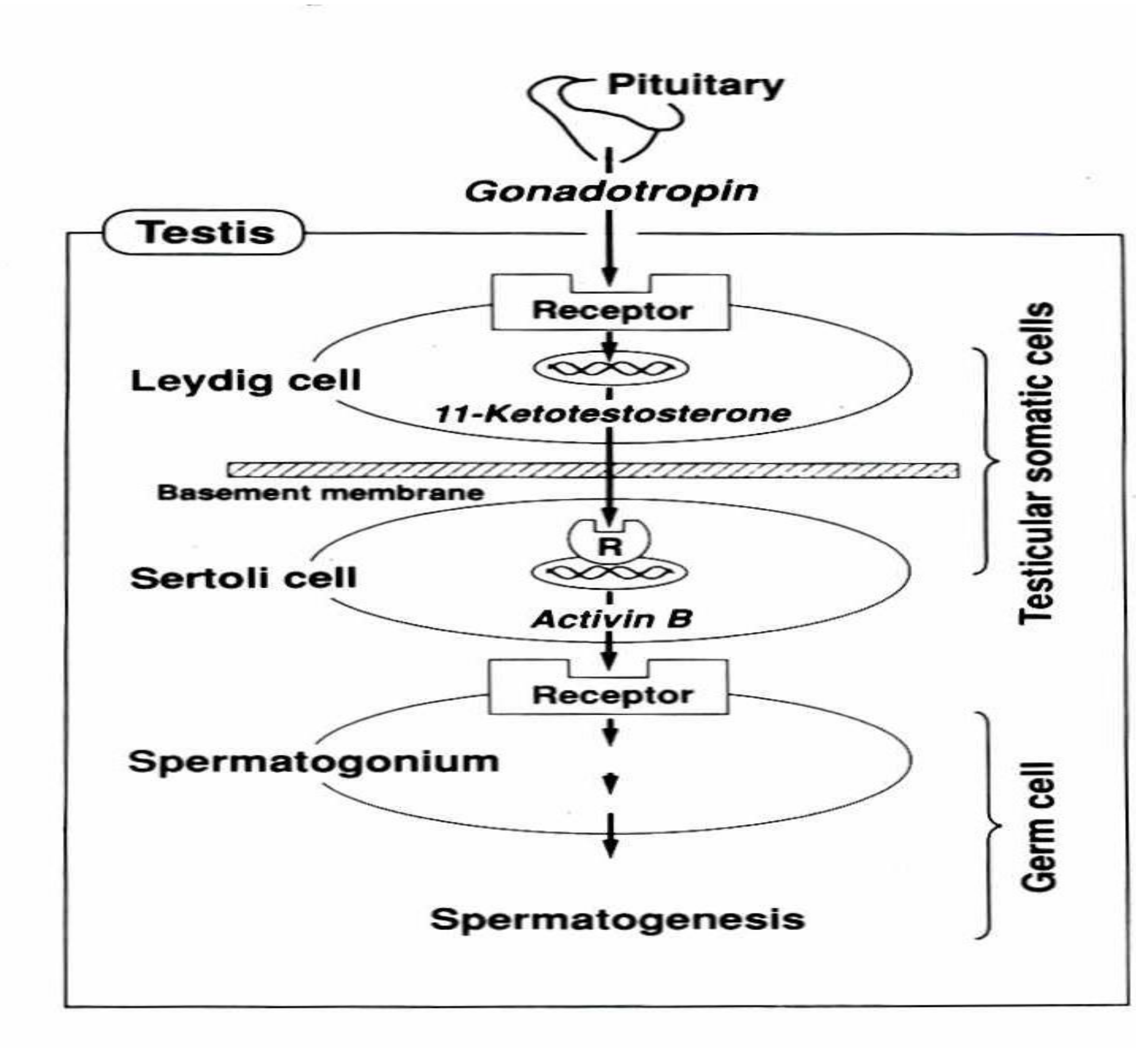

Fig. 3. Schematic representation of hormonal regulation of spermatogenesis in teleosts.

Adopted by Nagahama, 1994

In teleost fish models, the levels of 11-KT and T increased in plasma to promote testicular development and growth (Borg, 1994; Norberg et al., 2001). *In vivo* and *in vitro* studies showed that 11-KT as a potent androgen for spermatogenesis (Miura et al., 1991) whereas T act as efficient activator of hypothalamo and hypophyseal axis (Amano et al., 1994; Xiong et al., 1993; Montero et al., 1995) for the activation of testicular steroidogenesis. Thus, 11-KT is considered as the principal androgen in teleosts (Borg, 1994) which is first characterized from plasma of male sockeye salmon *Oncorhynchus* *nerka*, (Idler et al., 1960) and T is converted to 11β-hydroxytestosterone by 11β-hydroxylase (Ozon, 1972; Nagahama, 1994; Kobayashi et al., 1998; Rajakumar and Senthilkumaran, 2016) and then to 11-KT by 11β-hydroxysteroid dehydrogenase (Rajakumar and Senthilkumaran, 2016). For maturation of both sperm and egg 17α, 20β-Dihydroxy-4-pregnen-3-one (17α, 20β-DP) acts as a maturation including steroid (Miura et al., 1991; Nagahama, 1994; Sreenivasulu and Senthilkumaran, 2012). During spawning season of salmonids, 17α, 20β-DP was found to be high (Vizziano et al., 1996) and also involved in meiotic maturation (Miura et al., 2006; Senthilkumaran, 2011). These mechanisms direct testicular development, growth as well as to advance the process, which are under the control of changing centralizations of ligands, for example, hormones and growth factors (Hughes, 2001). The reports on the gene regulatory mechanism, engaged with these movements, related to testicular advancement in teleosts are limited (Zeng et al., 2016). Among various molecules, Wnt signaling seems essential for the process of gametogenesis (Gifford, 2015; Windley et al., 205) as it is pivotal for cellular development.

Wnt signaling molecules

Wnt signaling molecules are conserved pathway throughout evolution due to their role in the developmental process from embryogenesis. Wnt is named from fusion of two terms, wg derived from the *Drosophila* segment polarity gene *wingless* and *int* resulting from the proto-oncogene *integration-1* (Wodarz et al., 1998). There are about 19 Wnts in mammals and in general, Wnt siganaling pathways are canonical or Wnt/β-catenin dependent pathway and noncanonical or β-catenin-independent pathway which is further divided to planar cell polarity (PCP) and Wnt/Ca^{2+} pathway (Habas et al., 2005). Wnt plays a crucial role in cell fate differentiation, movement of the cell, cell polarity, axis formation, organogenesis, and stem cell renewal process. In teleosts, 14 Wnt forms have been identified yet their role in spermatogenesis has not been explored in detail except in zebrafish and few other species (Verkade and Heath, 2008). Report from our laboratory unveiled the importance of *wnt4/5* in regulating teleostean oogenesis (Prathibha and Senthilkumaran, 2014)

Wnt proteins bind to Frizzled (Fz) receptor family, which is a seven transmembrane- G-protein coupled receptor. Ten Fz receptors have been identified so far in human and they contain N-terminal extra-cellular cysteine-rich domain, which determines the Wnt glycoproteins binding capacity (He et al., 2004; Schulte and Bryja, 2007). The Wnt canonical pathway requires low-density-lipoprotein-related protein5/6 (LRP5/6) as a co-receptor along with Fz for the cascade mechanism (Cadigan et al., 2006) and then it bifurcates as canonical, and non-canonical Ca^{2+} or PCP pathways for the induction of gene expression in cells (Wallingford and Habas, 2005). The schematic representation of Wnt canonical and non-canonical pathways is represented in figure 4

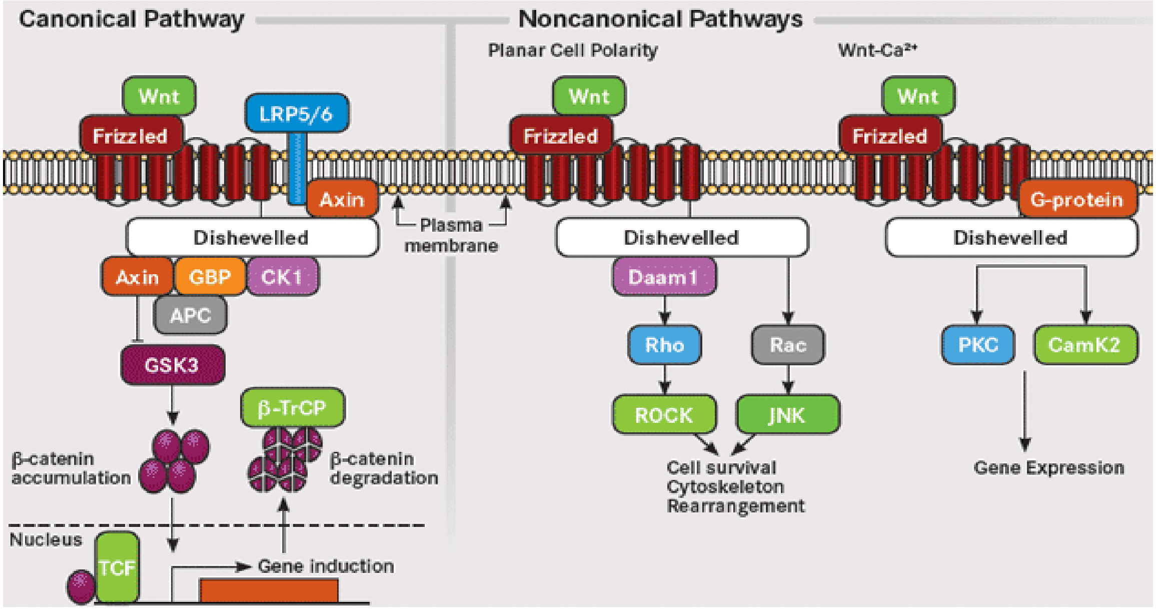

Fig. 4. Wnt canonical and non-canonical pathways in vertebrates.

Adapted from Prunskaite-Hhyyrylainen et al. 2014.

Role of Wnt signaling molecules in testicular function

In zebrafish, Wnt canonical pathway is known to play a vital role in steroidogenesis while Wnt non-canonical pathway plays a prominent role in development for the convergence of organ primordia (Matsui et al., 2005). In vertebrates, among all forms of Wnts, Wnt4, Wnt5, and Wnt8 are recognized to be indispensable for testicular differentiation and embryogenesis (Novin et al. 2015; Wikramanayake et al., 2004). Before gastrulation, five *wnt* genes (*wnt1*, *wnt4*, *wnt5*, *wnt8*, and *wnt16*) are expressed maternally during embryogenesis, Incidentally, *wnt4*, *wnt5* and *wnt8* with different ligands execute overlapping or similar functions and substitution for one another for the gene expression for embryo formation in sea urchin (Cui et al., 2014), while their role in the gonadal reproductive cycle a recrudescence is unclear.

In vertebrates, Wnt4 is known for its role in embryogenesis and sex-determination/differentiation (She et al., 2014). In human, Wnt4 is necessary for male fetal reproductive tract development and also activates *Sry* gene, which is important for male sex determination. Loss of Wnt4 in early genital ridge resulted in lower level of Sertoli cell differentiation (Jeays-Ward et al., 2004). In females, it plays a major role in oocyte selection, follicle formation, and maturation (Jaaskelainen et al., 2010; Prunskaite-Hyyryläinen et al., 2014). Cosidering these reports, it is essential to understand the role of Wnt4 in teleostean spermatogenesis.

The putative role of signaling molecules, Wnt5, and Wnt8 have been proved to stimulate spermatogonial stem cell maintenance in mouse (Yeh et al., 2011). Their role has been identified in mice from postnatal to adult stage along with their transcripts localization in spermatocytes and spermatids which validated their significant role in spermatogenesis (Kerr et al., 2013). In mammals, it also showed synchronized role with Wnt4 for the initiation of meiosis for ovarian follicular growth (Naillat et al., 2010). In vertebrates, Wnt4 and Wnt5 act via the non-canonical pathway while Wnt8 involves through the canonical signaling pathway (De, 2011). In teleosts, *wnt4/5* regulates ovarian steroidogenesis (Prathibha and Senthilkumaran, 2014), yet Wnt signaling pathways are still not known.

In vertebrates, after the activation of Wnt/β-catenin pathway, β-catenin translocates to nucleus which binds to transcription factor for the activation of steroidogenesis and spermatogenesis. Steroidogenic factor-1 (SF-1 in mammals, *ad4bp/sf-1* in fishes) is a transcription factor, known for their role in gonadal development as well as in the progression of spermatogenesis (Hoivik et al., 2010; Murugananthkumar and Senthilkumaran, 2016). The process of steroidogenesis and spermatogenesis begin when, steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) mobilizes cholesterol to mitochondria of steroidogenic cells. START and StAR/StarD7 maintain the steroidogenesis which invariably supports spermatogenesis. Later, the activated Wnt/β-catenin pathway binds to SF-1, which inturn activates StAR/StarD7 for proper steroidogenesis and spermatogenesis (Martin et al., 2013). Figure 5 depicts the probable role of Wnt signaling in steroidogenesis.

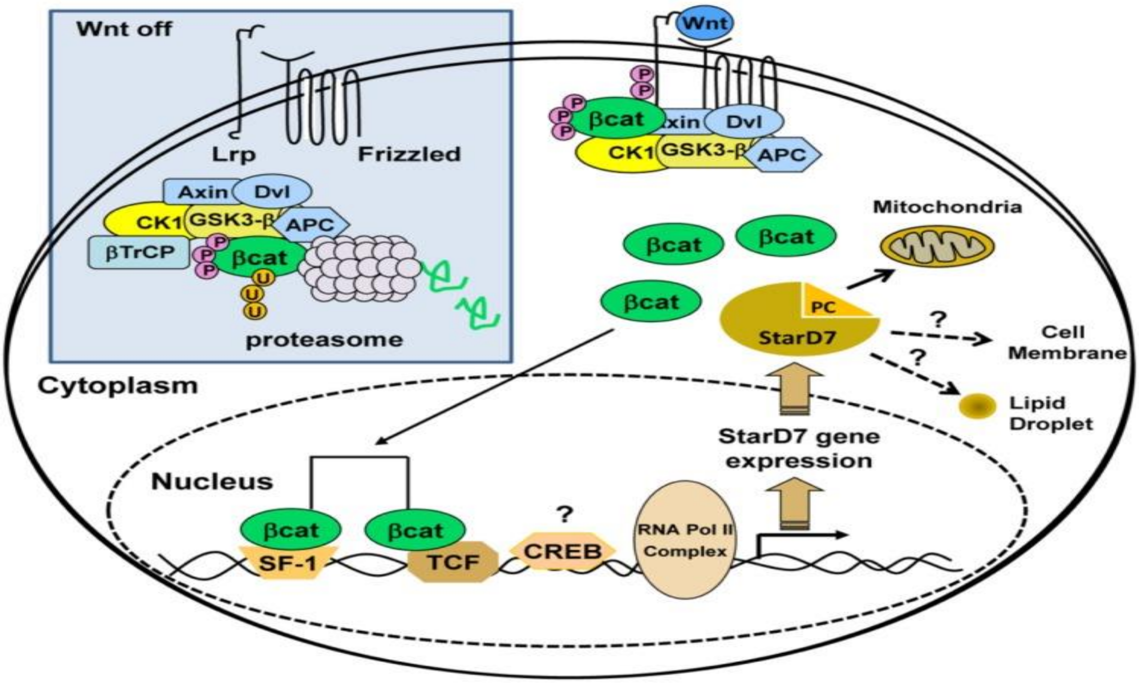

Fig. 5. The proposed model shows Wnt signaling involved in steroidogeneses induced gene expression, based on current data.

Adapted from Martin et al., 2013.

Role of Zn in testicular function

In addition to signaling molecules, trace element Zinc (Zn) also plays crucial role in spermatogenesis or testicular function. In mammalian male reproductive system, Zn involvement is necessary during mitotic and meiotic divisions for the conversion of spermatogonia to spermatocytes. Zn is known to involve in cell proliferation and acts as a co-factor for both deoxyribonucleic acid (DNA)-ribonucleic acid (RNA) polymerase and RNA-dependent DNA polymerase activities. In rats, Zn-deficient testis, showed decreased RNA, DNA, and protein levels due to decline of total RNA and protein content in spermatozoa along with declined fertility rate (Hidiroglou et al., 1984). Decreased levels of Zn in the testis lead to spermatogenic failure and few reports state that lack of Zn causes gonadal dysfunction like a reduction of testicular weight and shrinkage of seminiferous tubules (Hidiroglou et al., 1984; Fallah et al., 2018). The role of Zn in spermatogenesis is given in table1.

Spermatogenesis	**Functions of Zinc**
Initiation of spermatogenesis	Involves in ribonuclease activity
During spermatogenesis	Participate in spermatozoa maturation
End of spermatogenesis	Enhance sperm motility

Table 1. Table represents the functions of Zn during different phases of spermatogenesis

Adapted from Hidiroglou et al., 1984.

In mammals, deficiency of Zn causes low levels of LH receptor, decreased steroid production, and cell damage in Leydig cells (Kellokumpu et al., 1981; Prasad, 1985). Presence of Zn seems to increase in emerging spermatocytes in meiosis phase for DNA condensation (Kundu et al., 1996). It also involves in DNA package to generate spermatids, and after post ejaculation, it maintains the spermatozoa throughout life span in mammals (Pradeepa, 2007).

In mice, Zn was localized in Leydig cell membrane which suggests a role for Zn in the endocrine regulation of spermatogenesis (Croxford et al., 2012). In the Japanese eel, Zn has been identified as an essential trace element for spermatogenesis and showed higher concentration of Zn on day 9 after induction, additionally profound levels of Zn was seen in germ cells other than interstitial and Sertoli cells, where the accumulation of Zn is found in germ cells which involve in meiosis phase for the proliferation of spermatogenesis (Yamaguchi et al., 2009).

Inhibition of Zn with Zn specific chelator, N,N,N,N-tetrakis-(2-pyridyl methyl) ethylenediamine (TPEN) lead to down-regulation of canonical Wnt/β-catenin signaling pathway in mouse C17.2 neural stem cells. (Zhao at al., 2015). This poses a genuine question of interactive role of Zn and Wnt signaling in teleosts.

Though deficiency of Zn affects testicular growth and spermatogenesis, excess level of Zn leads to disruption of spermatogenesis and testicular function in male rats (Ali et al., 2013). Few studies explain the adverse effects of Zn toxicity leading to oxidative stress to gill, skin, or through oral route (Yah et al., 2012; Gupta et al., 2016). Existing scientific reports proposes that Zinc Oxide nanoparticles (ZnO-NPs), due to its smaller size and surface area can cross the blood-barrier rand translocate to nucleus of testicular cells to generate adverse effects (Lan and Yang, 2012). Exposure of Zn at sublethal levels adversely affect haematological parameters, survival, and hatchability in fish (Hughes and Tort, 1975; Tuurala and Soivio, 1982; Kori-siakpere and Ubogu, 2008)Industrial effluents release of various NPs to the aquatic system causing defects in breeders which eventually affect the biomass of fish production. Among NPs, ZnO-NPs showed considerable toxic levels in several lakes and rivers in India (Ghorade et al., 2014; Jain et al., 2004; Goswami and Sanjay, 2014). Thus, it is noteworthy to analyze the importance Zn in terms of its regulatory or inhibitory role on testicular function using a teleost fish model.

Teleost as animal model

Unlike mammals, fishes unveil as a good experimental model organism to address functions in development, morphology, physiology and behaviour due to evolutionary conserved across phyla. Their conservation helps to study various development related genes and mechanisms between divergent taxa. Thus, they have been used as a good animal model for comparative analysis, which underlies the evolution of development and physiology (Harris et al., 2014). The whole phylogeny with bootstrap support values and names for supraordinal taxa is represented in figure 6.

In addition, among vertebrates, fishes are essential for human as a food source due to its economic value which paves the necessity to maintain fish population and improve fish stocks.

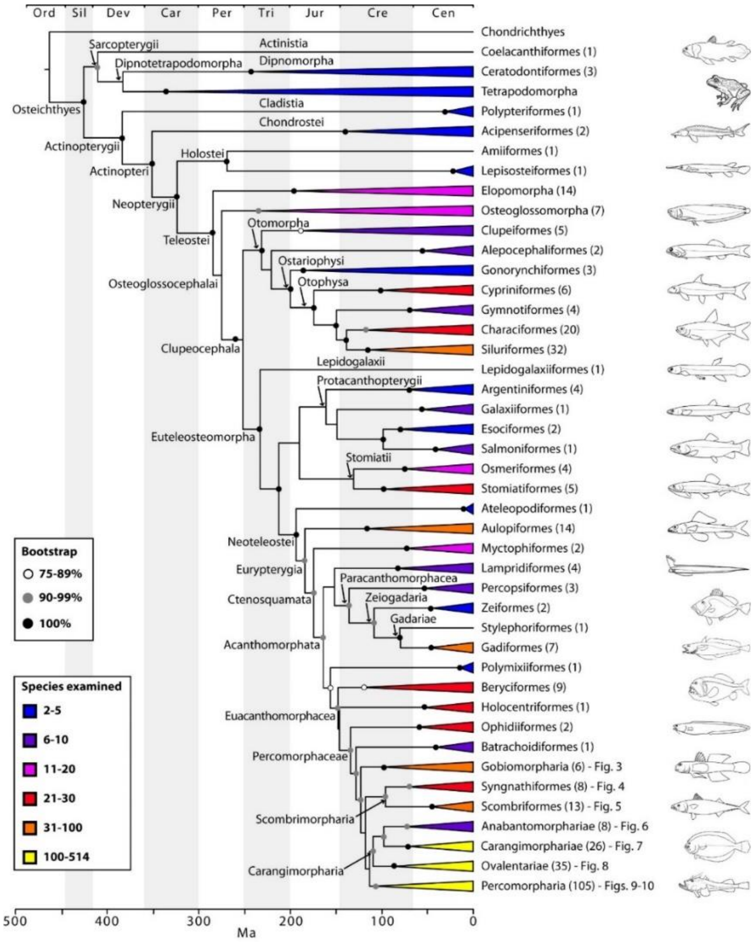

Fig. 6. Main phylogenetic hypothesis of bony fish groups collapsed to depict higher-level clades.

Adopted from Betancur et al., 2013.

Common carp: Animal model

The common carp, *Cyprinus carpio* used in this study an important food fish in India which belongs to the order *Cypriniformes* and the family *Cyprinidae*. The scientific classification was given in figure 7. It is the largest family of freshwater fish, and it inhabits in freshwater environments like ponds, lakes streams, and rivers, and also found rarely in brackish-water. In addition to their economic importance, common carp are also the best ‘ecological engineer' due to their ecological modification characteristics (Matsuzaki et al., 2009; Bajer and Sorensen, 2015). Common carp is one of the few freshwater species having multiple spawning in Indian aquatic habitats. Among teleosts (bony fishes), several model organisms are available due to variations in development and life cycle. In the present study, common carp has been chosen as animal model. Figure 7 shows common carp scientific classification.

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: *Cyprinus*
Species: *carpio*

Fig. 7. Scientific classification of common carp

Adopted from google image

Development of testis in common carp consists of six stages. It is difficult to distinguish sexes in stage I. Stage II testis comprises of multiple spermatogonia, and appearance of testicular duct. Stage III testis entails several layers of testicular duct. In stage IV testis, large primary spermatocytes, smaller secondary spermatocytes, and smallest spermatids could be observed. In stage V, testis releases a large number of sperm through spermiation. Stage VI testis contains only spermatogonia, some primary spermatocytes, and connective tissue reminiscent, after milt exudation. Testes then revert to stage III and redevelop from there for renewal during recrudescence (Koldras et al., 1990).

In view of this existing state of knowledge in teleostean male reproduction**,** the present research work was designed to understand the role of Wnt signaling and Zn in testicular development, steroidogenesis, and spermatogenesis using common carp as an animal model. Though several genes regulating the testicular growth and development has been studied in detail, but there is an inadequate information about the role of signaling molecules, transducing steroidogenic pathway in teleosts. Further, in teleosts, very less information are available to reveal the comprehensive interactive role of Wnt signaling and Zn in relation to gonadal development. As explained earlier, Zn is an essential trace element to promote spermatogenesis (Yamaguchi et al., 2009) yet it is not known whether it is possible to use administration of Zn as a strategy to promote spermatogenesis in immature or juvenile fish, physiologically. To this end, present study was also focused to analyze the impact of ZnO-NPs in comparison with Zinc sulphate ($ZnSO_4$) on testicular function in common carp.

Based on this, two major chapters have been drafted for present Ph.D thesis entitled ***"Role of Wnt signaling and Zn in common carp testicular function"*** as indicated below with a consolidated summary.

Chapter 1. Interactive role of Wnt signaling and Zn in regulating testicular function of common carp

Chapter2. Effects of ZnO-NPs and $ZnSO_4$ on the testis of common carp, *C. carpio*

The references have been compiled alphabetically in each chapter to accommodate specific citations pertaining to each chapter. Abbreviations mentioned in page nos. vii and viii were followed in the thesis chapters, however chapter-wise abbreviations were also done

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Chapter 1

Interactive role of Wnt signaling and Zn in regulating testicular function of common carp

Abstract

Present study identified significant involvement of *wnt4*, *wnt5* and *wnt8* in testicular growth of common carp. Wnt4 protein was localized in spermatocytes and interstitial cells of carp testis. Specific activation of Wnt signaling revealed that Wnt4 and Wnt5 might act through non-canonical while Wnt8 prefers canonical pathway. Impact of Wnt signaling was evident in regulating steroidogenic enzyme and testis-related genes upon Wnt blockade. Incidentally, Zn is an essential trace element to promote spermatogenesis in teleosts and mammals. This study demonstrated the interplay of Wnt and Zn in promoting spermatogenic progression. Single administration of different doses of Zn, *in vivo*, elevated the expression of *wnt* and *Zn transporter* genes. Single dose (30μg/g BW) Zn treatment elevated *steroidogenic* enzyme and *testis-related* genes which coincides with increased androgens. Conversely, single dose administration of Zn chelator to Zn administered (30μg/g BW) fish downregulated *wnt*, *steroidogenic* enzyme and *testis-related* genes, and androgens emphasizing the prominent role of Zn in testicular function perhaps through Wnt signaling. Similar effects were seen in *in vitro* experiments with Zn chelator. Bioaccumulation of Zn and histological analysis revealed the importance of Zn in spermatogenic progression and sperm motility. Various assays related to cell viability and proliferation, confirmed the role of Zn in

promoting spermatogenesis. Flow cytometric analysis confirmed Zn-induced elevation of *wnt* and *Zn transporters* genes in germ and other cells. Exposure of Wnt inhibitor, canonical, non-canonical activators and Zn confirmed that Zn promotes Wnt signaling. Taken together, Wnt signaling and Zn induction via Wnt specific pathways promote spermatogenesis in teleosts.

1. Introduction

Wnt signaling is evolutionary conserved among all vertebrates which involves β-catenin, the canonical and the non-canonical pathways which is subdivided in to PCP and Wnt/Ca^{2+} pathways (Komiya et al., 2008). Wnt proteins are secreted cysteine-rich proteins having an important role in several developmental processes and tissue homeostasis. Incidentally, the role of Wnts are not limited to cell proliferation, their requirement in PCP, cell fate specification and differentiation is well demonstrated (Logan et al., 2004). Although 14 members of Wnt family and specific pathways were identified in mammals (Croce et al., 2008), including fishes their functions were not specified in lower vertebrates. Among all forms of Wnts, Wnt4, Wnt5 and Wnt8 are known to be essential for embryogenesis and testicular differentiation in few vertebrates (Ward et al., 2004; Wikramanayake et al., 2004; Novin et al., 2015) yet their role during testicular development including reproductive cycle is not explored in lower vertebrates including teleosts.

Wnt4 is considered as one of the essential factors for embryogenesis and sex-determination/differentiation in vertebrates (She et al., 2014). Wnt4 is important for the development of male reproductive tract, with downstream of *Sry* and upstream of *Sox9* and *desert hedgehog*. In mammalian testis, Wnt4 mutant showed Sertoli cell differentiation with compromise implicating the pivotal role of Wnt4 during early genital ridge formation (Ward et al., 2004). In humans, it is required for oocyte selection, follicle formation and maturation (Jaaskelainen et al., 2010; Hyyryläinen et al., 2014). The significance of this gene in teleostean reproduction has not been explored in detail except being identified in zebrafish and few other species (Ungar et al., 1995; Wu and Chang, 2009; Jean et al., 2018; Kossack et al., 2018) during embryogenesis. More recent study from our laboratory revealed prominent role of *wnt4/5* in catfish ovarian development and oogenesis of catfish (Prathibha and Senthilkumaran, 2017).

Wnt5 and Wnt8 have been demonstrated to promote spermatogonial stem cell maintenance in mouse (Yeh et al., 2011). The roles of Wnt5 and Wnt8 have been identified from postnatal to adult stage and their transcripts were localized in spermatocytes and spermatids of mice (Kerr et al., 2013). The roles of these molecules in teleostean reproduction is not reported in detail, though it plays a coordinated role along with Wnt4 for the initiation of meiosis in mammals for ovarian follicular growth (Naillat et al., 2010). Wnt4 and Wnt5 act via the non-canonical pathway, while Wnt8 acts through the canonical pathway in *Drosophila*. However, De (2011) has shown that the canonical and non-canonical Wnt signaling pathways are difficult to classify.

Incidentally, in mammals, Wnt signaling seems to be regulated by various factors (Yang et al., 2016) and one of them being the co-factor or trace element, Zn. Recently, Zn deficiency resulted in downregulation of canonical Wnt/ β-catenin in the mouse C17.2 neural stem cells (Zhao et al., 2015). In addition, Zn is an essential trace element for normal growth and reproduction and it has vital roles in various biological activities. It is also required for the functionality of more than 300 enzymes and stabilization of DNA in humans (Frassinetti et al., 2006). In vertebrates, bioaccumulation of Zn as a trace element is higher in testis than other tissues (Bedwal et al., 1994). Marginal deficiency of Zn in human causes reproductive health issues (Roohani et al., 2013) and low level of Zn intake causes defects in sexual development, and gonadal dysfunction which further delays pregnancy and lactation in mammals (Bedwal et al., 1991; Saxena et al., 1993). In mammals, Zn plays a major role in regulating LH, FSH, prolactin, gonadal differentiation, testicular steroidogenesis and gene expression of several steroid hormone receptors (Bedwal and Bahuguna, 1994). In vertebrates, Zn gets transported in to cells through metallothione (*mt*), which has chaperone role in maintaining Zn in cytoplasm. Among Zn transporters, *mt* and *znt1* have a crucial role in transportation of Zn to the cells and they are also known as Zn biomarkers, especially in testicular development (Maret, 2011; Maret, 2012). In human, the presence of Zn in prostate and semen warrants significant role in male reproduction. Zn is abundant in all types of germ cells of testis and gets involved in the process of spermatogenesis in addition to maintenance of sperm chromatin structure in mammals while absence of Zn in testis lead to spermatogenic arrest (Turgut et al., 2003; Bjorndahl et al., 2010; Kumari et al., 2011). In accordance with these reports, progression of spermatogenesis, and the regulation of sperm motility orchestrated by Zn in the Japanese eel has been documented (Yamaguchi et al., 2009). However, it remains to be seen whether Wnt signaling is mediated through Zn.

In view of this, we made an attempt to understand the role of Wnt signaling during testicular development of the common carp, *C. carpio* through expression analysis under different testicular development stages as well by in depth pathway analysis using specific inhibitor of Wnt and activators of non-canonical and canonical pathways. Present study also analyzed the role of Zn in carp spermatogenesis. Considering the lack of information on Wnt signalling and interplay of Zn, comprehensive analysis has been performed under *in vivo* and *in vitro* conditions with additive and deficient routes using various high-end methods.

2. Materials and methods

2.1 Fish maintenance and sampling

Different age groups of *C. carpio* were obtained from Karnataka Veterinary Animal and Fisheries Sciences University, Bangalore, India. All fishes were acclimated for a fortnight under ambient photothermal conditions (12 h L/12 h D; 20 ± 2 °C) and fed commercial carp food, *ad libitum,* (*Tubifex* worms, Taiyo feed mill PVT. LTD., Chennai, India). Different tissues were collected (n=5) during gonadal recrudescence from adult male (testis, liver, kidney, brain, and muscle) and female (ovary) common carp for tissue distribution analysis. Testis samples were also collected (n=5) for ontogeny analysis from 60, 80, 100, 120, 140,160 180 and 250 days post hatch (dph). In brief, the mesonephric gonadal complex was isolated using fine sterile forceps under a stereoscopic dissection microscope from carp hatchlings at 60, 80 and 100 dph. In common carp, male and female gonads could be identified from 120 dph onwards, hence the testis samples were collected from 120–200 dph. Testis from pre-spawning, spawning and post-spawning periods were collected (n=5) for reproductive phase expression analysis.

2.2 Identification of different forms of Wnt through transcriptome analysis and cloning of *wnt4*, *wnt5* and *wnt8* from carp testis

Differentially expressed genes were obtained by sequencing with Illumine Nex Seq 500 sequencer with 75PE chemistry. Six forms of Wnt gens (*wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt10* and *wnt11*) were obtained from transcriptome data. Using TRI reagent® (Sigma) total RNA was isolated from common carp testis following manufacturer's instructions and cDNA synthesis was done using verso cDNA synthesis kit (Thermo Fisher Scientific) protocol. Open reading frame (ORF) was obtained for *wnt5* and *wnt8,* and partial cDNA fragment of *wnt4* was cloned by using the specific primers (table 1) designed from the transcriptome sequence data (Gene Expression Omnibus: Series accession number GSE112157) obtained earlier (Anitha et al., 2019). In addition, other specific primers used for various genes including for 18srRNA specific primers qPCR analysis are mentioned in Table 1.

Table 1. List of primers used for cDNA cloning and qPCR analysis

Gene name/symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
wnt2	CTATCTGTCCGTGGCAAT	AGCATCGCGGGATGAGCC
wnt4	TCACCGACATGTGTGCATC	ATCATAATTTGTTAACAAAT
wnt5	ATCGAGATCTGCAAACTA	GAGGTCTTTGGGACGAGCCG
wnt8	ATGGATCATCTGTGCAAC	CCCGTTGGGAGCTCTCCC
wnt10	AGTGACACCGTTCGCTGC	GTTAGTGTACTTCGAG
wnt11	CTGCAAACGGAACCTCG	TCAGGGGTGAAGTGAG
vasa	CCATGGTGATCGGGAGCA	GTTCCCACAGCGTCCGGT
nanos	ACATGATCAGAGGCATGCAG	TCTCTCCGTTGTGTTTGCAG
mt	GGATCCTTGCGATTGCGC	AGCAGCTGGAGCCGCAGG
znt1	CGCCTGCTGTGCATGCTG	CAGCGAAGCGCACCGCTA
hllb	GGCAGTGGAGCGAATGCTGAA	GCACCCCGGGGAACCTTGAGC
hsd11b	ATCACAGGGTGCGACTCGGGTTTCG	CGGCTGAGTGATGTCCACCTA
hsd20b	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA
sf-1/ad4bp	CGGACCTGGAAGAGTTGT	GGTTCTCTTGGCATGCAG
wt1	ACACACAGGAGAGAAACCGTACCA	TATGTGTCCGGGTGTGGGTCT
dmrt1	GCAGCCCAGGTGGCGTTA	GCCGTTCCATGCTGGAGG
sox9	TCTGGCGGCTGCTGAATGAAGG	CTCGGTATCCTCGGTTTCACC
18s rrna	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG

2.3 Histological analysis of testicular reproductive phase

Histological analysis was performed for pre-spawning, spawning and post-spawning phases of carp testis to ascertain gonadal stage. In brief, testis were dissected out and fixed in Bouin's solution (15:5:1) for overnight at room temperature (RT). Tissues were then washed with 70% ethanol to remove excess fixative and dehydrated using graded series of ethanol followed by xylene treatment to embed in paraplast (Sigma). Using rotatory microtome (Leitz, Wetzlar, Germany) sections were cut at 5 µm thickness. The tissue section slides were treated with xylene to deparaffinise followed by rehydration with a series of ethanol and then stained using haematoxylin and eosin. The slides were mounted with DPX mountant (SRL) after dehydrating with graded series of ethanol. All sections were observed under Olympus CX41 bright field light microscope attached with a Micropublisher (Model-MP3.3) cooled CCD camera (Q-imaging, BC, Canada) and photographed.

2.4 Immunohistochemistry (IHC) and Immunofluorescence (IF)

IHC and IF with Wnt4 antibody was performed in pre-spawning phase of carp testis by using the protocol of Rajakumar and Senthilkumaran (2014). In brief, tissues were dissected out and kept in fixative 4 % paraformaldehyde in phosphate buffered saline (PBS, Sigma) for overnight at 4 °C. Further, it is embedded using cryomedium (OCT compound medium, Leica Microsystems) after rinsing with PBS. With the help of cryoblock testis was sectioned at 7 µm thickness on to Poly-L-Lysine coated glass slide using a cryostat (Leica CM1850, Leica Microsystems). The sections were hydrated using PBS followed by blocking with 10 % normal goat serum (Merck Bangalore Genei) for 1 hr at RT. Polyclonal antibody of Wnt4 (1: 1,000) or pre-adsorbed antibody (for negative control) was added on to sections and kept for overnight at 4°C. Next day, the sections were rinsed with PBS for three times and incubated with HRP-conjugated secondary antibody (1: 5,000; Merck Genei) for 1 hr at RT and ABC reagent (avidin-biotinylated horseradish peroxidase complex) supplied in VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, CA) for 30 min in humid chamber. The slides were developed using substrat, 3′, 3′-diaminobenzidine (DAB) as chromogen and H_2O_2 (Vector Laboratories) after PBS wash. After developing sections were washed in PBS followed by counterstain with haematoxylin (Qualigens fine chemicals, Mumbai, India). Finally the slides were mounted using DPX after dehydration using a graded series of ethanol. In the case of IF, FITC-conjugated anti-mouse secondary antibody (Vector Laboratories) was used and further counterstained with DAPI (Vector Laboratories) and mounted using VECTASHIELD® mounting media. For IHC, microphotographs were taken with a CX41 Olympus microscope (Olympus Corporation, Tokyo, Japan) fitted with a Micropublisher 3.3 RTV-CCD camera through Q capture Pro 6 software (Quantitative Imaging Corporation), while for IF, an IX81 Olympus Microscope (Olympus Corporation.

2.5 qPCR

Total RNA was prepared from various tissues and testis samples at different stages and after various experiments described below. Similarly, total RNA was also prepared from various primary cell culture or flow cytometry sorted samples explained below. The samples were treated with TURBO™ DNase (Thermo Fisher Scientific) at 37°C for 30 min to eliminate genomic DNA. The quantity and quality of total RNAs were assessed using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For all qPCR experiments, 1 µg of total RNA was used with Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and each experiment was carried in triplicate with n=5 biological samples. The first strand cDNA was amplified using verso® reverse transcriptase with random hexamers by following supplier's protocol. qPCR was performed by using atleast one or both primers designed at exon-intron boundary with the amplicon size of ~ 150–250 bp. Universal thermal cycle conditions was programmed according to the ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) machine. Considering 18SrRNA as endogenous control, the expression was normalized for ΔCt value (ΔCt = Target gene Ct -18S rRNA Ct). The experiment was validated by 2^- ΔCt formula to get relative expression. To check the PCR amplification, melting-curve was analysed. qPCR analysis was done to analyse the expression of *wnt4*, *wnt5*, *wnt8*, *mt*, *znt1*, *vasa*, *nanos*, *h11b*, *hsd11b*, *hsd20b*, *ad4bp/sf-1*, *wt1*, *dmrt1* and *sox9* with different tissue samples as explained earlier. Primary cell culture samples control as well as various treatments mentioned below under 2.7 were also used for preparing total RNA.

2.6 Western blot analysis of Wnt4 and β-catenin

The antibodies used to carry out western blot analyses are anti-mouse Wnt4 (Santa Cruz biotechnology) and anti-mouse anti-β catenin Antibody (Sigma). The Wnt4 monoclonal and β-catenin polyclonal antibodies raised against c-terminal regions of mouse Wnt4 and β-catenin showed 90 and 99% homology, respectively to their counterparts of carp. Western blot was done using Wnt4 and β-catenin to test canonical and non-canonical pathways, respectively for the control and experimental samples mentioned under 2.8 and 2.9. Same set of samples were used for anti-β-tubulin (Santa Cruz biotechnology) antibody as control. All the western blot analysis was performed by using the method described in Rajakumar and Senthilkumaran (2014). In brief, testis and primary cells were homogenized with homogenization buffer containing 50 m M Tris-HCl, pH 7.4, 150 m M NaCl, 1 m M DTT, and

ProteoBlockTM protease inhibitor cocktail (Thermo Scientific). The 12% SDS-polyacrylamide gel was ran with a homogenate concentration of 100 μg of protein and then transferred on to a nitrocellulose membrane (Pall Life sciences), 5% skimmed milk in Tris-buffered saline (TBS) was used for blocking after transfer and incubated for 1 hr at RT and was washed 7 times for 7 min each in TBS with 0.1% Tween 20 (TBST). Later, incubating with a 1: 5,000 dilution of Wnt4 and β-catenin antibody respectively for overnight at 4 °C. The next day, membranes were incubated with HRP conjugated goat anti-mouse IgG secondary antibody (Merck Genei) for 1hr at RT. Following washing, blots were developed with commercial ECL western blotting substrate as per the manufacturer's instructions (Promega). The densitometry was not carried put for the western blot analysis.

2.7 Carp primary testicular cell culture

Testicular culture was prepared by following the protocol of Yamaguchi et al. (2009) and Murugananthkumar and Senthilkumaran (2016) after minor modifications. In brief, mature male carps were dissected using sterile forceps and scissors, and washed thrice with cell culture grade of PBS, to remove blood. The testes were minced thoroughly with scissors and blunt end of syringe to make testis in to small pieces for suspension in L15 medium (Sigma) which was then resuspended with 1 ml of 0.5% trypsin (Sigma) with 0.8% collagenase type lV (Sigma), 0.0015% DNase (Sigma) and incubated at RT for 30 min. The minced tissues were filtered using corning strainer 40 μm (Sigma), the cells collected were centrifuged at 3000x g for 3 min. PBS was added to the pellet to remove debris, later the cells were resuspended in 2ml medium mixture of dulbecco's modified eagle Medium (Sigma), 10% fetal bovine serum (Sigma), antibiotic and antimycotic (Sigma) agents. Cells (~ 40,000 cells/well) were then seeded in a 96-well plate and incubated at 37○C for 24 h in a 5% CO_2 incubator

(CO_2/Tri-Gas Incubator SCA/SMA-165, ASTEC, Kasuya-Fukuoka, Japan). The viability of primary cells-was observed by checking its quality using giemsa fluorescent dye (SRL). This method is used for the subsequent experiments related to testicular cell culture. All the primary testicular cell culture based experiments mentioned below were done in six wells per each treatment/concentration, which was repeated thrice.

2.8 Treatment with Wnt inhibitor and activators

Carp testicular cell culture was prepared as per the protocol explained earlier and incubated with Wnt antagonist IWP-2 (Sigma), Wnt canonical activator, alsterpaullone (AP, 5 mM, Cat. No: I0161, Sigma) and Wnt non-canonical activator, calcium ionophore A23187 (Iono, 10 mM, Sigma) at three different doses 2, 5 and 10 µM, after dissolving in dimethyl sulfoxide (DMSO, Sigma) for 24 hrs, in a 5% CO_2 incubator (ASTEC, Kasuya-Fukuoka, Japan and the expression analysis (qPCR) was performed for each treatment mentioned below with *wnt4*, *wn5*, *wnt8* and *β-catenin*. Further these samples were also used to test Wnt4 and β-catenin proteins by western blot analysis.

Two control groups of primary testicular cell were maintained for each set of experiment to identify Wnt specific pathways. To test the non-canonical pathway three groups of primary testicular cells treated with10µM IWP-2 was maintained for 24hrs. After 24hrs 1st group received vehicle while the second and third group received 10µM AP group and 10µM Iono, respectively and maintained for another 24 hrs. One more group of primary testicular cells was treated with 10µM Iono for 24hrs and then 3µg/ml Zn was added and maintained further 24hrs. Thus, the entire duration of experiment was 48hrs for all the experimental or control samples.

To test the canonical pathway three groups of primary testicular cells treated with10μM IWP-2 was maintained for 24hrs. After 24hrs, 1st group received vehicle while the second and third group received 10μM AP group and 10μM Iono, respectively and maintained for another 24 hrs. One more group of primary testicular cells was treated with10μM AP for 24 hrs and then 3μg/ml Zn was added and maintained further 24hrs. Thus, the entire duration of experiment was 48hrs for all the experimental or control samples.

2.8.1 Ca^{2+} influx analysis after Wnt non-canonical activation

The control and 10 μM Iono treated cells were stained with acid fuchsin calcium salt (Sigma) to observe Ca^{2+} influx under a light microscope, olympus cx41 light microscope (Olympus corporation, japan) fitted with a mp3 micropublisher 3.3 RTV (Q-imaging, BC, Canada) software.

2.9 Effects of single administration of different doses of Zn and Zn chelator on carp testis

Forty adult 250 days old male carp (n=5) was divided in to 8 groups (n=5). First three groups received of 10, 20, and 30 μg/g BW (body weight) Zn (Sigma), injection once intraperitoneally and then maintained for 14 days. Group 4 was received vehicle treatment to serve as control. Groups 5, 6 and 7 received Zn specific chelator N,N,N',N'-tetrakis-(2-pyridylmethy) ethylenediamine (TPEN, Sigma) at the doses of 0.1, 1 and 10 μM. Group 8 received only vehicle instead of Zn chelator to compare as control. All groups were maintained in the aquarium tanks separately with proper replenishment of water and fish feed for the duration of the experiment. After treatment, the fishes were sacrificed and testis was dissected out from all the groups to perform real-time PCR (qPCR) to analyse the gene expression of *wnt4*, *wnt5*, *wnt8*, *mt*, *znt1*, *hllb*, *hsd11b* and *hsd20b*, histology and Inductive coupled plasma resonance spectroscopy (ICP-OES) experiments. These samples were also used for western blot analysis of Wnt4 and β-catenin. Blood was drawn to obtain serum for T and 11-KT assays. Fish maintanance, experiments and sacrifices were performed by following the general guidelines of institutional animal ethical committee, University of Hyderabad (CPCSEA, Inst. Reg.No.151/1999 dt.22.07.1999).

2.10 Enzyme immunoassay (EIA) of androgens

Blood was collected from control, Zn 30 μg/g BW and pre-treated 30μg/g BW Zn-10 μM TPEN group and then centrifuged at 10,000 x g for 10 minutes in 4° C to obtain serum for the measurement of T and 11-KT using EIA kit (Cayman) manufacturer's protocol. The intra- and inter-assay variations were calculated, and the assay validation was done using the method described by Swapna et al. (2006).

2.11 Histology

Histological analysis of all treated samples (control, 30 μg/g BW Zn and pre-treated 30μg/g BW Zn-10 μM TPEN group) were performed as per the method explained earlier. Randomly, forty lumens (n=5) of the testis of control, 30 μg/g BW Zn and pre-treated 30μg/g BW Zn-10 μM TPEN groups were analyzed to determine the percentage of spermatocytes, and spermatids/sperm.

2.12 Sperm motility test

Sperm motility test was performed as per the method described by Kagawa et al. (2009). In breif, testis were dissected to obtain milt (1:1000 dilution) and 1μl of diluted milt was addded to the activation medium, which contained the mix of 1 ml of 450 mM sodium chloride buffered and 20 mM of HEPES at pH 7.6 and placed at 4 ° C. The milt was placed on microsciopic slide using the tip of the dissection needle to obtain a homogenous mixture for motility analysis. Temperature range was chosen based on the preliminary data for sperm motility in carp (Dadras et al., 2016). Sperm motility of control, 30µg/g BW Zn and pre-treated 30µg/g BW Zn-10 µM TPEN group induced samples were observed under olympus cx41 bright-field light microscope, using 4X objective eye piece and sperm motility was scored. The motile spermatozoa was considerd based on forward movement of the spermatozoa and the percentage of motile spermatozoa was calculated based on the method described by Viveiros et al. (2003), which is denoted as: 0 as no cells movement, 1 as 25% motile cells, 2 as 50% motile cells, 3 as up to 75% motile cells and 4 as more than 75% motile cells. The score values were calculated at least in 20 different fields for each sample (n = 5) to obtain mean and standard error of mean.

2.13 Estimation of bioaccumulation of Zn by ICP-OES and localization of Zn using ZnAF-2DA after administration of Zn and Zn chelator

Bioaccumulation of Zn in testis was estimated by ICP-OES (ULTIMA 2 ICP Optical Emission Spectrometer, Horiba Scientific, Edison, NJ) in control, 30µg/g BW Zn and 10 µM TPEN administered 30µg/g BW Zn pre-treated fishes as per the method described by Ashoka et al. (2009). In brief, the samples were weighed and added in 15 ml falcon for digestion containing 2 ml hydrogen peroxide and 3 ml nitric acid, which was immediately held in water bath for 2 hrs at 85°C to get dissolution of the metal from the testicular tissue. The dissolute samples were subjected to ICP-OES in order to estimate the level of Zn in the tissue. The operating conditions of the machine and the final readings are given in table 2. Each samples were carried triplicate for each time and the R^2 value was calculated (≤ 2.2 % and ≤ 2.5 %). Mean intra- and inter-assay co-efficient of variations was measured respectively. The obtained results are represented in the units of parts per billion (ppb).

Detection of Zn in testis was further authenticated by performing Zn specific probe ZnAF-2DA and staining as per the manufacturer's instructions (Sigma) to observe under fluorescent microscope (model BX-51-34FL microscope; Olympus).

Table 2: Operating conditions of the ULTIMA 2 ICP Optical Emission Spectrometer.

Specifications	Conditions
RF power	1.0 kW
Wavelength	213.856
Plasma gas flow rate	12LPM
Auxiliary gas flow rate	0.5LPM
Nebulizer gas flow rate	0.8LMP
Nebulizer	Concentration Nebulizer
Spray chamber	Cyclonic spray chamber

2.14 Flow cytometric analysis (FACS) of control and Zn 30 µg/g BW exposed primary testicular cells after sorting

Primary testicular cells of control and 3µg/ml Zn exposed cells were sorted using flow cytometry by following the protocol of Zhao et al. (2016). Using Epics Altra (Beckman-Coulter, Hialeah, FL), argon gas was operated in the range of 488-nm, the sheath pressure was 8.5 psi, and the flow rate did not exceed 1500 cells/sec, the forward scattered (FS), side scattered (SS) signals were amplified and the cells were sorted based on the size and granularity to acquire germ cells (A gate) and other cells types (B gate) from the cell suspension. In order to identify the cell types obtained by FS and SS dependent flow cytometry, qPCR was performed relative to marker genes for germ cells (*vasa* and *nanaos*).

Both control and 3µg/ml Zn exposed sorted germ and other cells were washed with PBS, then centrifuged at 3000x g for 3 min to obtain the pellet. Those were further processed for total RNA preparation and c-DNA synthesis subsequently, qPCR analysis of various genes. The calculation was done as per the method explained earlier.

2.15 Spermatogonial cell proliferation after exposure of Zn and Zn chelator

2.15.1 Cell viability assay

Primary cells were exposed to 1, 2 and 3µg/ml of Zn and 0.01, 0.1 and 1 µM TPEN groups were incubated separately for 48 hrs. To each treated groups, 20 µl of 5 mg/ml MTT (Sigma) dissolved in DMSO was added and incubated for 4 hrs. The plate was then read in an ELISA microplate reader at 570 nm (Bio-Rad). The experiment was done in triplicate for each set (n = 5). The relative cell viability (%) was calculated and expressed in percentage relative to untreated cells.

2.15.2 DNA proliferation (BrdU) assay

Control, 3µg/ml Zn and 1µM TPEN exposed primary cells were incubated in 96-well microtiter plates in 37°C, 5 % CO_2 for 72 hrs and to this BrdU (Novus biologicals) was added (10 µM final concentration) and kept for 30 min in RT and then anti-BrdU was added and incubated at RT at 60 min. After three washing cycles, chemiluminol was added and the readings were measured using the multiplate reader at 570 nm (Bio-Rad). Integration time was measured at 1000ms and gain 130 and the results were expressed as relative fluorescence units.

2.15.3 Cell cycle analysis

For cell cycle analysis, control, 3μg/ml Zn and 1μM TPEN exposed primary testicular cells were digested by trypsin and washed with ice cold PBS and then fixed using 70% ethanol and kept at 4°C for 24 hrs. The following day, cells were washed with PBS and treated with RNase (Sigma) at 37°C for 30 mins. Further, cells were stained with propidium iodide (Sigma) along with 0.5% tween-20, to analyse for cell cycle phases (BD Biosciences, Franklin Lakes, NJ, USA) with flow jo software.

2.15.4 DNA fragmentation (tunnel) assay

Genomic DNA was prepared from control, 3μg/ml Zn and 1μM TPEN groups using GSure® Fast Tissue Kit (GCC Biotech) and the isolated genomic DNA sample was loaded in 1.5% agarose gel to check fragmentation of DNA, if any. The protocol was followed according to the manufacturer's instructions (Takara).

2.16. FACS with specific antibodies Wnt4 and β-catenin for identification of Wnt specific pathways

Two control groups of primary testicular cell were maintained for each set of experiment to identify Wnt specific pathways. To test the non-canonical pathway two groups of primary testicular cells treated with 10μM IWP-2 was maintained for 24hrs. After 24hrs 1st group received vehicle while the second received 10μM Iono and maintained for another 24 hrs. Another group of primary testicular cells was treated with10μM Iono for 24 hrs and then 3μg/ml Zn was added and maintained further for 24hrs. Thus the entire duration of experiment was 48hrs for all samples.

To test the canonical pathway two groups of primary testicular cells treated with10µM IWP-2 was maintained for 24hrs. After 24hrs, 1st group received vehicle while the second group received 10µM AP group and maintained for another 24 hrs. Another group of primary testicular cells was treated with10µM AP for 24 hrs and then 3µg/ml Zn was added and maintained further for 24hrs. Thus, the entire duration of experiment was 48hrs for all the experimental samples.

FACS analysis was performed using Wnt4 and β catenin antibodies to identify Wnt signaling pathways by following the protocol of Bassity et al. (2012). In brief, cells were maintained at 4°C throughout staining protocol until FACS. Each treated and control cells were stained with Wnt4 and β catenin antibodies for 30 min at the dilution (1:10) respectively, followed by secondary antibodies goat anti-mouse FITC (Thermo scientific) at 1:100; and goat anti-rabbit Alex 488 (Cat. No: 112058001A, Merk) at 1:100 for 30 min. Cells were centrifuged at 300 x g for 5 mins and the pellet was washed with PBS. Using Epics Altra (Beckman-Coulter, Hialeah, FL), argon gas was operated in the range of 488nm, with logarithmic amplifiers. The florescent data were collected and the binding capacity was calculated using flow Jo software.

2.17 Statistical Analysis

All data were represented as mean ± standard error of mean (SEM). All data were compared by using one-way ANOVA followed by Student–Newman–Keuls (SNK) post hoc test. Statistically significant data were considered with probability of $P \leq 0.05$.

3. Results

3.1 Cloning of *wnt4*, *wnt5* and *wnt8* from carp testis

Differentially expressed *wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt10* and *wnt11* genes, were obtained from transcriptome analysis, where in partial cDNA fragments of 670 bp of *wnt4***,** ORF of ~1 kb and ~1 kb of *wnt5* and *wnt8* were cloned from carp testis using RT-PCR by designing specific primers (table 1) transcriptome data (Anitha et al., 2019). *wnt4* was not attempted for full length cloning of cDNA, as the partial fragment did possess specific signature domains to carry out expression analysis needed for the present study.

3.2 Tissue distribution, ontogeny and gonadal phase expression analysis

Expression analysis of *wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt10* and *wnt11* (Fig. 1A) in testis and ovary of adult carp was performed to compare the isoforms. Among all the forms, *wnt4*, *wnt5* and *wnt8* showed dominant expression in ovary and testis when compared to *wnt2*, *wnt10* and *wnt11*. Tissue distribution analysis was performed for *wnt4*, *wnt5* and *wnt8* in different tissues of carp that includes testis, ovary, kidney, liver, brain and muscle (Fig. 1B) wherein *wnt4* showed dominant expression in ovary and testis followed by kidney, liver, brain and muscle. The level of expression was high in testis followed by ovary, muscle, brain, kidney, and liver for *wnt5*. The expression of *wnt8* was high in ovary followed by testis, brain, liver, muscle and kidney. The expression analysis of ontogeny study (Fig. 2A-C) at 60, 80, 100, 120, 140, 160, 180 and 250 dph for *wnt4*, *wnt5* and *wnt8* revealed synchronous elevated expression levels from 140 to 250 dph. Incidentally, 60 to 100 dph samples were undifferentiated gonads (mesonephric gonadal complex) while 120 to 250 dph were testis. Thus, elevated expression of *wnt* isoforms from 140 to 250 dph indicated a positive correlation with testicular development. During testicular cycle (Fig. 3A), *wnt4* showed high expression in pre-spawning phase followed by spawning and post-spawning phase and *wnt5* showed high expression in spawning phase, followed by pre-spawning and post-spawning phases, while *wnt8* expression levels increased from pre-spawning to spawning to post-spawning phases.

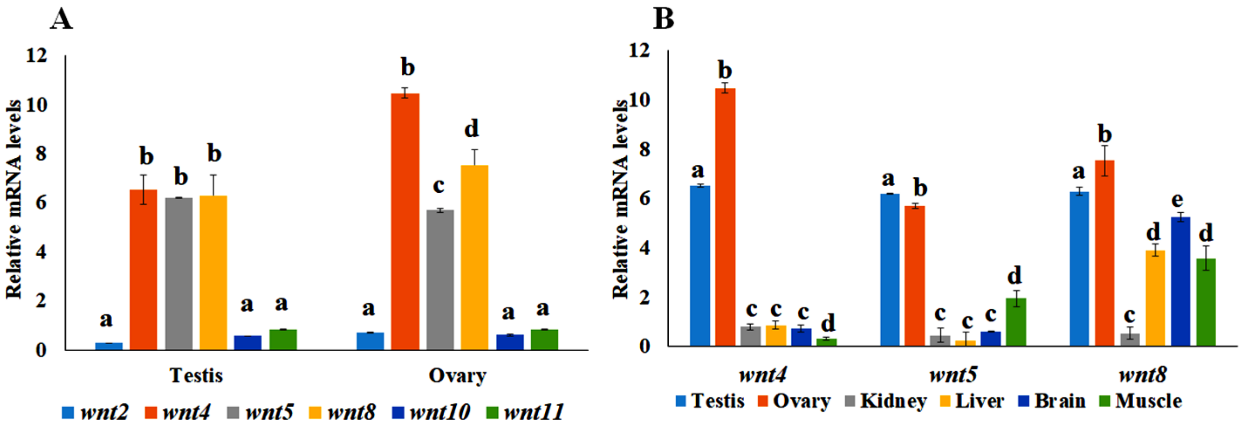


Fig. 1. Relative mRNA levels of (A) *wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt10* and *wnt11* in testis and ovary of carp and *wnt4*, *wnt5* and *wnt8* (B) in different tissues of adult carp. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post hoc test). All the comparisons were made specifically for tissue type (A) or each isoform (B).

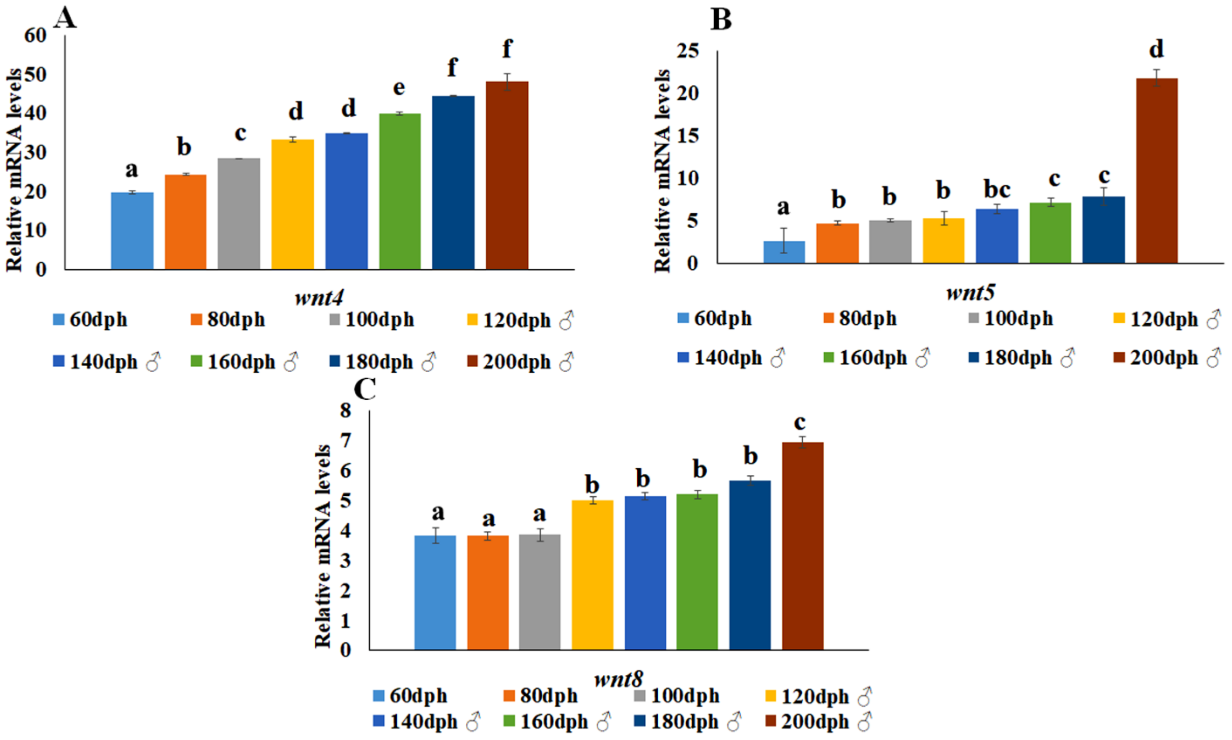


Fig. 2. Expression analysis of *wnt4* (A), *wnt5* (B) and *wnt8* (C) in different stages of gonad development from 60, 80, 100, 120, 140, 160, 180 and 250 dph. Note: The samples from 60 to 100 dph were undifferentiated gonads (mesonephric gonadal complex) while 120 from 250 dph samples were testis. Abbreviation: dph- day post hatch. The statistical data was calculated as in the Fig.1.

3.3 Histology of reproductive phases of testis

Testicular cycle of male carp was assessed by histology to determine the stages. In all three phases (Fig. 3B-D), testis showed well organised basal lamina or interstitial layer, spermatocytes and densely packed spermatids/sperm, however, stage-specific pattern was precisely evident. The pre-spawning phase (Fig. 3B) showed a greater number of spermatocytes and spermatids/sperm. In spawning phase (Fig. 3C), the number of spermatids/sperm increased to fill the lumen, however, the number of spermatocytes decreased. While in the post-spawning phase (Fig. 3D) testis showed a smaller number of spermatocytes and spermatids/sperm. All these results were explained qualitatively by using photo microphotographs of testis shown in Fig. 3B-D.

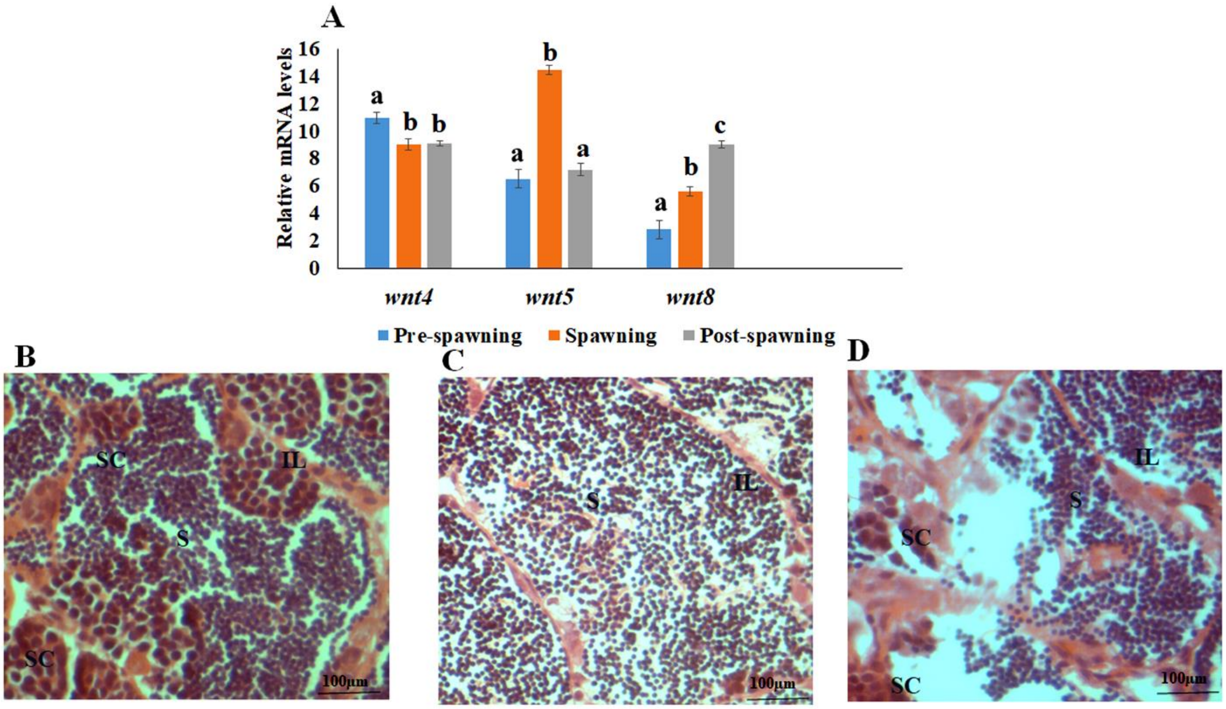


Fig. 3. Relative mRNA levels of *wnt4*, *wnt5* and *wnt8* (A) in different phases of (pre-spawning, spawning and post-spawning) testicular reproductive cycle. Haematoxylin and eosin (B) staining of testis showing different testicular phases of carp. Abbreviation: IL - Interstitial layer SC- spermatocytes and S- spermatid/sperm. The statistical data was calculated as in the Fig.1.

3.4 Immunoreactivity of Wnt4 in carp testis

IHC and IF was performed with Wnt4 antibody in testis at pre-spawning phase of adult carp testis (Fig. 4 A-E). Wnt4 protein could be detected in spermatocytes and interstitial layer (Fig. 4A), while negative control having pre-absorbed antibody with excess Wnt4 antigen counterstained with haematoxylin displayed no immunoreactivity in carp testis (Fig. 4B). It was further confirmed by IF staining which showed similar results in the adult testis of pre-spawning phase (Fig. 4 C) where spermatocytes and interstitial cells stained using FITC with DAPI as counter stain (Fig. 4 D-E).

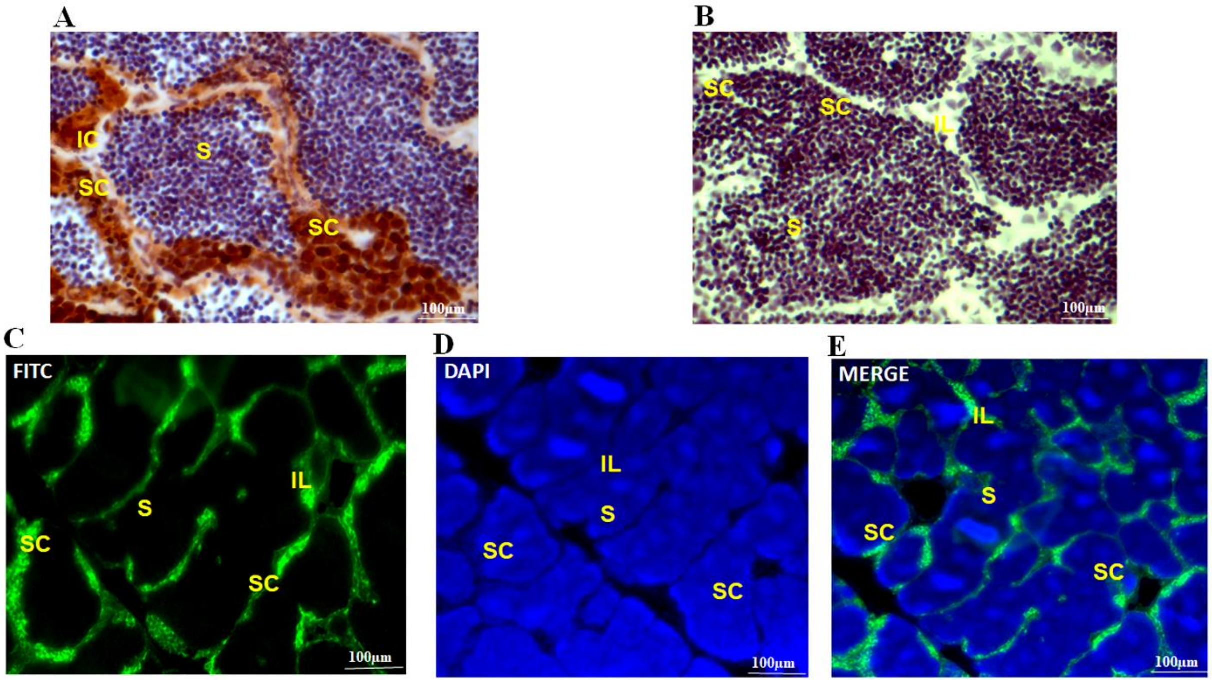


Fig. 4. Localization of Wnt4 protein in pre-spawning phase of carp testis. Immunoreactivity (IHC) of Wnt4 protein (A) was observed in spermatocytes and interstitial cells whereas (B) negative control (pre-adsorbed antibody) showed no signal. IF stained with FITC (C) indicates positive signal for spermatocytes and interstitial cells and DAPI was used as a counterstain (D) and MERGE (E) was shown to depict signal further with the counter stain. Abbreviation: IL-interstitial layer, SC-spermatocytes and S-spermatid/sperm (Scale bar indicates A-B 100 µm).

3.5 Analysis of canonical and non-canonical pathways

The expression levels of *wnt4*, *wnt5*, *wnt8* and *β-catenin* showed significant downregulation in 2, 5 and 10 μM IWP-2 groups compared to control and DMSO groups (Fig. 5A). Expression levels of (Fig. 5B) steroidogenic enzyme (*hllb*, *hsd11b* and *hsd20b*) and transcription factor (*sf-1/Ad4B*, *wt1*, *dmrt1* and *sox9*) genes decreased significantly in 10 μM IWP-2 treated group compared to control and DMSO groups (Fig. 5C). Among the AP exposed groups (Fig. 6A), *wnt4* expression elevated significantly in 10 μM AP group compared to control, DMSO, 2, 5 μM AP groups while *wnt5* expression was significantly high in 5 and 10 μM AP compared to control, DMSO and 2 μM AP groups. Expression of *wnt8* increased in a dose-related manner in AP exposed groups, compared to control and DMSO groups. Expression of *β-catenin* elevated significantly in 5 and10 μM AP groups, compared to control, DMSO and 2 μM AP groups. Expression levels of *wnt4*, *wnt5* and *wnt8* (Fig. 6B) was high in 2, 5 and 10 μM Iono groups compared to control and DMSO groups. Expression of *β-catenin* was higher in 10 μM Iono group compared to control, DMSO, 2 and 5 μM Iono groups.

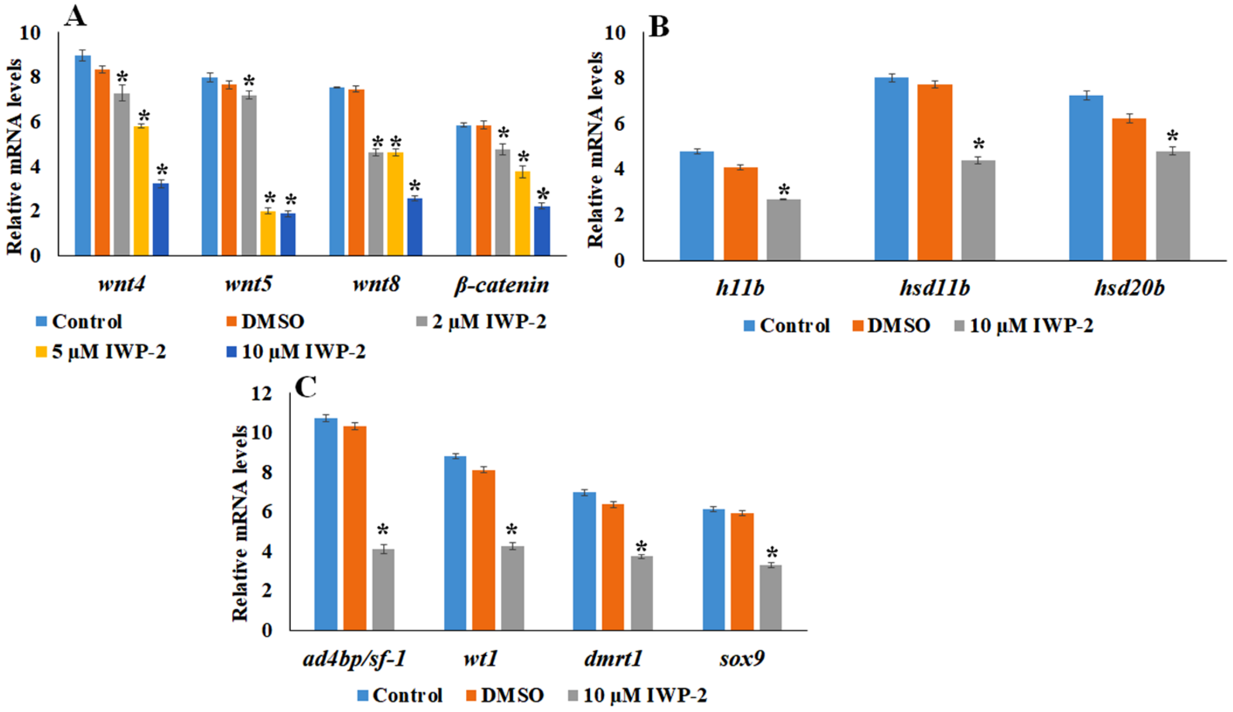


Fig. 5. Expression analysis of *wnt4*, *wnt5*, *wnt8* and *β-catenin* (A) control, DMSO, 2, 5, 10 µM IWP-2 groups (B) steroidogenic enzyme *hllb*, *hsd11b* and *hsd20b* and (C) transcription factor *ad4BP/sf-1*, *wt1*, *dmrt1* and *sox9* genes of control, DMSO and 10 µM IWP-2 groups. The statistical data was calculated as in the Fig.1.

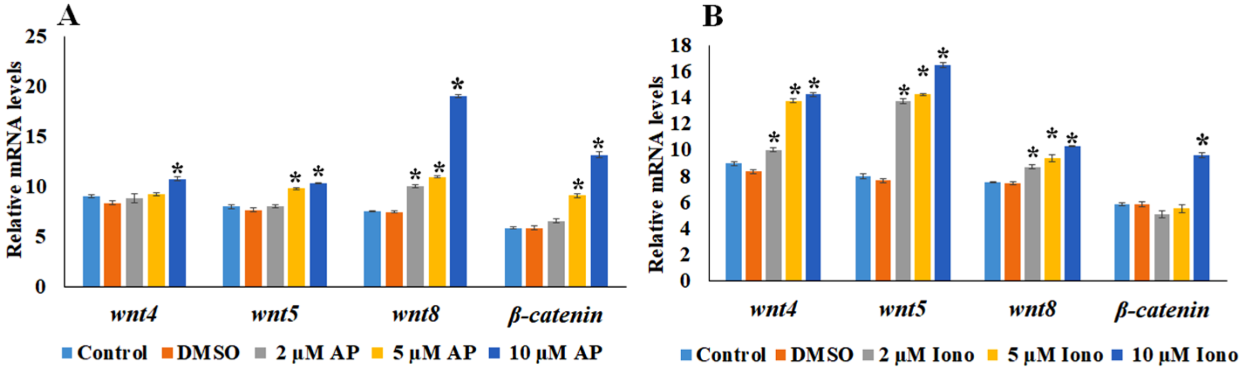


Fig. 6. Expression analysis of *wnt4*, *wnt5*, *wnt8* and *β-catenin* (A) control, DMSO, 2, 5, 10 µM AP groups and (B) control, 2, 5, 10 µM Iono groups. Abbreviation: AP- Alsterpaullone, Iono- Ionophore A23187. The statistical data was calculated as in the Fig.1.

3.6 Effects of Zn and Zn chelator of Wnt and Zn transporter genes

Expression pattern of *wnt4* was significantly ($P < 0.05$) upregulated (Fig. 7A) in 30 µg/g BW Zn treated group when compared to control, 10 and 20 µg/g BW Zn treated groups. Expression of *wnt5* was found to be elevated in 20 and 30 µg/g BW Zn treated groups when compared to control and 10 µg/g BW Zn groups.

Expression of *wnt8* was significantly high in 30 µg/g BW Zn group compared to control, 10 and 20 µg/g BW Zn groups. The expression of *mt* was predominantly higher in 20 and 30 µg/g BW Zn groups compared to control and 10 µg/g BW Zn groups. While, *znt1* expression was high in 10, 20 and 30 µg/g BW Zn groups in a dose-related manner when compared to control. Whereas, significant ($P < 0.05$) downregulation of all those genes (Fig. 7B) were observed in pre-treated 30 µg/g BW Zn of 0.1, 1 and 10 µM TPEN in a dose-related manner.

Further, pre-treated 30 µg/g BW Zn of 0.1, 1 and 10 µM TPEN treated groups (Fig. 7C) showed significant downregulation of Wnt4 protein levels in a dose-related manner while the endogenous control, β-tubulin showed constitutive expression in all the samples including control.

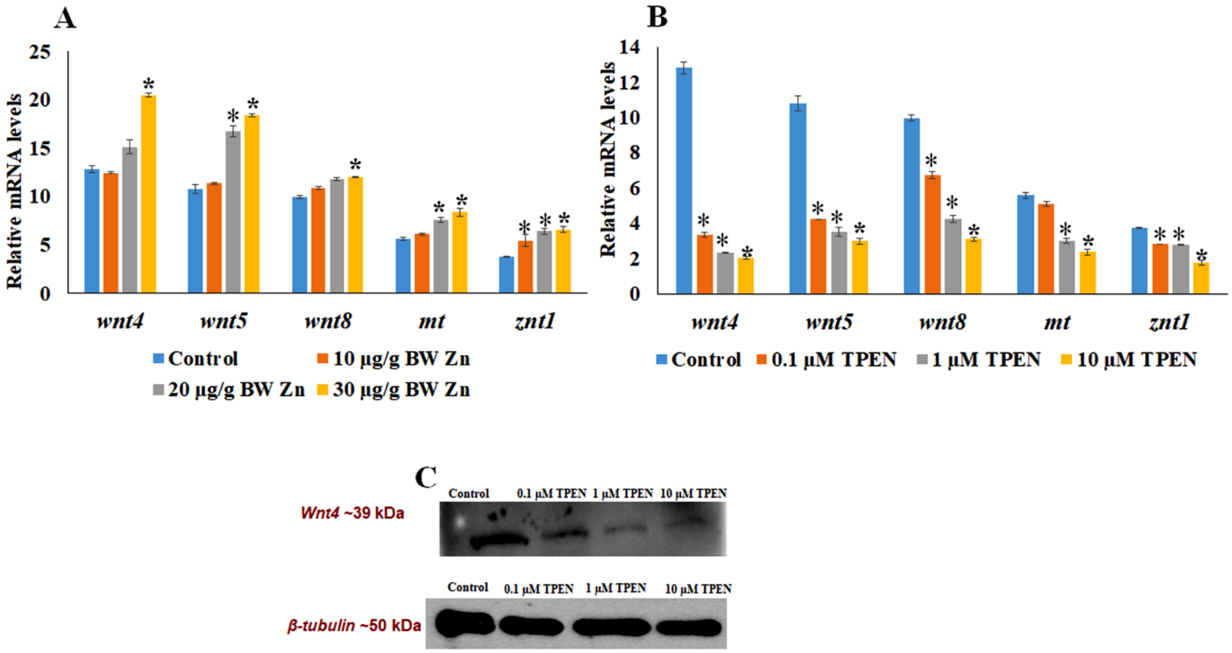


Fig. 7. Expression analysis of *wnt4*, *wnt5*, *wnt8*, *mt* and *znt1* (A and B) in control, 10, 20 and 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn of 0.1, 1 and 10 µM TPEN treated groups. (C) Western blot analysis of Wnt4 protein in control, pre-treated 30 µg/g BW Zn of 0.1, 1 and 10 µM TPEN groups. Abbreviation: gram body weight- g BW. The statistical data was calculated as in the Fig.1.

3.7 Expression analysis of few steroidogenic enzyme and transcription factor genes in testis after the administration of Zn and Zn chelator and estimation of androgen levels

The expression levels of steroidogenic enzyme (*hllb*, *hsd11b* and *hsd20b*) genes were elevated in 30 µg/g BW Zn group (Fig. 8A) compared to control and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. In other words, expression of all those steroidogenic enzyme genes was downregulated in pre-treated 30 µg/g BW Zn-10 µM TPEN group compared to control and 30 µg/g BW Zn groups. Similar pattern of expression was observed in transcription factor (*ad4BP/sf-1*, *wt1*, *dmrt1* and *sox9*) genes with increased expression in 30 µg/g BW Zn group (Fig. 8B) compared to control and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. Whereas, decreased expression of those transcription factor genes was observed in pre-treated 30 µg/g BW Zn-10 µM TPEN group compared to control and 30 µg/g BW Zn groups.

The androgens, T and 11-KT were elevated in 30 µg/g BW Zn group while in the pre-treated 30 µg/g BW Zn-10 µM TPEN group (Fig. 8C and D) the levels declined.

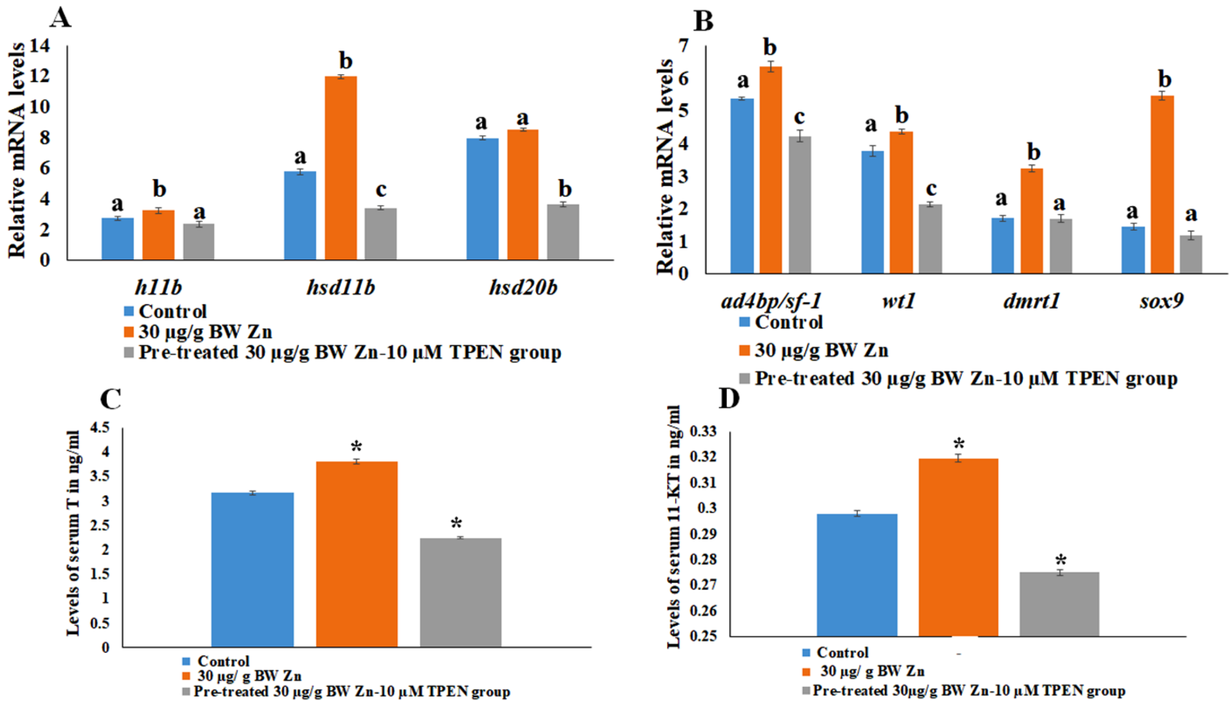


Fig. 8. Expression analysis of (A) steroidogenic enzyme (*hllb*, *hsd11b* and *hsd20b*) and (B) transcription factor (*ad4BP/sf-1*, *wt1*, *dmrt1* and *sox9*) genes in control, 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. Estimation of 11-KT and T levels (C and D) in control, 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. The statistical data was calculated as in the Fig.1.

3.8 Histological analysis of testis after Zn and TPEN treatments

Histological analysis of control testis showed pre-spermiating stage of testis (Fig. 9A) with appropriate number of spermatocytes and spermatids/sperm as per the stage. In 30 µg/g BW Zn administered testis group (Fig. 9B), number of spermatocytes and spermatids/sperm were increased significantly compared to control and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. While, pre-treated 30 µg/g BW Zn-10 µM TPEN group (Fig. 9C) showed decreased number of spermatocytes and spermatids/sperm when compared to 30 µg/g BW Zn administered group. Counting of spermatogenic cells converted to percent revealed elevated percentage of (Fig. 9D) spermatocytes and spermatids/sperm in 30 µg/g BW Zn group compared to control and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. In pre-treated 30 µg/g BW Zn-10 µM TPEN group of spermatocytes and spermatids/sperm percentage significantly decreased when compared to 30 µg/g BW Zn group.

3.9 Sperm assessment of control and Zn exposed testis

Sperm motility assement was carried out in control, 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn-10 µM TPEN groups (Fig. 9E). Significant increase ($P < 0.05$) of motile spermatozoa was evident in 30 µg/g BW Zn group compared to the control group. Whereas, in pre-treated 30 µg/g BW Zn-10 µM TPEN group showed significant decrease ($P < 0.05$) of motile spermatozoa compared to control group.

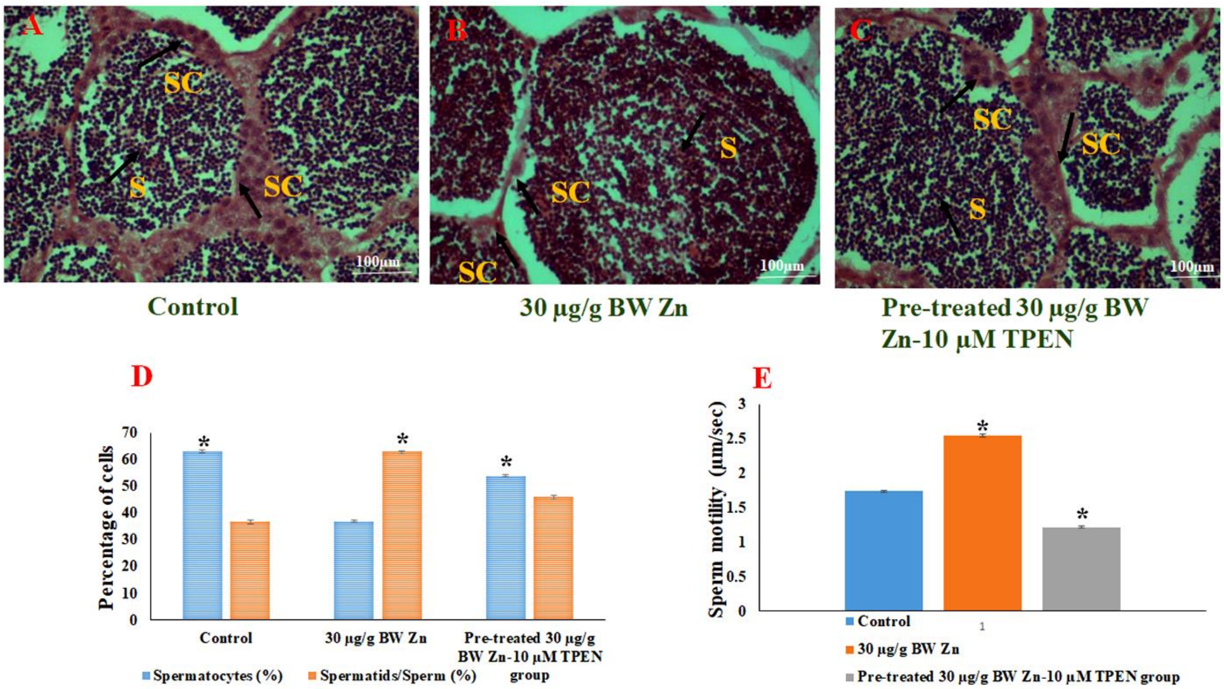
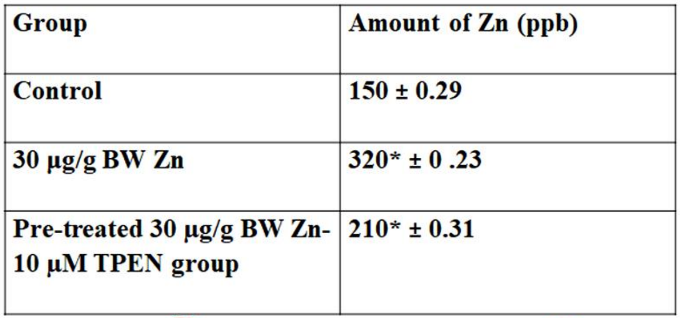


Fig. 9. Representative histology images of testis of (A) control (B) 30 µg/g BW Zn (C) pre-treated 30 µg/g BW Zn-10 µM TPEN of common carp stained with haematoxylin and eosin. The percentage of (D) spermatocytes and spermatids/sperm were represented and the histogram graph shows sperm motility (E) of control, 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn-10 µM TPEN groups. Abbreviation: SC- spermatocytes and S- spermatid/sperm. (Scale bar indicates A-C 100 µm). The statistical data was calculated as in the Fig.1.

3.10 Estimation and detection of Zn using ICP-OES and localization of Zn in testis

Concentration of Zn was estimated in control, 30 µg/g BW Zn and pre-treated 30 µg/g BW Zn-10 µM TPEN groups (Table 3) to estimate the level of bioaccumulation in testis. Further, 30 µg/g BW Zn administered sample showed significant bioaccumulation of Zn ($P < 0.05$) when compared to control. In accordance to these results, the control testis (Fig. 10A) showed less emission of fluorescence when compared to 30 µg/g BW Zn group indicating only the presence of endogenous Zn. The 30 µg/g BW Zn tissue (Fig. 10B) showed more intense fluorescence when compared to the control and pre-treated 30 µg/g BW Zn-10 µM TPEN testis. On the contrary, pre-treated 30 µg/g BW Zn-10 µM TPEN group (Fig. 10C) showed very low fluorescence when compared to control and 30 µg/g BW Zn exposed testis.



Group	Amount of Zn (ppb)
Control	150 ± 0.29
30 µg/g BW Zn	320* ± 0 .23
Pre-treated 30 µg/g BW Zn-10 µM TPEN group	210* ± 0.31

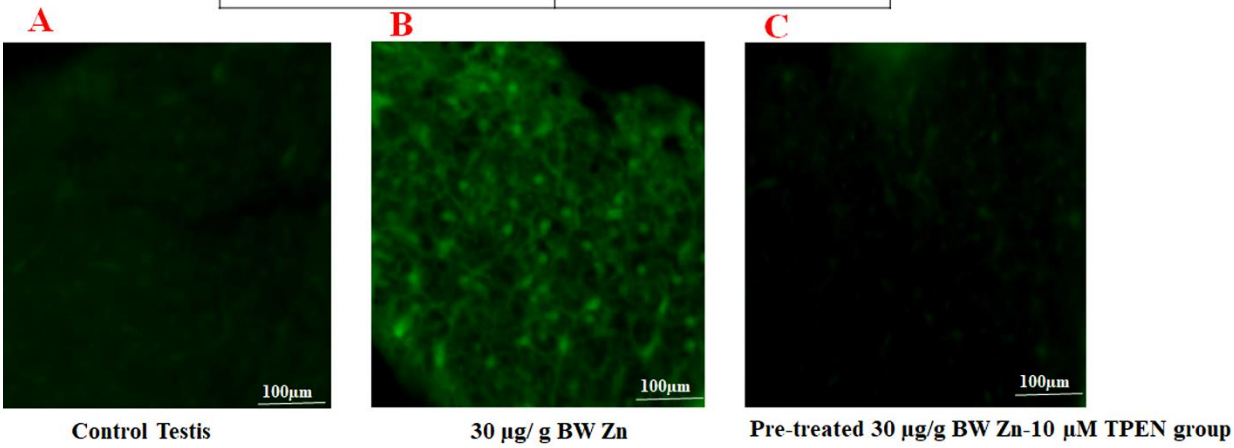


Fig. 10. Table-3 represents bioaccumulation of Zn in testis of control, 30 µg/g BW Zn, pre-treated 30 µg/g BW Zn-10 µM TPEN by ICP-OES (Inductive coupled plasma resonance spectroscopy). Illustrative images stained with Zn dye (ZnAF-2DA) in (A-C) control, 30 µg/g BW Zn, pre-treated 30 µg/g BW Zn-10 µM TPEN treated testis. (Scale bar indicates A-C 100 µm). The statistical data was calculated as in the Fig.1.

3.11 Assessment of spermatogonial cell proliferation after exposure of Zn and Zn chelator

3.11.1 Cytotoxicity assay

Primary testicular cells were exposed to 1, 2 and 3 μg/ml of Zn and 0.01, 0.1 and 1 μM of TPEN to determine whether Zn and TPEN treatments affects cell viability by MTT assay. All Zn treated groups (Fig. 11A) showed viable cells like that of control, while all TPEN groups showed less viability in a dose-related manner compared to control and all the three concentrations of Zn treated groups.

3.11.2 Bromodeoxyuridine (BrdU) incorporation assay

In order to determine DNA proliferation, if any, in Zn exposed cells, BrdU incorporation into the growing DNA strands was measured in control and treated samples (3 μg/ml Zn and 1 μM TPEN). Treatment with 3 μg/ml Zn exposed cells showed significant elevation of BrdU incorporation in newly synthesised DNA when compared to control group. While, 1 μM TPEN group showed decreased BrdU incorporation (Fig. 11B) compared to control and 3 μg/ml Zn groups.

3.11.3 Cell cycle analysis

Further, to clarify DNA synthesis, cell cycle distribution of the primary cells was analysed using flow cytometry. Control group (Fig. 11C) did not show cell propagation in S phase compared to 3 μg/ml Zn group. While 3 μg/ml Zn exposed cells (Fig. 11D) showed increased S phase propagation compared to control and 1 μM TPEN treated groups. In the 1 μM TPEN

(Fig. 11E) exposure group, S phase propagation significantly declined compared to control and 3 µg/ml Zn groups.

3.11.4 Tunnel assay

DNA fragmentation was further investigated by tunnel assay running into agarose gel electrophoresis of cells from control, 3 µg/ml Zn and 1 µM TPEN groups (Figs. 11F). In 3 µg/ml Zn exposed and control cells showed intact genomic DNA without any fragmentation while, 1 µM TPEN treated group of cells showed laddereing/fragmented DNA compared to control and 3 µg/ml Zn groups.

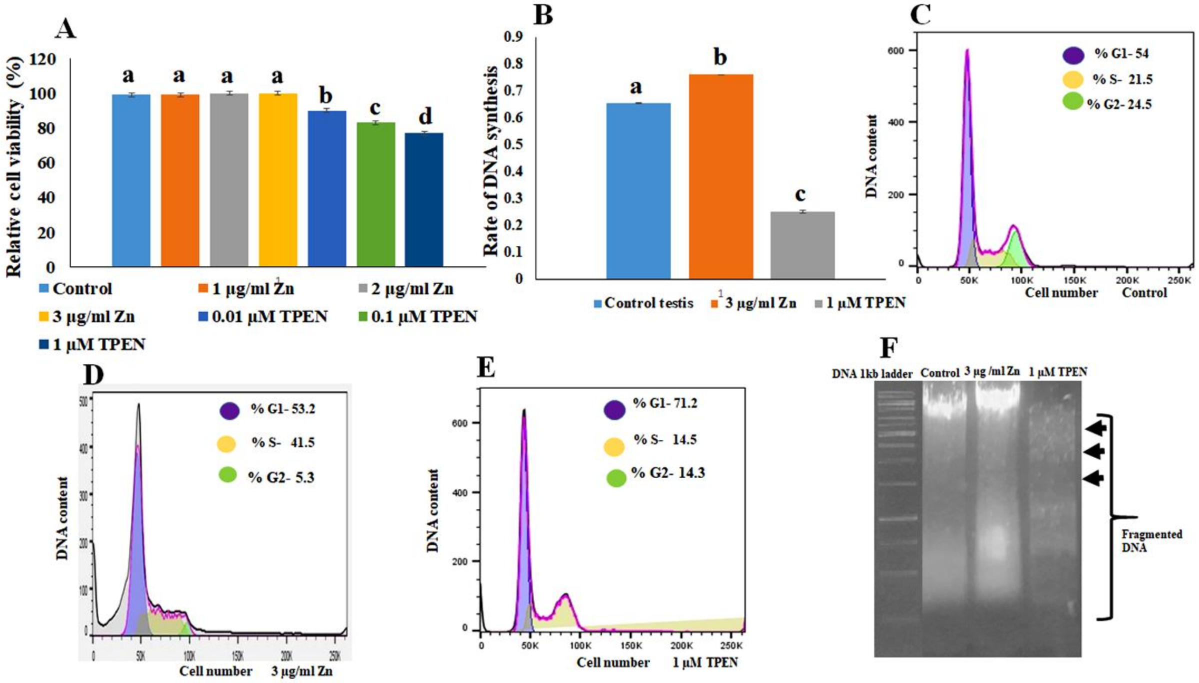


Fig. 11. Relative cell viability (%) was determined (A) for control, 1, 2 and 3 µg/ml Zn, 0.01, 0.1 and 1 µM TPEN treated groups by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. Histogram graph represents BrdU incorporation based proliferation analysis of (B) control, 3 µg/ml Zn and 1 µM TPEN exposed groups. FACS

analysis of (C, D and E) control, 3 µg/ml Zn and 1 µM TPEN exposed groups showed cell cycle phases of carp testicular cells, following DNA fragmentation (F) of control, 3 µg/ml Zn and 1 µM TPEN treated groups. Abbreviation: FACS - Fluorescence-activated cell sorting. The statistical data was calculated as in the Fig.1.

3.12 Analysis of FACS sorted control and Zn exposed cells by performing qPCR of *wnt* isoforms and Zn transporter markers

Viability of primary cells tested using giemsa fluorescent stained cells showed (Fig. 12A) normal spermatocytes and spermatids/sperms. Primary cells sorted by FACS as control (Fig. 12B) showed standard number of A and B gate cells. Whereas, 3 µg/ml Zn exposed primary testicular cells (Fig. 12C) showed increased number of cells in both A and B gate panel after FACS sorting. Primary cells of control isolated from the A gate showed significant high expression of germ cell marker genes, *vasa* and *nanos* compared to B gate indicating that the former has more germ cell population (Fig. 12D). Increased expression of *wnt4*, *wnt5*, *wnt8*, *mt* and *znt1* (Fig. 12E) were observed in 3 µg/ml Zn exposed group of both A and B gate cells indicating the presence of these isoforms at similar levels in both the cell population sorted by FACS.

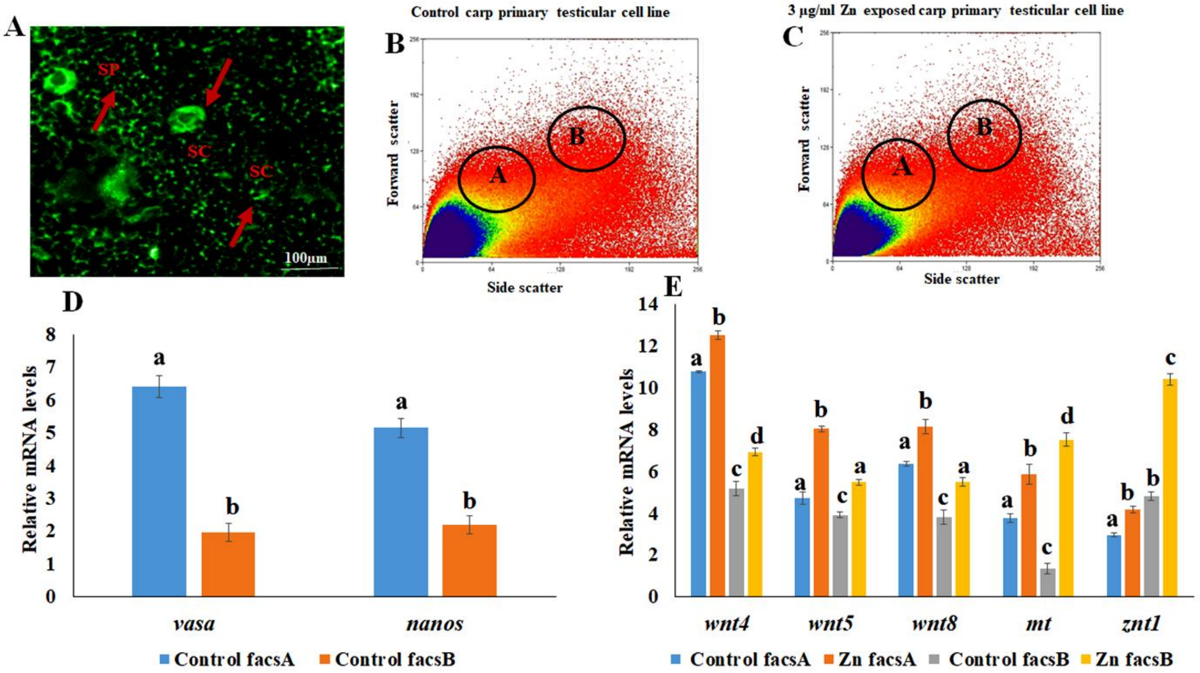


Fig. 12. Representative image of primary testicular cells (A) stained with giemsa fluorescent dye. Representative images of cells sorted by FACS of (B) control primary testicular cells and (C) 3 µg/ml Zn exposed groups. Expression analysis of (D) *vasa* and *nanos* sorted FACS A (germ cell) and FACS B (supporting cells). Expression analysis of *wnt4*, *wnt5*, *wnt8*, *mt* and *znt1* in control, FACS A, 3 µg/ml Zn exposed FACS B, control FACS B and 3 µg/ml Zn exposed FACS B cells. The statistical data was calculated as in the Fig.1.

3.13 Identification of canonical and non-canonical pathways

In order to determine canonical and non-canonical pathways of Wnt signaling, specific activators of Wnt signaling were used in combination with Zn induction. Combination of 3 µg/ml Zn with 10 µM Iono group significantly elevated expression of *wnt4* and *wnt5* (Fig. 13A) compared to all other groups. Whereas, combination of 3 µg/ml Zn with 10 µM AP group showed significant high expression of *wnt8* and *β-catenin* (Fig. 13B) compared to all other groups.

The protein level of Wnt4 (Fig. 13C) was elevated like that of mRNA levels in the combination of 3 µg/ml Zn with 10 µM Iono treated cells compared to control and all other treated groups. While, IWP-2 group showed decreased Wnt4 protein level when compared to control and all other groups. The protein level of β-tubulin in all those treated groups showed similar expression levels as that of control.

Western blot analysis of β-catenin protein (Fig. 13D) showed elevated expression in the combination of 3 µg/ml Zn with 10 µM AP treated group compared to control and all other treated groups. On the other hand, 10 µM IWP-2 treated group showed decreased β-catenin protein level when compared to control and all other treated groups. The protein levels of endogenous loading control, β-tubulin showed no significant changes in all groups including control.

Wnt non-canonical pathway was further verified by staining with calcium specific dye (acid fuchsin calcium dye). The control cells (Fig. 13E) showed less purple coloured stain, whereas, the 10 µM Iono treated group (Fig. 13F) showed dark nucleated cells when compared to control cells indicating calcium flux.

Further, the binding ability of Wnt4 and β-catenin protein was reconfirmed using FACS and immunostaining (Fig. 14A-H) in control and all other combination group of cells. Wnt4 protein binding capacity (Fig. 14A-D) was significantly elevated in the combination of 3 µg/ml Zn with 10 µM Iono group when compared to all other groups. Whereas, the binding capacity of β-catenin protein (Fig. 14E-H) showed high affinity in 3 µg/ml Zn with 10 µM AP group compared to all other groups.

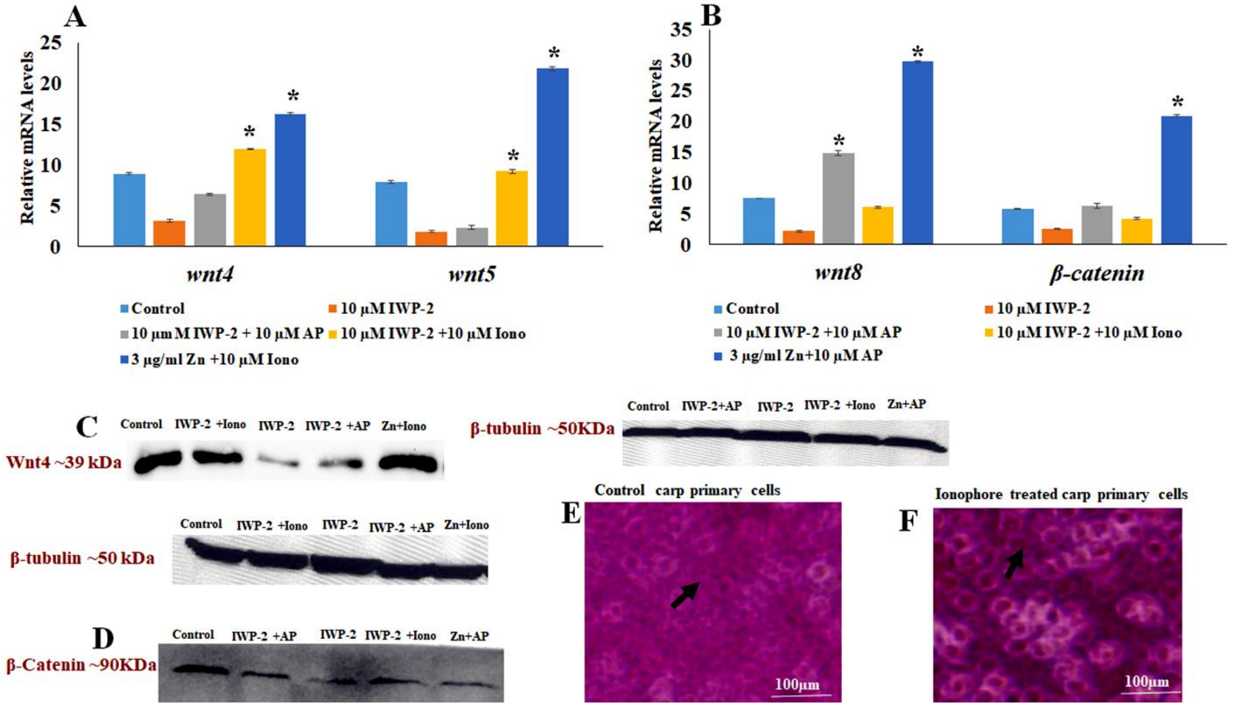


Fig. 13. Relative mRNA levels of (A) *wnt4* and *wnt5* in control, 10 µM IWP-2, 10 µM IWP-2 with 10 µM AP, 10 µM IWP-2 with 10 µM Iono and 3 µg /ml Zn with 10 µM Iono, (B) *wnt8* and *β-catenin* in control, 10 µM IWP-2, IWP-2 with 10 µM AP, 10 µM IWP-2 with 10 µM Iono and 3 µg/ml Zn with 10 µM AP. Western blot analysis of Wnt4 protein (C) in control, 10 µM IWP-2 with 10 µM Iono, 10 µM IWP-2, 10 µM IWP-2 with 10 µM AP, and 3 µg/ml Zn with 10 µM Iono and (D) β-catenin in control, 10 µM IWP-2 with 10 µM AP, 10 µM IWP-2, 10 µM IWP-2 with 10 µM Iono and 3 µg/ml Zn with 10 µM AP. Microscopic images were showed in (E and F) control primary testicular cells and 10 µM Iono treated cell stained with calcium dye (acid fuchsin calcium dye), arrows indicates dark colour nucleus. The statistical data was calculated as in the Fig.1.

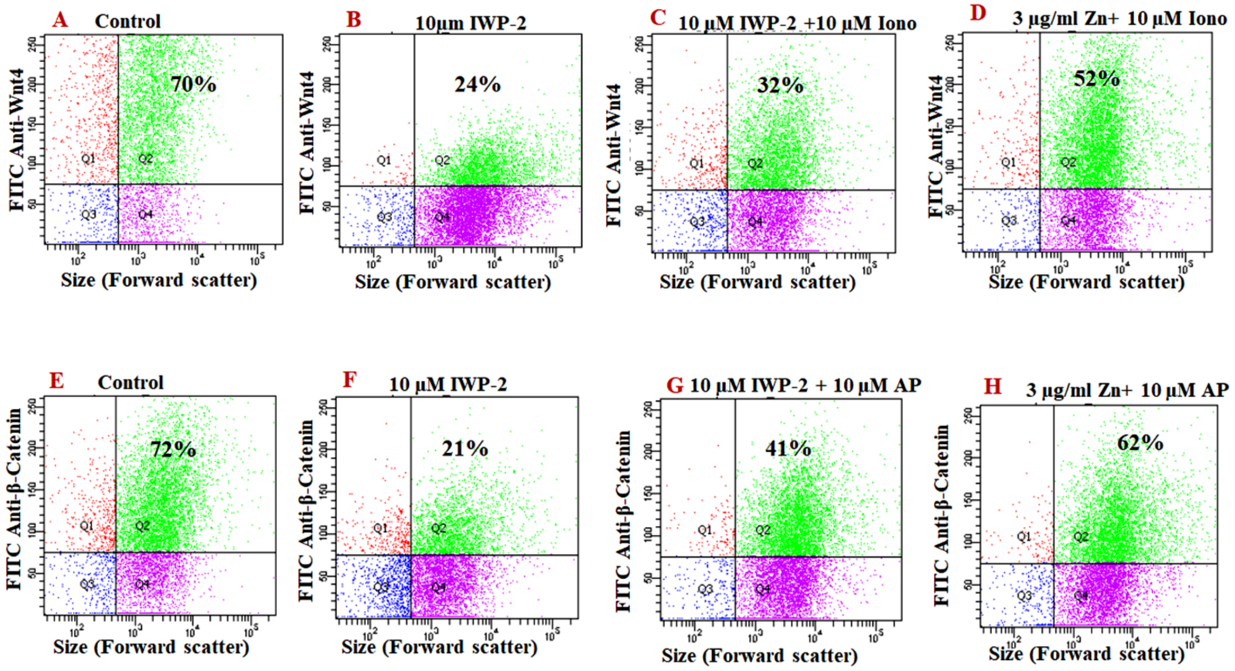


Fig. 14. FACS analysis of carp primary testicular cell culture of (A) control (B) 10 µM IWP-2 (C) 10 µM IWP-2 with 10 µM Iono (D) 3 µg/ml Zn with 10 µM Iono exposed groups with Wnt4 antibody, (E) control (B) 10 µM IWP-2 (C) 10 µM IWP-2 with 10 µM AP (D) 3 µg/ml Zn with 10 µM AP exposed groups with β-catenin antibody with FITC tagged secondary antibody.

4. Discussion

Present report demonstrated the significance of six wnt genes *wnt2*, *wnt4*, *wnt5*, *wnt8*, *wnt10* and *wnt11* isolated through gonadal transcriptome analysis (Anitha et al., 2019). Expression analysis of six wnt genes of *wnt4*, *wnt5* and *wnt8* revealed prominent expression in the testis which signifies their pivotal role in development till maturation. Tissue distribution of *wnt4*, *wnt5* and *wnt8* expression depicted higher expression in ovary and testis indicating their importance in gonadal function, representing a conserved role reported in Spotted knifejaw (*Oplegnathus punctatus*) and mammals (Vainio et al., 1991; Pailhoux et al., 2002;

Jaaskelainen et al., 2010; Du et al., 2017). Though the levels of expression are comparatively less in other tissues than gonads, the presence of *wnt* transcripts may indicate certain role in development as reported in zebrafish (Matsui et al., 2005). Ontogeny analysis of these three *wnt* (*wnt4*, *wnt5* and *wnt8*) genes indicates its role from the critical stage of gonadal differentiation indicating its importance in gonadal development as that of the other transcription factors like *foxl2*, *ad4bp/sf-1* and *sox9b* analyzed in catfish (Sudhakumari and Senthilkumaran, 2013). Concomitant elevation of expression of *wnt4*, *wnt5* and *wnt8* during the testicular development stages (120 dph onwards) warrants a key role in spermatogenesis (Komen et al., 1992). The histological analysis revealed cytological changes in pre-spawning, spawning and post-spawning phases of carp which was in correlation with the expression pattern *wnt4* and *wnt5* were predominant in all the stages of testicular reproductive cycle more specifically during testicular recrudescence, while, *wnt8* showed a lower expression at pre-spawning phase when compared to the other phases. The coordinated role of *wnt4/5a* in relation to the follicular development in mammals (Naillat et al., 2010) and fish (Prathibha and Senthilkumaran, 2017) supports such an analogous role in testicular development of common carp. Since, Wnt signaling facilitates progression of spermatogenesis, sperm maturation and normal female fertility in mice (Boyer et al., 2010; Kerr et al., 2014), robust expression of *wnt4* and *wnt5* in spawning phase indicated a similar role in carp. Prominent expression of *wnt8* in the testis of common carp undergoing post-spawning phase and different expression of *wnt4* and *wnt5*, indicated that all the three forms of *wnt* might participate in the regenerative role of spermatogenesis for the next cycle. In accordance to these findings, Wnt4 protein was localized in spermatocytes and interstitial layer of the testis indicating its role in spermatogenesis or in testicular growth as that of mouse, probably (Chen et al., 2016). These results together with other reports demonstrated an important role for *wnt4*, *wnt5* and *wnt8* in both ovarian and testicular development (Ramel et al., 2004; Yu et al., 2006; Chawengsaksophak et al., 2012; Prathibha and Senthilkumaran, 2017). In accordance to this, there are many reports validating role for that Wnt in embryogenesis, sex determination and differentiation (Sreenivasan et al., 2014). Wnt signaling also have an essential role in maintaining germ cell survival (Liu et al., 2010). Present study for the first time highlighted the role of *wnts* (*wnt4*, *wnt5* and *wnt8*) in testicular function. In order to validate the role of Wnt, it is important to analyze signaling pathways of *wnt* isoforms. In view of this, a series of experiments was performed to test Wnt signaling pathways related to testis. Before attempting to delineate specific pathways of Wnt signaling, it is essential to test the existence of Wnt signaling cascade in carp testis. Earlier reports on this line showed that IWP-2 blocks Porcn which adds a palmitoyl group to Wnt proteins for their signaling ability vis-à-vis Wnt secretion in vertebrates (Amberg et al., 2013). Downregulation of *wnt4*, *wnt5*, *wnt8* and *β-catenin* expression after exposure of IWP-2 in primary testicular cell culture authenticated the existence of Wnt signaling cascade in carp testis. Exposure of IWP-2 to primary testicular cell culture decreased the levels of expression of steroidogenic enzyme (*h11b*, *hsd11b* and *hsd20b*) and transcription factor (*ad4bp/sf-1*, *wt1*, *dmrt1* and *sox9*) genes which perhaps indicate a link between *wnts* and steroidogenesis in testis. Comprehensively AP acts as a canonical activator which accumulates the β-catenin by blocking the activity of glycogen synthase kinase-3β in vertebrates (Leost et al., 2000). AP induction resulted in selective elevation of *wnt8* as well as *β-catenin* but not *wnt4* and *wnt5* suggested that *wnt8* is indeed activated through canonical pathway. In mammals, Wnt canonical pathway lead to accumulation of β-catenin in the nucleus (Bain et al., 2003), wherein *wnt8* is involved in secondary axis formation in *Xenopus* (Sokol et al., 1991; Cui et al., 1995) and operates through the canonical pathway (Croce and McClay, 2006). On the contrary, treatment with Iono elevated the expression of *wnt4* and *wnt5* but not *wnt8* demonstrated that *wnt4* and *wnt5* acts through non-canonical pathway like that of other vertebrates (Chen et al., 2009; Tanigawa et al., 2011). In general, Iono acts on non-canonical pathway to trigger intracellular Ca^{2+} and activates Protein Kinase C in vertebrates and mammals (De, 2011; Tabar et al., 2014). Earlier reports in mammals and few teleost species indicated that both canonical and non-canonical pathways of Wnt play a key role in gonadal differentiation and growth (Matsui et al., 2005; Maatouk et al., 2008; Wu and Chang, 2009; Nicol and Guiguen, 2011). Data presented in this report demonstrated the role of *wnt4*, *wnt5* and *wnt8* signaling in the regulation of testicular function (steroidogenesis) with specific pathways for the first time in common carp.

Other than the signaling molecules, some of the co-factors or trace elements do contribute for development. Among all trace elements, Zn has been identified as an essential trace element for spermatogenesis in fish and mouse (Yamaguchi et al., 2009; Foresta et al., 2014). In mouse C17.2 neural stem cells Zn deficiency resulted in downregulation of canonical Wnt/β-catenin (Zhao et al., 2015). On the other hand, Wnt signaling play vital role in spermatogenesis, and the loss of Wnt signaling lead to male infertility in mice (Kerr et al., 2014). Hence, it is important to analyze the importance of Wnt signaling and Zn interaction on the progression of testicular development and growth including spermatogenesis. On this perspective, present report on exposure of Zn advancing testicular growth and spermatogenesis authenticated this phenomenon. To test this further, the impact of Zn inhibition using Zn chelator was also analyzed. Upon one time injection of 10, 20 and 30 μg/g

BW Zn, expression levels of *wnt4*, *wnt5*, *wnt8*, *mt* and *znt1* were elevated in 30 μg/g BW Zn. Thus 30 μg/g BW Zn dose was selected for throughout the study, *in vivo* and 0.1, 1 and 10 μM TPEN treatments. All those genes were downregulated in 10 μM TPEN group which further confirmed the use of one specific dose for Zn inhibition. Treatment with Zn specific inhibitor TPEN blocked the intracellular Zn which resulted in germ cell death, further the action was reverse after adding the Zn to the culture (Yamaguchi et al., 2009). Testicular steroidogenesis is an important event for androgen production and spermatogenesis, which can be monitored by steroidogenic enzyme (*h11b*, *hsd11b* and *hsd20b*) and transcription factor (*ad4BP/sf-1*, *wt1*, *dmrt1* and *sox9*) genes, signaling (*wnt4*, *wnt5* and *wnt8*) molecules as evidenced from earlier studies (Borg, 1994; Raghuveer and Senthilkumaran, 2009; Prathibha and Senthilkumaran, 2017). Incidentally, Zn treatment, upregulated all the genes mentioned above which in turn might have elevated the levels of androgens. Whereas Zn inhibitor treatment downregulated expression levels of all those genes vis-à-vis decreased androgen levels. These results might implicate direct or indirect effects of Zn to promote testicular function. To clarify the action of Zn in testicular function, *mt* and *znt1* expression analysis was performed in Zn treated and inhibited samples. In testis, Zn is regulated by some of the Zn regulating proteins like Mt and Znt1 which transports Zn in to the cells. *mt* and *znt1* is also known as Zn specific markers in mouse testis which was localized in spermatocytes and spermatids (Elgazar et al., 2005). Expression levels of *mt* and *znt1* showed higher expression after Zn induction while Zn chelation downregulated. These results suggested that *wnt*, *steroidogenic* enzyme and *transcription factor* genes were upregulated through the induction of Zn. Furthermore histological analysis, revealed the progression of spermatocytes to spermatids/sperm in the testis. In addition, sperm motility experiment also demonstrated elevated motility of sperm in Zn exposed testis which was inhibited considerably upon Zn chelation. It has been reported that Zn supplementation increased sperm volume and motility with normal morphology in infertile mouse whereas, the inhibition of Zn using Zn chelator decreased spermiation vis-à-vis infertility (Kumari et al., 2011., Zhao et al., 2016., Harchegania et al., 2018). Further, ICP-OES and Zn specific fluorescent probe analysis of Zn and TPEN exposed group authenticated the bioaccumulation of supplemented Zn in the testicular tissue. Taken together, our current study and previous reports suggested that Zn accumulated in the testis which might play a key role in the regulation of spermatogenesis. The study was further extended to validate direct effects of Zn by *in vitro* analysis using primary testicular cells of carp. Upon Zn exposure, cell viability, DNA proliferation, tunnel assay and cell cycle analysis indicated that Zn is essential for spermatogonial cell proliferation. Concomitantly, TPEN exposure hampered spermatogenesis cell proliferation as evidenced by performing various analysis mentioned above. Zn functions as a signal transmitter including cellular events, cell proliferation, differentiation and apoptosis. Zn inhibition lead to decreased *wnt* genes expression in rat and neural stem cells (Nakatani et al., 2000; Zhao et al., 2015). Thus Zn might promote Wnt signaling to impart its essential role in testis. To test this, cell level analysis was performed in the present study. FACS analysis based sorting of germ and other cells was validated by increased expression levels of germ cell marker genes *vasa* and *nanos* in sorted germ cells than other supporting cells. Zn exposed cells revealed their prominent role in germ and supporting cells with increased expression levels of *wnt4*, *wnt5*, *wnt8*, *mt* and *znt1* suggesting the role of *wnt* genes and Zn in germ and other cells maintenance. Hence, *wnt* genes and Zn are essential for the progression of spermatogenesis, which authenticated the present hypothesis about the interactive role of *wnt* and Zn in testicular growth of teleost fish. The mechanism underlying Zn inhibition mediated downregulation of Wnt signaling remains unclear. Nevertheless, present study revealed the importance of Wnt signaling through Zn to contribute for testicular growth and spermatogenesis. The results authenticated Zn induced advancement of spermatogenesis.

To evaluate the correlative or synergistic role of Wnt signaling and Zn-induction, treatment of Zn (3µg/ml) was done to primary testicular cells in combination with Wnt pathway specific stimulator for canonical (AP) and non-canonical (Iono) after inhibiting through Wnt-blocker (IWP-2). Specific upregulation of *wnt4/5* and *wnt8* under non-canonical and canonical pathway stimulation, respectively after Zn treatment with IWP-2 warranted the interactive role of Zn and Wnt signaling. Western blot results on β-catenin and Ca^{2+} imaging confirmed these findings. FACS analysis using specific antibodies for Wnt4 and β-catenin convincingly validated the results of Zn-Wnt signaling interaction in testis. However, precise or direct binding of Zn to stimulate Wnt signaling remains to be elucidated. Nevertheless, Wnt signaling cascade is positively influenced by Zn to promote testis function. In summary, *wnt* isoforms, *wnt4*, *wnt5* and *wnt8* signaling plays a crucial role in carp testis, more emphatically steroidogenesis. Trace element Zn, augment Wnt signaling to promote testicular growth and spermatogenesis.

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Chapter 2

Effects of ZnO-NPs and $ZnSO_4$ on the testis of common carp, *C. carpio*

Abstract

Having identified the essential role of Zn in promoting testicular spermatogenisis as well as steridogensis, it is imperative to analyze the effect of Zn as a long term potential element in common carp. On the basis of this, present study analyzed the effects of Zn in the testis of six months of old male carp *C. carpio* exposed to three different doses (10, 50 and 100 µg/L) of ZnO-NPs (nanopowder) and $ZnSO_4$ (soluble form) for 21 days. Characterization of ZnO-NPs was done after sonication, and the size and shape of ZnO-NPs were determined as ~20-30 nm spherical structure with a zeta potential of +26.0 mV. After the treatment, expression of certain transcription factors and steroidogenic enzyme genes were quantified in both control and treated testis, which showed significant down regulation after treatments. Measurement of oxidative stress-related enzymes such as catalase, superoxide dismutase, and glutathione-S-transferase revealed significant elevation in the testis of treated groups when compared to control. Histological analysis of testis showed defective lumen and slow progression of spermatogenesis. The effect of both forms of Zn was further analyzed by *in vitro* using TM3 Leydig cell culture. Upon treatment, loss of adhesion and clumping of cells, decrease in the viability of cells, and significant increase of apoptotic cells were seen. In addition, comet and ROS assays authenticated the DNA damage and significant increase of ROS generation in both the treatments. Taken together, exposure of both the forms of Zn even at minimal dose adversely affects testicular growth in common carp. Incidentally,

histological analysis after treatment withdrawal showed the tendency of testis indicating the plasticity of testicular development in carp to rescue the effect.

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Effects of zinc oxide nanoparticles and zinc sulfate on the testis of common carp, *Cyprinus carpio*

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1. Introduction

Data presented in the chapter 1 described the influence of Wnt signaling and Zn interaction to promote spermatogenesis if exposed at minimal dose for a shorter duration in adult fish. It is then imperative to analyze whether, Zn promotes testicular growth and spermatogenesis if exposed for a longer duration. To this end, present report analyzed the effect of Zn in both forms (NPs and salt) during testicular development in the common carp, *C. carpio*, a fresh water food fish in India This type of nanotechnology based approach requires brief description on NPs.

Nanotechnology has developed significantly in the last decade and emerging as one of the most promising and exciting technologies owing to its wide applications in agriculture, medicine and drug delivery (Emerich and Thanos, 2005; Suri et al., 2007). Despite to its need, due to distinct physicochemical and electrical properties of nano-sized materials, it had gained considerable attraction, yet its uniqueness and even the size differences cause toxicity in living organisms (Handy et al., 2008). Inevitable discharge of NPs in the form of powder, cluster, and crystal, and their byproducts released into the aquatic system either directly or indirectly imparts adverse effect and/or toxicity to aquatic organisms (Lei et al., 2016). NPs also form an aggregate with the organic matter or any other natural particles present in freshwater and seawater (Handy et al., 2008). Further, NPs influence the abiotic factors such as pH, salinity and ultimately affect the biotic environment.

Fishes serve as excellent experimental models for aquatic toxicology study as they are regarded as one of the best bio-indicators of pollutants (Authman et al., 2015). Several studies warrant that compounds accumulating in the aquatic ecosystem through several industrial and other effluents, especially in fish could pose a risk not only to the aquatic fauna but also be detrimental to human and other organisms due to bio-magnification (Amin et al., 2011). In addition, fishes are susceptible to any pollution including pesticides and metal ions. Existing scientific reports proposes that NPs can cause oxidative stress-dependent toxic effects to gill, skin or through oral route (Gupta et al., 2016). Interestingly, several studies have validated that NPs can cross the blood-testis barrier, leading to testicular accumulation NPs and translocation to testicular cells and/or nucleus to generate adverse effects (Lan and Yang, 2012; Murugananthkumar et al., 2016). Zn is a well-known essential trace element and also used as NPs. Incidentally, Zn helps in regulating various physiological functions including spermatogenesis in fish and mammals. Deficiency of Zn in the Japanese eel disrupts the spermatogenesis and sperm motility (Yamaguchi et al., 2009) while in mammals exposure of diet with Zn deficiency leads to defect in testicular function and increase in the production of free radicals (Merker and Gunther, 1997). ZnO-NPs are used in sunscreens and for industrial coating to protect wood, plastics, and textiles (Abbasalipourkabir et al., 2015) which can pose a risk as a pollutant by flow through reaching water bodies. Although beneficial effects of Zn are understood well, the adverse or toxic effects of Zn, if any is not completely studied in particular in lower vertebrates. Exposure of ZnO-NPs in male mouse results in disruption of spermatogenesis and testicular function (Talebi et al., 2013). Sublethal levels of Zn exposure adversely affect hatchability, survival and hematological parameters of fish (Kori-siakpere and Ubogu, 2008). This might become crucial as any defect in breeders will eventually affect the fish population biomass. Despite these information, studies related to metal nanotoxicity including Zn on any edible fresh water fishes of India are scanty. Thus, it is essential to analyze the role of Zn in terms of beneficial verses detrimental effects. To this end, use of common carp as an animal model may provide lead information on ZnO-NPs and $ZnSO_4$ toxicity, if any in teleost due to its wide distribution (Bongers et al., 1998). Three different doses of ZnO-NPs and $ZnSO_4$ such as 10, 50 and 100 μg/L were selected, which is less than the reported LC50 values in common carp i.e. 4.897 mg/L (Subashkumar and Selvanayagam, 2014) and other teleosts 4.92 mg/L and 1.58 mg/L (Xiong et al., 2011; Kiyani et al., 2013) and far less by considering the presence of Zn in several rivers of India (Jain et al., 2004; Goswami and Sanjay, 2014). After exposure of ZnO-NPs and ZnSO4 to six-month-old male carp, the expression of transcription factor (*dmrt1*, *activin B*, *dax1*, *foxl2*, *wnt5* and *ad4bp/sf-1*) and few steroidogenic enzyme genes (*20βhsd*, *cyp19a1*) related to testicular development and levels of T and 11-KT were quantified in testis of control and treated groups. Biochemical analysis of oxidative stress enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) were also measured. In addition, histological examination of control and treatment testis was done to support our findings. The study was further extended to analyze the effects of both forms of Zn using TM3 Leydig cell culture. The purpose of investigating the effect in the *in vitro* system is to understand the damage at the cellular level and to compare the cellular toxicity with respect to different doses exposed to fish. Relative cell viability and Zn-induced apoptotic responses were analyzed using MTT and dead cell apoptosis assay, respectively. Further, comet assay and intracellular reactive oxygen species (ROS) measurement were performed to validate the results. To check the efficacy of restoration of testicular development after treatment withdrawal, by histological analysis was done on testis.

2. Materials and Methods

Preparation and physicochemical characterization of ZnO-NPs

One mg of ZnO nanopowder (Sigma) and $ZnSO_4$ (SRL) was dissolved individually in 1 ml of ultrapure water (Milli-Q). The dissolved ZnO-NPs was sonicated for 15 min using a narrow-stepped titanium probe of an ultrasonic homogenizer (model 300V/T, Biologics Inc. VA, USA). The sonication amplitude was set at 5 µm for 30 sec with an interval of 1 min between consecutive pulses. The particle size of ZnO-NPs is <50 nm, white powder nearly spherical shape, and purity ~ 99%. Measurement of their zeta-potential determined the surface charge of ZnO-NPs, showed that the addition of Alginic acid increased their negative surface charge, which could be contributing to the improved dispersion in the water. Structural and morphological properties of the ZnO-NPs was observed under scanning electron microscopy (FE-SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA) operated at 20KV, energy dispersive X-ray analysis (EDX), Transmission Electron Microscopy (TEM), X-ray Diffraction (XRD) (Rikagu Mini-2 using CuKα1, $\lambda = 0.15406$ nm), Fourier Transform Infra-Red spectroscopy (FTIR), (FE-SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA), operated at

20KV and FEI Technai G2 S-Twin (TEM,) operated at 80 KV. $ZnSO_4$ dissolved in water was used without any sonication methods.

2.1. Animal maintenance and treatments

All the details about animal maintenance including breeding and rearing and dissection of testis were described earlier in Chapter 1. Six-month-old common carp of similar size and weighed 2.5 ± 0.35 g were used for the experiment. The fishes were exposed to three different doses (10, 50, and 100 µg/L) of ZnO-NPs and $ZnSO_4$ while one group was maintained as a control. For treatment withdrawal studies, 100 µg/L ZnO-NPs and $ZnSO_4$ treatment and control groups were used. These groups of fishes were initially treated and later as per experimental methods described earlier and later 21 days withdrawal period was given. All the fishes were kept in 50 L glass aquarium tank equipped with a static renewal flow-through system and dechlorinated tap water was used. They were acclimated for a fortnight under ambient photothermal conditions and fed with commercial carp food, *ad libitum*. Fishes were exposed to freshly prepared (daily) solution of ZnO-NPs and $ZnSO_4$ (10, 50 and 100 µg/L of final concentration) individually for 21 days, while the control group received only vehicle control. Replenishment of water was done every day (including the control group) before the treatment was given, and the procedure was followed until the end of the treatment period. After treatment, the fishes were sacrificed and testis was dissected out from all the groups to perform real-time PCR (qPCR), histology and FTIR analysis. Blood was collected using sterile plastic syringes from the caudal peduncle before sacrifice. Serum was separated by centrifugation at 5000rpm for 10 min at 4 °C for hormone analysis (T and 11-KT). Testis samples of treatment withdrawal groups was collected for histology only. All the animal (fish) experiments were performed by following the general guidelines of the

Institutional Animal Ethics Committee, University of Hyderabad (CPCSEA, Inst. Reg.No.151/1999 dt.22.07.19

2.3 qPCR

Total RNA isolation, reverse transcription and qPCR were done as described in the chapter 1. qPCR primers were designed for the amplicon length of ~250 bp and all reactions were performed in triplicate for three different samples using gene specific primers (Table 1). Changes in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

Table 1. List of primers used for cDNA cloning and qPCR analysis

Gene Name/symbol	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
20βhsd	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA
cyp19a1	GAGCAGGTCATCTGCTGT	GGATGTCCACCTGTCCCT
dmrt1	GCAGCCCAGGTGGCGTTA	GCCGTTCCATGCTGGAGG
Actvin b	GGCAGTGGAGCGAATGCTGAA	GCACCCCGGGGAACCTTGAGC
dax1	CCAGATGTTGCAGGGCTGC	CCTCCATGTTGACAGCGCC
foxl2	GCGTCTCACGCTGTCCGG	GCCGGTAGTTGCCCTTCT
wnt5	ATCGAGATCTGCAAACTA	GAGGTCTTTGGGACGAGCCG
wnt4	TCACCGACATGTGTGCATC	ATCATAATTTGTTAACAAAT
ad4bp/sf-1	CGGACCTGGAAGAGTTGT	GGTTCTCTTGGCATGCAG
18S rRNA	GCTACCACATCCAAGGAAGGCA GC	CGGCTGCTGGCACCAGACTTG

2.4 Histology

Testis of control, ZnO-NPs and $ZnSO_4$ treated juvenile carp (n=5) were dissected out for histological analysis which was explained well in chapter 1.

2.5 Measurement of oxidative stress

Tissue samples were homogenized with 1 ml of 1 × PBS, 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 at pH 7.4, and centrifuged at 10,000 ×g (15 min at 4 °C). The supernatant was transferred into a microtube and then frozen at −80 °C till used. The total protein was estimated by Bradford (1976) method and used for the following assays.

2.5.1 CAT

CAT activity was performed according to the procedure of Beers and Sizer (1952) and it was assessed by measuring the rate of H_2O_2 consumption. Therefore, in this reaction H_2O_2 absorbance gets decreased and turned into O_2 and H_2O_2. The reaction was started by adding the sample into the quartz cuvettes with substrate contained 50 mM potassium phosphate buffer (pH 7.0) and 12.1 mM H_2O_2 in a total reaction volume of 3 ml. At 240 nm consumption of peroxide (extinction coefficient (mM) of 0.04 mM−1 cm−1) was monitored using a UV-vis spectrophotometer (UV-1601, Shimadzu, Germany). At 15 s intervals throughout a 180-s the absorbance was measured, and results are given as μM of H_2O_2 consumed/min/mg of protein. For validation of the assay, bovine catalase solution was taken as a positive control to prepare standard graph.

2.5.2 SOD

SOD was performed by using a method derived from Kostyuk and Potapovich, (1989). Auto-oxidation of quercetin (Sigma) was blocked by the homogenate containing SOD. SOD was directly correlated with the degree of inhibition of quercetin oxidation where the quercetin oxidizes at pH 10, and this was a free radical chain reaction involving superoxide function for SOD. The total reaction mixture of 1 ml contains 0.016 M phosphate buffer and N,N,N',N'-tetramethylethylenediamine buffered with 0.08 mM EDTA. The reaction was initiated by addition of 0.1 ml of 0.015% quercetin solution. To estimate SOD in the supernatant of tissue homogenates, in 1 ml of final volume mixture, 20 µg of protein was added and monitored using UV-vis spectrophotometer (Shimadzu). At 406 nm the absorbance was decreased due to inhibition of auto-oxidation of quercetin and monitored the values. The results were expressed as U/mg protein

2.5.3 GST

Estimation of GST was done by using the method of Jakob et al. (1980) to measure the activity of GST at 340 nm, though the conjugate of glutathione (GSH) and dichloro-2, 4-dinitrobenzene (CDNB) was measured. For the assay, 100 mM CDNB (Sigma) and reduced 200 mM GSH, (Sigma) was prepared in 1 × PBS as a substrate solution. To the thiol group of the glutathione, CDNB was conjugated with GST to make CDNB-GSH adduct. The adduct of CDNB (S-2, 4-dinitrophenyl glutathione) was measured at the absorbance of 340 nm using UV-vis spectrophotometer (UV-1601, Shimadzu, Germany). Based on molar coefficient 9 m/M/cm, the enzyme activity was calculated. Results were determined as µM of the adduct formed/min/mg of protein.

2.6 Enzyme immunoassay (EIA)

T and 11-KT levels in serum were estimated by EIA kit (Cayman) as per the manufacturer's protocol and it was explained in chapter 1.

2.7 TM3 Leydig cell culture (*Mus musculus*) and Cell viability assay

For *in vitro* experiments, TM3 Leydig cells (*Mus musculus*) was purchased from NCCS, Pune, India and were cultured in DMEM supplemented with 10% FBS (Invitrogen), 1× glutamine and antibiotic and antimycotic solution (Gibco-BRL) at 37 °C and 5% CO_2. The viability of cells–was observed using trypan blue and culture was passaged to obtain required cells which were then maintained for 24 hrs and later used for the experiments. For cytotoxicity studies, ZnO-NPs and $ZnSO_4$ were dissolved at a final concentration of 0.1, 0.5, and 1 µg/ml. Cell viability assay was performed as explained in chapter1

2.8 Membrane permeability / Dead cell apoptosis assay

Determination of cellular apoptosis or necrosis level was done using Vybrant Apoptosis Assay Kit #4 (Molecular Probes, Eugene, OR) as per the manufacturer's protocol. YO-PRO®-1 stock solution (Component A) which binds specifically to apoptotic cells and PI stock solution (Component B) that binds specifically to necrotic cells were added to each 1 ml of cell suspension (~ $1X10^6$ cells) and incubated on ice for 20–30 min. The results were analyzed using BD LSR Fortessa™ cell analyzer (BD Biosciences).

2.9 Comet assay

DNA damage following 24 hrs exposure of ZnO-NPs and $ZnSO_4$ at concentrations of 0.1, 0.5, and 1 µg/ml on TM3 Leydig cells were evaluated using single cell gel electrophoresis assay (Raisuddin and Jha, 2004). Control and treated cells were embedded in 0.5% low melting agarose (Sigma) layer between 1% normal melting agarose (Sigma) and 0.5% normal melting agarose. The cells were lysed with high salt and detergent concentrations 100 mM EDTA (Sigma), 2.5M NaCl (Sigma), 10 mM Tris base (Bio-Rad), 1% Triton X-100 (Sigma), adjusted to pH 10 for 1 hr. DNA was allowed to unwind 1mMEDTA, 10% DMSO (Sigma), 300 mM NaOH (Sigma), pH 13 for 20 min and then subjected to electrophoresis in the same solution as for unwinding (25 V, 300 mA) for 15 min. After electrophoresis, the alkalis in the gels were neutralized by rinsing the slides in a neutralization buffer (0.1 M Tris pH 7.5) for 5 min. The slides were treated with methanol for 10 minutes, stained with 45 µl of 20 µg/ml ethidium bromide solution and viewed under a fluorescent microscope (Olympus Optical Co.

Ltd). 1000 cells were analyzed and % tail DNA was measured to evaluate the extent of DNA damage. Results were expressed as means of three replicates for each sample (n=5) ± standard error of the mean.

2.10 ZnO-NPs and $ZnSO_4$ induced ROS generation

Intracellular ROS, generated due to treatment of different ZnO-NPs and $ZnSO_4$ (0.1, 0.5, and 1 μg/ml), were evaluated using 2′, 7′-dichlorodihydrofluorescein diacetate oxidation (DCFH-DA, Invitrogen) using multimode fluorescence microplate reader (Synergy H1 hybrid, Biotek, USA) by following the protocol adapted by Kang et al. (2012). Cells were seeded in a 96-well plate at a density of 5000 cells/well, followed by incubation in a CO_2 incubator for 24 h. The cells were then washed using PBS buffer and treated with three different concentrations (0.1, 0.5, and 1 μg/ml) of ZnO-NPs and $ZnSO_4$ and 25mM DCFH-DA dye for 1 h. The generated ROS level was detected by measuring the fluorescence intensity at 523 nm with an excitation at 502 nm. Levels of ROS variation was determined by comparing the obtained fluorescence intensity of treated samples with control. Triplicate experiments were performed to determine the standard error of the mean.

2.11 Determination of Zn concentration in tissues by ICP-OES spectrometry

The concentration of Zn in the testis of carp treated and control groups was measured by ICP-OES and it was explained in chapter 1.

2.11 Statistical Analysis

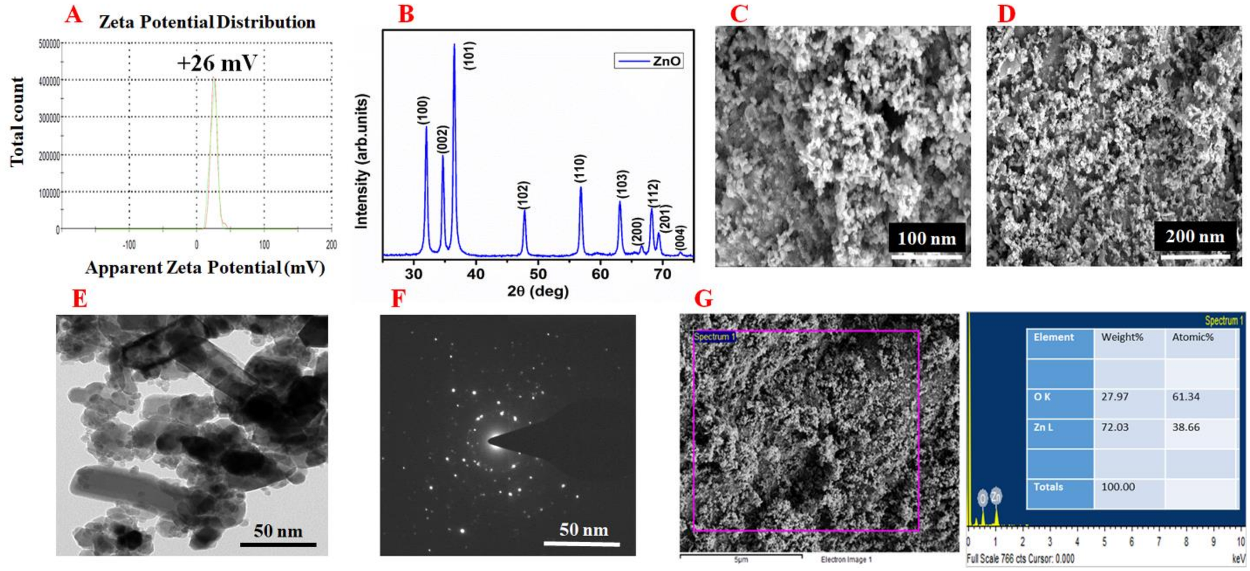
All *in-vitro* and *in-vivo* studies were carried out in triplicate. Data were represented as a mean ± standard deviation. The significance of the difference between two groups was analyzed by using student's t-test and between multiple groups were analyzed by one-way ANOVA. The level of significance was stated as $P < 0.05$.

3. Results

3.1 Characterization of ZnO-NPs after sonication

Characterization of ZnO-NPs was performed through zeta potential, FESEM, TEM, and EDS (Fig. 1a-g). ZnO-NPs was sonicated for 15 min to reach the desired size and shape and to make the uniform distribution of sample for an effective biological response. After sonication, the zeta potential of ZnO-NPs was measured around +26.0 mV while the surface of ZnO-NPs contains negative charge (Fig. 1a). In general, a colloidal solution with high zeta potential showed high stability and vice versa, while the prepared sample showed a moderate zeta potential value which can be considered stable. XRD pattern of the ZnO-NPs after sonication also displayed the sharp and broad crystalline peaks which confirmed the ZnO-NPs nature (Fig. 1b). The mean ZnO-NPs size was measured from the full-width half maxima of the most intensive peak (101) of Zn. The 2θ values from x-axis, 31.67^0, 47.40^0 and 36.14^0 corresponding to 100, 102, and 101 planes of the face-centered spherical shaped structure of metallic Zn. The intensities of all reflections are well matched with standard data which conforms ZnO-NPs have high purity and no impurities were not observed. The average size distribution was conformed as 30–40 nm which is well matched with TEM particle size analysis. The particle size was also evident in FESEM (Fig. 1c-d) and TEM (Fig. 1e-f) images. In FESEM, the average particle size of ZnO-NPs showed 30-40 nm with well-defined spherical shaped morphology. TEM images are conforming analogous particle size of ZnO-NPs with respect to FESEM images. In addition, elemental analysis of ZnO-NPs using EDS showed the purity of ZnO-NPs with no other particle contamination (Fig. 1g).

Stability of ZnO-NPs and $ZnSO_4$ were determined by FTIR study and analysis of spectra was done by OMNIC series software (Fig. 2a-d). Baseline correction has been done after obtaining spectra. ZnO-NPs was annealed at 300 °C and 500 °C. A pinch of ZnO-NPs and lyophilized testis samples were directly kept under the IR probe and scanned from 500 cm^{-1} to 4000 cm-.The fingerprint region of upper peaks were observed around 534.39 and 601.43 cm^{-1} whereas the distinct vibration of ZnO and the lower peaks were absent around 534.39 and 601.43 cm-1 in the control testis sample (Fig. 2a). The lower peak of 10 µg/l of ZnO-NPs samples were correlated with the ZnO peak around (534.39 and 601.43 cm-1) 547.11 and 604.11 cm-1 (Fig. 2b). The lower peak of 50 µg/l of ZnO-NPs samples were correlated with the ZnO peak around (534.39 and 601.43 cm-1) 530.76 and 616.73 cm-1 (Fig. 2c). The lower peak of 100 µg/l of ZnO-NPs samples were correlated with the ZnO peak around (534.39 and 601.43 cm-1) 547.11 and 616.57 cm-1 (Fig. 2d).



Element	Weight%	Atomic%
O K	27.97	61.34
Zn L	72.03	38.66
Totals	100.00	

Fig. 1. Characterization of ZnO-NPs. (A) Zeta potential of ZnO-NPs (B) XRD analysis of ZnO-NPs (C & D) FESEM images of ZnO-NPs (E & F) TEM images of ZnO-NPs (G) EDX spectrum contains the atomic and weight percentage of ZnO-NPs.

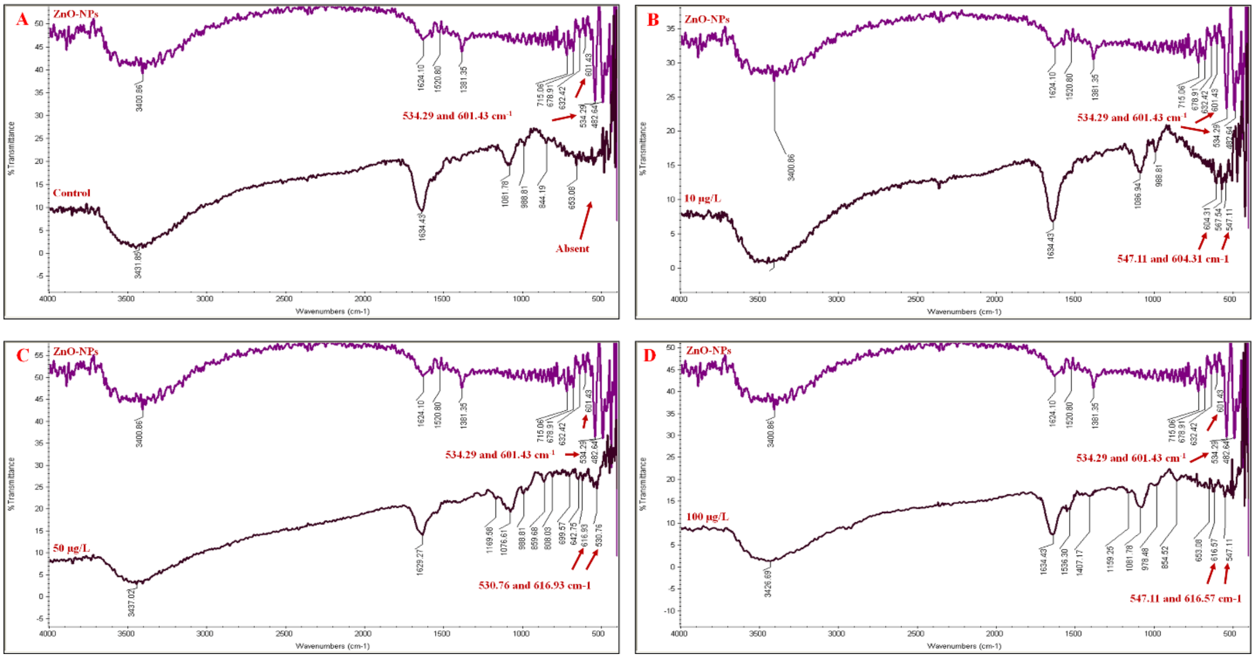


Fig. 2. FTIR overlay spectra of ZnO-NPs and testis exposed to 10, 50 and 100 μg/L of ZnO-NPs. (A) ZnO-NPs and control testis (B) ZnO-NPs and 10 μg/L ZnO-NPs treated testis (C) ZnO-NPs and 50 μg/L ZnO-NPs treated testis (D) ZnO-NPs and 100 μg/L ZnO-NPs treated testis. The spectra obtained from ZnO-NPs was compared for each group of tissue

3.2 Expression analysis of several transcription factor and steroidogenic enzyme genes and estimation of androgen levels in control and treated groups

The expression level of the transcription factors (*dmrt1*, *activin B*, *dax1*, *foxl2*, *wnt5*, and *sf1*) and steroidogenic enzyme (*20β-hsd* and *cyp19a1*) genes were quantified in the testis of control and treated groups (Fig. 3a and b). The expression levels were quantified for all the groups in comparison to control while the change in percentage decrease represented here is for the more significant group of the respective gene. Irrespective of the different levels of exposure of Zn, the testis of common carp in the treated groups showed significant ($P < 0.05$) downregulation for all the genes analyzed when compared to control. The expression level of steroidogenic enzyme genes (*20β-hsd* and *cyp19a1*) showed significant ($P < 0.05$) decrease of 50 and 12 % in 100 µg/L ZnO-NPs treated groups when compared to the control. Significant ($P < 0.05$) down-regulation in the expression levels of *dmrt1* (21%), *activin B* (19%), *dax 1* (45%), *foxl2* (31%), *wnt5* (53%) and *sf1* (62%) in for 100 µg/L dose group of ZnO-NPs exposed groups when compared with the control. Similarly, *20β-hsd* and *cyp19a1* showed a significant ($P < 0.05$) decrease of 46 and 89 %, respectively in 100 µg/L in $ZnSO_4$ treated groups in comparison to the control. The levels of transcripts of transcription factor genes *dmrt1, activin B, dax 1, foxl2, wnt5, and ad4bp/sf-1* displayed significant ($P < 0.05$) 80, 67, 84, 79, 84, and 49 % decrease respectively in 100 µg/L dose group of both the Zn exposed groups in comparison with the control.

Estimation of androgens (T and 11-KT) was done for the control and ZnO-NPs and $ZnSO_4$ treated groups (Fig. 3c and d). A significant decrease ($P < 0.05$) in the levels of T and 11-KT was evident in both the groups when compared to the control. However, among the groups, $ZnSO_4$ treatment showed more significant ($P < 0.05$) decrease in the levels of expression of the genes analyzed and androgens.

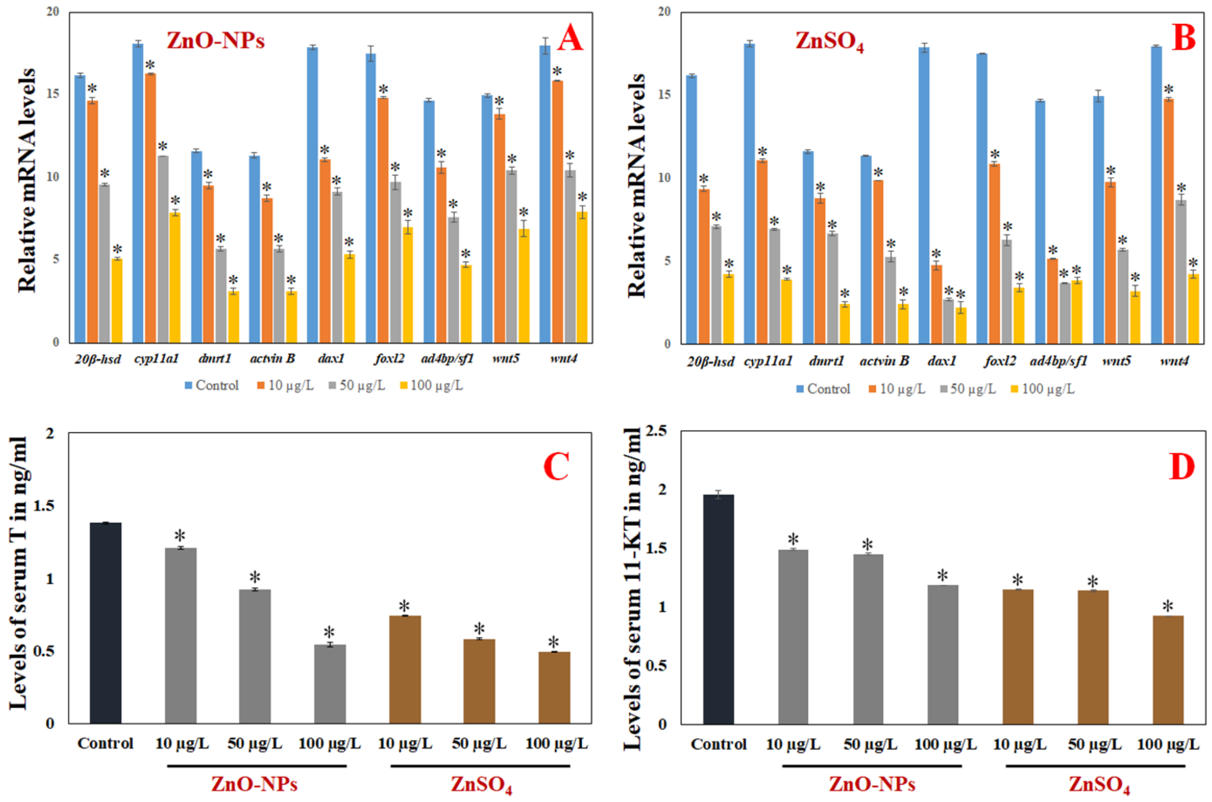


Fig. 3. Expression analysis of several transcription factor (*dmrt1*, *activin B*, *dax1*, *foxl2*, *wnt5* and *ad4bp/sf-1*) and steroidogenic enzyme genes (*20β-hsd* and *cyp11a1*). (A & B) estimation of androgen levels (T and 11-KT; C & D) in control and both the treated groups. Panel of A and B are ZnO-NPs, while C and D are $ZnSO_4$. The relative expression was calculated and normalized with the expression of reference gene, 18SrRNA. Data (n = 5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post-hoc test). C=Control & Arabic number = Dose treated.

3.3 Effects on the levels of antioxidant enzymes (CAT, SOD, and GST) between control and treated groups

The levels of antioxidant enzymes (CAT, SOD, and GST) were determined in the control and ZnO-NPs and $ZnSO_4$ treated groups of common carp (Fig. 4a-c). In both the treated groups, CAT was significantly higher in the testis of common carp. The amount of H_2O_2 (substrate) utilized was directly measured as the units of catalase produced. Common carp exposed to both the groups of treatment exhibited a significant ($P < 0.05$) increase in the level (of H_2O_2 consumed/min/mg protein) of substrate utilized in the testis (Fig. 4a). Similarly, the levels of SOD (Fig. 4b) and GST (Fig. 4c) showed a significant increase ($P < 0.05$) when compared to the control group. The increased activity of oxidative stress enzymes suggested an increase in the production of ROS.

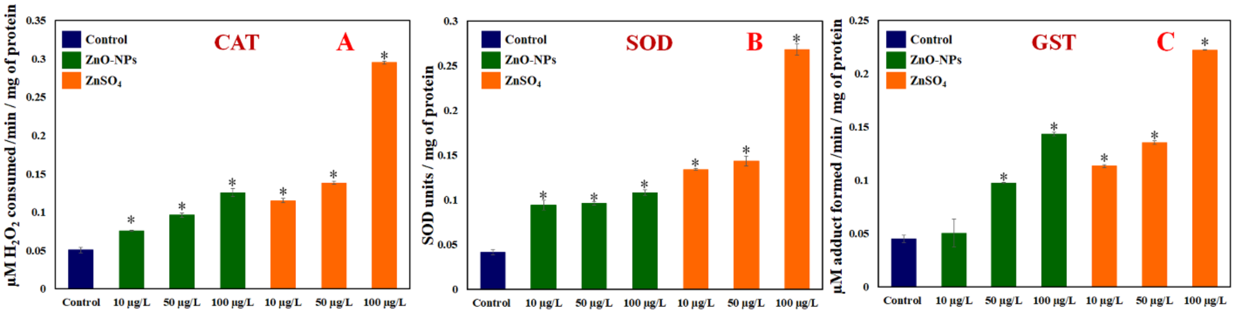


Fig. 4. Estimation on the levels of antioxidant enzymes (A) CAT, (B) SOD and (C) GST between testis of control and treated groups ZnO-NPs and $ZnSO_4$ of common carp. Data (n = 5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post-hoc test).

3.4 Histology of control and treated testis of common carp

Histology of control testis stained with hematoxylin and eosin displayed normal appearance of spermatocytes (SC), spermatids/sperm (S) and interstitial layer (IL). The control testis also showed a normal progression in spermatogenesis including spermatogonia and dark nucleated spermatocytes. The spermatids and/or sperm were detected in the lumen of the testis (Fig. 5). In the treated groups, distortion in the lumen, as well as spermatocytes, was detected. Treatment with a lower concentration (10 µg/L) showed alteration in the architecture of testis lumen, however, the presence of sperm was observed with spermatocytes stained with dark nuclei. Disruption in basal lamina or interstitial layer was clearly seen in the testis after the treatment with 50 µg/L ZnO-NPs and $ZnSO_4$. Incidentally, the presence of spermatids/sperm was found to be less in the testis (Fig. 5). Distortion of spermatocytes and spermatogonia were also seen. Treatment with 100 µg/L concentration of ZnO-NPs and $ZnSO_4$ showed similar symptoms with an accumulation of irregular or disrupted spermatocytes (Fig. 5). The interstitial layer of the lumen was completely damaged with no detection of sperm. Histology of common carp testis after 21 days withdrawal of treatment (both the groups) exhibited the presence of spermatids/sperm indicating the regeneration of testicular cells and progression of spermatogenesis (Fig. 6).

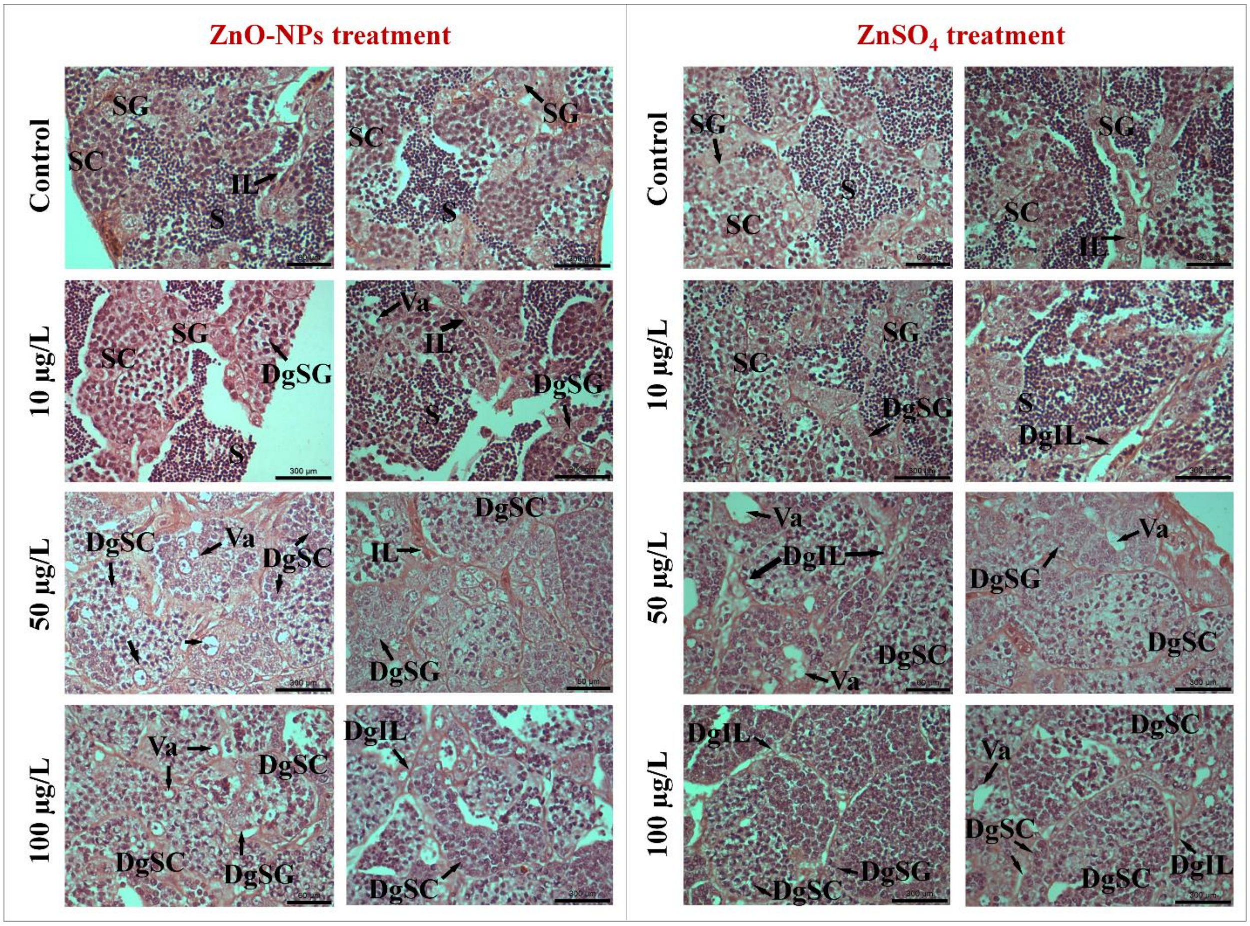


Fig. 5. Representative histology images of control, ZnO-NPs and $ZnSO_4$ treated testis of common carp stained with hematoxylin and eosin. (IL – interstitial layer or basal lamina; SC – Spermatocytes; SG – Spermatogonium; S – Sperm and/or spermatids). Scale bars were indicated inside the microphotographs.

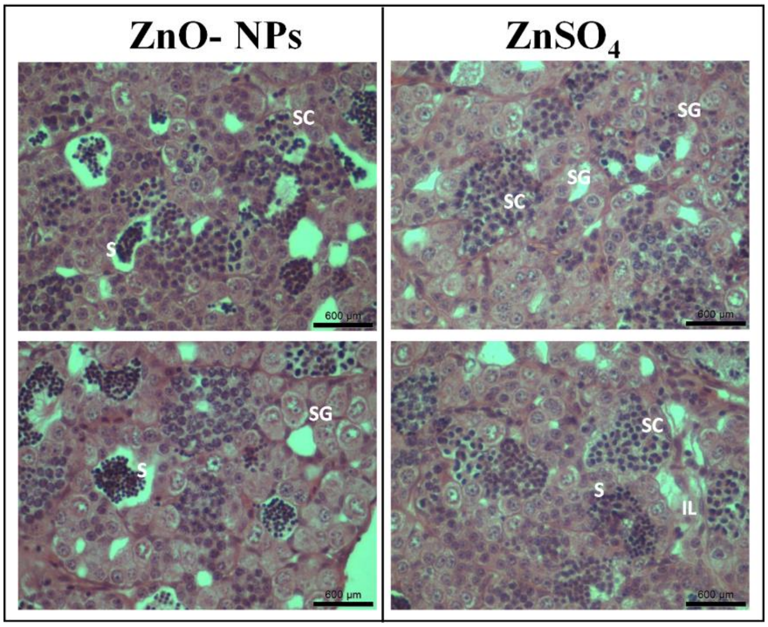


Fig. 6. Histological analysis of common carp testis after 21 days withdrawal of treatment (ZnO-NPs and $ZnSO_4$). (IL – interstitial layer or basal lamina; SC – Spermatocytes; SG – Spermatogonium; S – Sperm and/or spermatids). Scale bars were indicated inside the microphotographs.

3.5 Cytotoxic and morphological analysis of TM3 Leydig cell culture upon Zn treatments

TM3 Leydig cells were exposed to 0.1, 0.5, 1 µg/ml of ZnO-NPs and $ZnSO_4$ to check the cell viability was calculated by MTT assay (Fig. 7a). The cell morphology for control and the treated groups were examined under light microscope. Control group showed normal characteristic feature of TM3 Leydig cell culture with adherent while less cellular toxic effects were seen in 0.1 ug/ml treated batches of ZnO-NPs and $ZnSO_4$ (Fig. 7b). The treatment groups (ZnO-NPs and $ZnSO_4$) of 0.5 and 1 µg/ml doses showed loss of adherence, clumping and floating of the cells were observed indicating the signs of apoptosis/necrosis. The effect seems to be high after $ZnSO_4$ treatment groups when compared to ZnO-NPs groups (Fig. 7b).

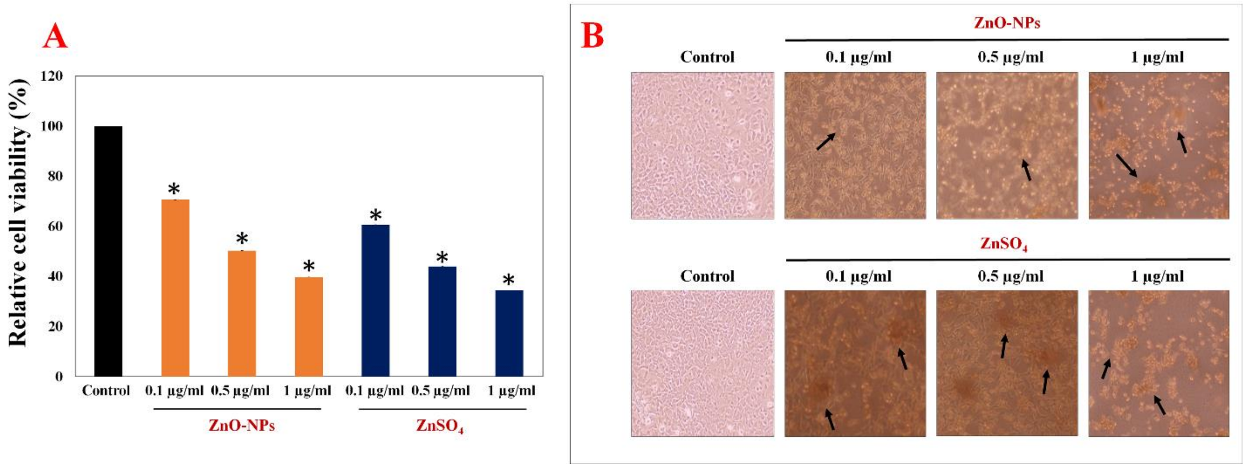


Fig. 7. Cytotoxic and morphological analysis of TM3 Leydig cell culture upon Zn treatments after 48 hrs incubation. (A) Relative cell viability (%) was determined for cells exposed to PBS (control) and ZnO-NPs and $ZnSO_4$ treated groups by MTT assay. (B) Representative cell culture images were taken under a light microscope for the assessment of toxicity. All the results were expressed as total percentage of viable cells with mean ± SEM of three independent determinations with (n = 5) for each point (*$P < 0.05$; statistically significant difference compared with control). Arrows indicated the signs of toxicity with loss of adhesion and cell clumping in the treated groups. Arrow heads indicate healthy control cells.

3.6 Flow cytometric analysis of TM3 Leydig cell culture exposure to Zn

Zn-induced cellular toxic responses were further examined in the treated groups of ZnO-NPs and $ZnSO_4$ through the addition of YO-PRO-1 and PI dyes and the difference in normal and apoptotic/necrotic cells were analyzed by flow cytometry (Fig. 8a-h). Further, the results were also compared with the control groups without ZnO-NPs and $ZnSO_4$ (Fig. 8a and e). Results demonstrated that apoptotic/necrotic cells were increased with the concentration of ZnO-NPs (Fig. 8 b-d) and $ZnSO_4$ (Fig. 8 f-h) in the treated groups when compared to control. The cell group observed in the quadrant (Q3) is represented as live cells. The percentage of cells stained with YO-PRO-1 dye is represented as early apoptotic cells (Q4 or low right quadrant), while in the upper right quadrant (Q2) late apoptotic cells stained with both YO-PRO-1 and PI were seen. The upper left quadrant (Q1) denotes the dead cell population, and each quadrant cell percentage was calculated and indicated in the table 2. In general, the percentage of cells in the quadrants of Q2 and Q4 increased correspondingly to the concentration in both the groups.

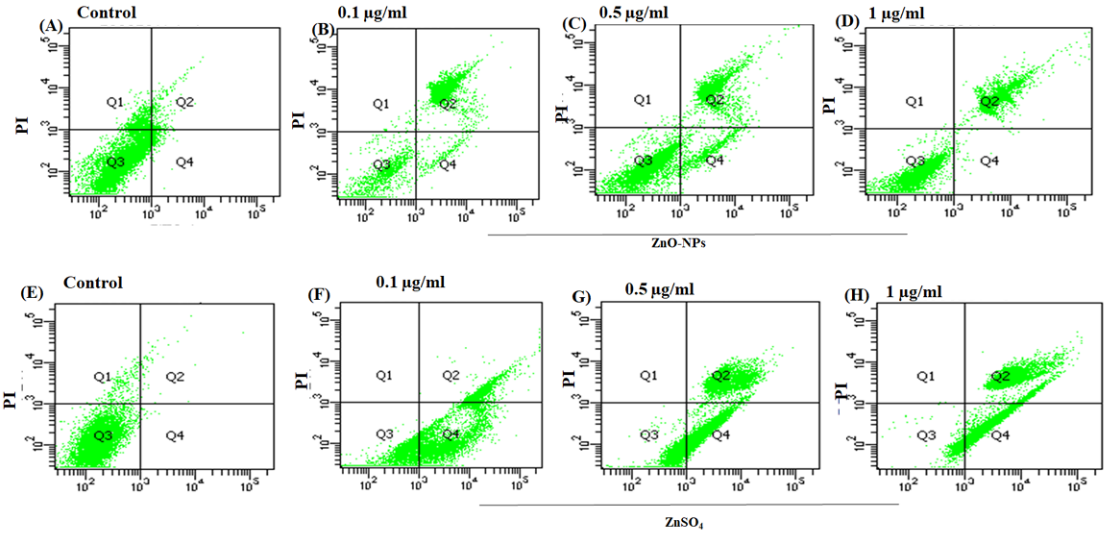


Fig. 8. Flow cytometry analysis of TM3 Leydig cell culture exposed to increasing concentration of ZnO-NPs and ZnSO4 and estimated using YO-PRO-1 and PI labeled dye. Early apoptotic cells stained with YO-PRO-1 dye was indicated in the lower right quadrant (Q4) and late apoptotic cells stained with both YO-PRO-1 and PI were shown in the upper

right quadrant (Q2).

Treatment	Concentration of dose (µg/ml)	Normal dead cells (Q1) Upper left quadrant (%)	Late apoptotic cells (Q2) Upper right quadrant (%)	Live cells (Q3) Lower left quadrant (%)	Early apoptotic cells (Q4) Lower right quadrant (%)
TM3 Leydig cells					
$ZnSO_4$	Control	6.8	2.6	87.5	3.1
	0.1	0	23.8	32.5	43.7
	0.5	0	28.7	23.9	47.4
	1	0	43.0	5.4	51.6
ZnO-NPs	Control	2.1	0.6	97.2	0.2
	0.1	0.2	20.3	75.0	4.4
	0.5	0.2	40.4	51.3	8.1
	1	0.3	55.2	37.3	7.2

Table 2. Determination of percentage of live, dead and apoptotic cell population of *Mus musculus* TM3 Leydig cells exposed to ZnO-NPs and $ZnSO_4$ and analyzed through FACS analysis.

3.7 Estimation of DNA damage by comet assay

DNA damage by comet assay showed a significant increase in the tail length, tail intensity and olive movement in both the treated groups when compared to control while the representative images (Fig. 9a). All the indicated parameters were measured and calculated in the graph (Fig. 9b) were exhibited with DNA damage at the single cell level after the treatment. Concurrently, a dose-dependent response was evident which further indicated the presence of chromosomal irregularities in comparison to the control.

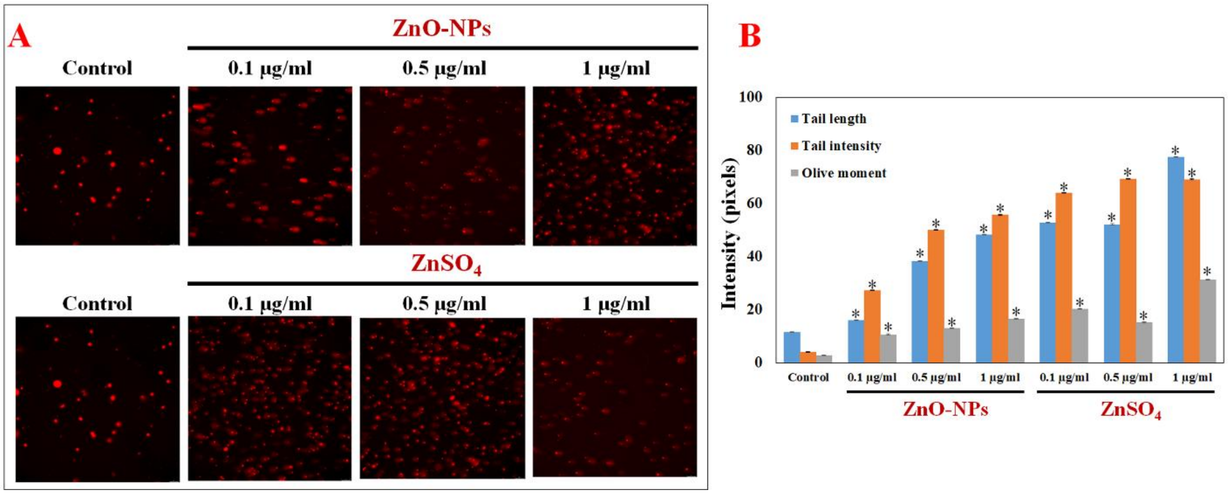


Fig. 9. Estimation of DNA damage by comet assay in the control and treated groups of ZnO-NPs and $ZnSO_4$. (A) Representative images of TM3 Leydig cells of control, ZnO-NPs and $ZnSO_4$ treatment after 48 h. (B) The comet parameters (tail length, tail intensity, olive moment) as measured using image J comet assay plugin software (shown in histogram), for ZnO-NPs and ZnSO4 treatment groups and the untreated cells (control). The statistical data was calculated same as fig.3.

3.8 Measurement of ROS in TM3 Leydig cells exposed to Zn treated groups

Intracellular ROS was measured in TM3 Leydig cells after the exposure of ZnO-NPs and $ZnSO_4$ groups and compared with the control (Fig. 10a-b). Significant ($P<0.05$) increase in the percentage of ROS and time-dependent increase in the intracellular ROS levels were seen in both the treated groups when compared to the control (Fig. 10a). The flow cytometry analysis showed a significant increase in the intensity of FITC (73 to 91.1%) at concentrations 0.1 µg /ml -1 µg as compared to control (20 %) and the percentage of cumulative ROS generation was calculated and tabulated for each group (Fig. 10b).

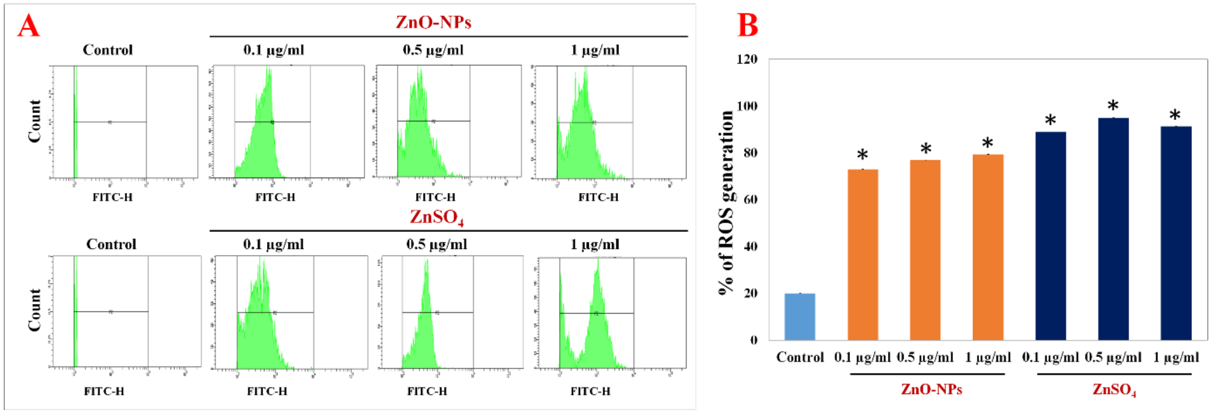


Fig. 10. Measurement of intracellular ROS generation by FACS between control and treated groups of ZnO-NPs and $ZnSO_4$. (A) Intracellular ROS in TM3 Leydig cell culture exposed to the concentrations 0.1, 0.5 and 1 µg/ml of ZnO-NPs and $ZnSO_4$ groups (B) Percentage of ROS generation was calculated between control and the treated groups.

3.9 Measurement of tissue Zn concentration in testis of control and treated groups

Concentration of Zn was estimated in the control and both the treated groups. All the results were expressed in ppb. The amount of Zn in the testis was increased significantly ($P < 0.05$) with the increasing concentration of Zn treated groups and also in comparison to the control ($P < 0.001$). The operating conditions were given in chapter 1. The results of all the groups were provided in the table 3.

Group		Amount of Zn (ppb)
Control		**50 ± 0.29**
ZnO-NPs*	**10 µg/ L**	**885 ± 0.33**
	50 µg/L	**4665 ± 0.31**
	100 µg/L	**5359 ± 0 .23**
$ZnSO_4$*	**10 µg/ L**	**993 ± 0.34**
	50 µg/L	**4773 ± 0.44**
	100 µg/L	**5596 ± 0.12**

Table 3. Determination of Zn in testis of control and treated groups. All the results were expressed in ppb with mean ± SEM

4. Discussion

The present study analyzed the effects of Zn in both forms exposed to 6-month-old common carp for 21 days to determine its role in terms of dose and duration. Physico-chemical characterization of NPs is essential for any aquatic toxicological study. The analysis showed relatively negative charge on the surface of NPs. Toxicity and stability of NPs depends on size and Zeta potential as well as tissue uptake and interstitial trafficking. The measured zeta potential was around +26.0mV. FTIR was used to assess potential interaction of the molecules. Though minor shift was observed in functional positions in treated groups when compared to ZnO-NPs, which is considered the change is negligible. Taken together, these results warrants the entry or presence of ZnO-NPs in the testis of treated groups. SEM reveals that the ZnO-NPs are spherical and having granular nature with very high purity and uniform nanostructure, and EDAX showed the percentage of ZnO which suggested the presence of more Zn in ZnO-NPs contains less toxic than $ZnSO_4$ (Choi et al., 2016). In TEM analysis, ZnO-

NPs displayed well-defined spherical shaped morphology and average particle size of ZnO-NPs was measured as 20-30 nm (Pietroiusti et al., 2011). In this regard, the potential mode of action of ZnO-NPs undergoes toxicity effect, and the assays were employed (Foresta et al., 2014). Overall, ZnO-NPs data validate for experimental use pertaining to the aim of the present study.

Several studies have authenticated the bioaccumulation, physiological and immunological responses of exposed ZnO-NPs in fish (Abdel-Khalek et al., 2015), however, the present study provided a comprehensive analysis on the reproductive status of male common carp upon the exposure of Zn in the form of nanopowder and $ZnSO_4$ soluble form. Since Zn is known as an essential trace element for spermatogenesis in human and animals (Yamaguchi et al., 2009; Foresta et al., 2014), the results were expected to have a positive effects on the progression of spermatogenesis. However, exposure of Zn in the current study showed a toxic response to the reproductive status of fish even at very low concentration. In addition, the study was further extended to authenticate the toxic effects of Zn by *in vitro* analysis using TM3 Leydig cell culture. Interestingly, all the results of treatment with $ZnSO_4$ have witnessed more toxicity when compared to ZnO-NPs. This might infer that the reproductive stage of the fish seems to be critical to evoke a positive or deleterious response by Zn. Testicular steroidogenesis is an important event for androgen production and spermatogenesis, which is essentially regulated by several transcription factor (*dmrt1, activin B, dax1, foxl2, wnt5* and *ad4bp/sf-1*) and steroidogenic enzyme genes (*20β-hsd* and *cyp19a1*) as evidenced from earlier studies (Nagahama et al. 1997, Sreenivasulu et al. 2012; Sudhakumari and Senthilkumaran, 2013, Murugananthkumar and Senthilkumaran, 2016, Prathibha and Senthilkumaran, 2017). Incidentally, treatment of both forms of Zn down regulated many of the genes mentioned above which might implicate the deleterious effects of Zn either directly or indirectly and also by reducing the levels of androgens. In order to analyze this further histological analysis of testis was performed.

Although the effect of Zn toxicity was assessed in several studies, this is the first report showing distinct damage of testis with increasing concentrations of ZnO-NPs and $ZnSO_4$ exposure by histology. Rats fed with Zn-deficient diet-induced necrosis of precursors of germ cells leading to tubular atrophy and affected differentiation of spermatids (Merker and Gunther, 1997). In the present study, common carp testis showed a severe damage in both the treated groups. Spermatocytes, sperm/spermatids, and basal lamina or interstitial layer with the well-defined architecture of testis were clearly seen in the control group. Treatment with ZnO-NPs and $ZnSO_4$ exhibited a defect in the testicular lumen as well as spermatogenesis progression and incidentally, the higher dosage groups showed no signs in the presence of spermatids/sperm. On the other hand, recovery after Zn treatment displayed the presence of spermatids/sperm which signifies testis recouping and regeneration of spermatogenesis. Several studies have validated the harmful effects of NPs especially in a reproductive system which adversely affects the spermatogenesis at various levels (Lan and Yang, 2012). Histological analysis of common carp after Zn treatment showed hemorrhage and tubular regeneration in the kidney, while inorganic exposure of mercury leads to histological damage and oxidative stress in gonads of zebrafish (Yu et al., 2013). Exposure of Cu-NPs in common carp showed altered oxidative stress enzymes and disruption in gills, liver, and kidney (Gupta et al., 2016). In addition, histology and TEM analysis on the effect of copper in catfish validated damage in the testicular cells and basal lamina (Murugananthkumar et al., 2016). As like copper and mercury, Zn, a heavy metal trace element also shares similar impact when exposed to common carp. In view of this, high levels of oxidative stress enzymes (CAT, SOD, and GST) authenticate the toxic effects. Overall down-regulation of several transcription factor and steroidogenic enzyme genes indicate the targeted effects of ZnO-NPs and $ZnSO_4$ on carp testis. Oxidative stress reflects disturbance between the ROS production and the biological response of an organism that detoxifies the mechanism. It is one of the most commonly reported mechanisms on any NPs toxicity and can be overcome through several enzymatic and non-enzymatic antioxidant systems (Manke et al., 2013). In general, increase in oxidative stress enzymes results in the production of more ROS. Exposure of Zn in common carp showed an increase in oxidative stress enzymes in both the treatments. In *Oreochromis niloticus* and *Tilapia zillii*, the impact of Zn-NPs showed a significant increase in levels of several antioxidant enzymes and gene expression in brain (Saddick et al, 2017). Similarly, adverse effects of Zn-NPs were seen in the liver and gills with an increase in the levels of oxidative stress enzymes (Abdel-Khalek et al., 2015). Hence, entry of Zn in common carp might have stimulated the signaling cascade mechanism and elevated the production of ROS generation. Present study indicated the entry of NPs into the testis of common carp, interaction with macromolecules to alter the levels of gene expression, androgen, and oxidative stress enzymes, which eventually affected the testicular architecture. Next, *in vitro* analysis was performed using TM3 Leydig cell cultures with increasing concentrations of ZnO-NPs and $ZnSO_4$ to assess cellular toxicity and apoptosis. Toxicity effect was less in 0.1 µg/ml dose while the effects increased at high doses. Morphological analysis of TM3 Leydig cells upon Zn treatment showed clumping of cells with loss of adherent. Comet assay upon Zn treatment showed damage to DNA which correlated well with the increase in oxidative stress enzymes in the common carp. In accordance with the present study, ZnO-NPs (<35 nm size) showed significant genotoxic damage in the human lymphoblastoid cell line (Demir et al., 2014). In addition, ROS mediated cytotoxic effect was similar to the observations in the *in vivo* analysis in the common carp, revealing the characteristics of DNA damage and apoptosis (Sharif et al., 2012; Bishop et al., 2007). Taken together, elevated concentrations of Zn cause adverse reactions and toxicity in common carp in a way similar to *in vitro* analysis.

The present report provided a comprehensive analysis of toxic effects of Zn in common carp testis. Exposure of Zn-NPs to common carp resulted in downregulation of several genes and androgens level affecting testicular function. Increase in oxidative stress-related enzymes supports the damage in testis and defect in spermatogenesis progression observed histologically. In addition, *in vitro* analysis on the effect of Zn showed signs of toxicity and cellular damage. Testicular histological observation after treatment withdrawal indicated probable recouping and plasticity in the common carp to counter the toxic effects of Zn. This may perhaps explain the property of Zn being considered as an important trace element for testicular function in teleost. Present study highlights the stage dependent effects of Zn excess in testicular function, which is different from Zn-defiency.

In general, comparing the results of the chapters 1 and 2, it is apparent that exposure of Zn at minimal dose for a shorter duration promotes spermatogeneis. In contarst, if Zn exposure is done for a longer duartion even at a minimal dose using ZnO-NPs and $ZnSO_4$ impairs testicular devlopment in common carp. Nevetheless, testis recouping after Zn (ZnO-NPs and $ZnSO_4$) treatment withdrawal warrents that Zn exposure might promote testicular devlopment and spermatogenesis if appropriate dose and duration is selected. Ideally Zn is an essential tarce element for promoting tesicular function only when appropriate dose, time and reproductive stage are chosen.

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Consolidated Summary

The present study represents the importance of *wnt4*, *wnt5* and *wnt8* during testicular development and regulation of spermatogenesis in common carp, *C. carpio*. In addition, comprehensive role of these genes in relation to testicular development and steroidogenesis were also analyzed using Zn as an essential trace element. Further, various analysis towards understanding of the interactive role of Wnt signaling and Zn in spermatogenesis proved that Zn acts as a global activator for both canonical and non-canonical Wnt signaling pathways. A detailed study was also carried out to test whether Zn at higher dose for longer duration can be used in common carp to promote spermatogenesis. Incidentally, upon exposure of ZnO-NPs to common carp resulted in downregulation of several genes and androgens level affecting testicular function. Increase in oxidative stress-related enzymes supports testicular damage and defect in spermatogenesis progression through histological analysis. In addition, *in vitro* analysis on the effect of Zn showed signs of toxicity and cellular damage. Testicular histological observations after treatment withdrawal indicated probable recouping and plasticity in the common carp. This may perhaps explain the property of Zn being considered as an important trace element for testicular function in teleosts. Present study highlighted the stage-dependent effect of Zn on testicular function. Desirable effects of Zn is not seen when it is given in excess or to juvenile fish. It seems the action of Zn to promote carp spermatogenesis is unequivocally demonstrated in this study, yet, the effect is dose-, duration- and stage-dependent. A brief overview of results were graphically represented in figure 1.

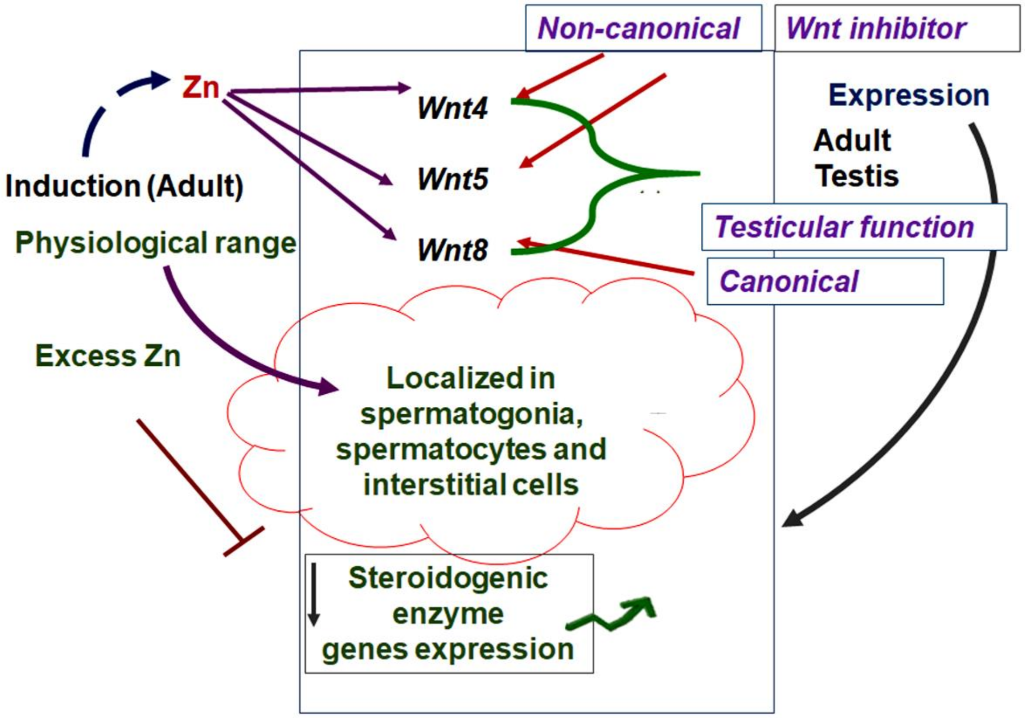

Fig. 1. Proposed mechanism of Wnt specific pathways with interactive influence of Zn to regulate steroidogenesis vis-à-vis testicular growth and maturation in common carp.

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Effects of zinc oxide nanoparticles and zinc sulfate on the testis of common carp, *Cyprinus carpio*

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ABSTRACT
The present study analyzed the effects of zinc oxide nanoparticles (ZnO-NPs) and zinc sulfate ($ZnSO_4$) in the testis of six-month-old common carp *Cyprinus carpio* exposed to three different doses, viz., 10, 50, and 100 µg/L for 21 days. Characterization of ZnO-NPs was done after sonication, the size and shape of ZnO-NPs were determined as ∼20–30 nm spherical structure measured zeta potential of +26.0 mV. After treatment, determination of zinc (Zn) concentration in the testes revealed desired impact of the exposure. Expression of several transcription factors and few steroidogenic enzyme genes in the treated testis showed significant downregulation than the control. Measurement of oxidative stress-related enzymes such as catalase, superoxide dismutase, and glutathione-S-transferase revealed substantial elevation in the testis of treated groups when compared to control. Histological analysis of testis exhibited dose-related response, defective lumen, and slow progression of spermatogenesis. Exposure of both the forms of Zn on TM3 Leydig cell culture displayed loss of adhesion, clumping with decreased viability, and a significant increase in the apoptotic cells. In addition, comet and intracellular reactive oxygen species (ROS) assays authenticated DNA damage upon treatment with a significant increase in ROS. Histological analysis after treatment withdrawal showed revival of testis in carp to rescue the effect. Thus, the present report highlights the adverse effect of Zn on the testis function in common carp as well as evident drastically toxic in *in vitro* cultures.

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1. Introduction

Nanotechnology has developed significantly in the last decade and emerging as one of the most promising and exciting techniques owing to its broad applications in agriculture, medicine, and drug delivery as biosensors (Emerich and Thanos 2005; Suri, Fenniri, and Singh 2007; Vigneshvar et al. 2016). On the other hand, inevitable discharge of nanoparticles (NPs) in the form of powder, cluster, and crystal, and their byproducts released into the aquatic system either directly or indirectly imparts adverse effect and/or toxicity to aquatic organisms (Krysanov et al. 2010; Jeevanandam et al. 2018). NPs can form an aggregate with the organic matter or any other natural particles present in water bodies, yet its uniqueness even the size difference can cause toxicity to living organisms (Handy et al. 2008). Further, NPs are known to influence the abiotic factors such as pH and salinity, and affect the biotic environment, ultimately.

Fishes serve as excellent experimental models for aquatic toxicology study as they are regarded as one of the best bioindicators of pollutants (Authman et al. 2015). Several studies warrant that compounds accumulating in the aquatic ecosystem through several industrial and other effluents, especially in fish could pose a risk not only to the aquatic fauna but also be detrimental to human and other consuming organisms due to biomagnification (Amin, Begum, and Mondal 2011; Ahmed et al. 2016). Interestingly, several studies have validated that NPs can cross blood–testis barrier leading to testicular accumulation NPs and translocation to testicular cells and/or nucleus to

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Gonadal transcriptome analysis of the common carp, *Cyprinus carpio*: Identification of differentially expressed genes and SSRs[☆]

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ABSTRACT

Common carp (*Cyprinus carpio*) is a world-wide freshwater fish of eutrophic waters. *C. carpio*, have various reproductive traits, including early sexual maturity, that may make them excellent, large, realistic, aquaculture model species. In the present work, *de novo* assembly of gonadal (testicular and ovarian) transcriptomes from juvenile common carp was performed to identify genes involved in gonadal development. A total of 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, were obtained from the immature testicular and ovarian transcriptomes, respectively. About 84,367 unigenes were constructed after removing redundancy involving representation of transcripts in both gonadal transcriptomes. Gene ontology (39,171 unigenes), clusters of orthologous group's analysis (6651 unigenes) and Kyoto encyclopedia of genes, and genomes automatic annotation server analysis (4783 unigenes) were performed to identify potential genes along with their functions. Furthermore, 18,342 (testis) and 8693 (ovary) simple sequence repeats were identified. About 298 differentially expressed genes were identified, of which 171 and 127 genes were up-regulated in testis and ovary, respectively. Quantitative real-time reverse transcription PCR was performed to validate differential expression of selected genes in testis and ovary. Nearly 809 genes related to reproduction were identified, sex-wise expression pattern of genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others

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Abbreviations: ad4bp/sf-1, adrenal 4 binding protein/steroidogenic factor-1; *amh*, anti-Mullerian hormone; *amhr2*, anti-Mullerian hormone receptor type 2; *amhy*, Y chromosome-linked anti-Mullerian hormone; *ar*, androgen receptor; *atm*, Ataxia telanglectasia mutated; COG, clusters of orthologous groups; Ct, cycle threshold; *ctnnbip1*, catenin beta interacting protein 1; *cux1*, cut like homeobox 1; *cux2a*, cut like homeobox 2a; *cyp11b1*, cytochrome P450, family 11, subfamily b, polypeptide 1; *cyp17*, steroidogenic cytochrome P450 17-hydroxylase/lyase; *cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1; *cxcl12*, C-X-C motif chemokine ligand 12; *cxcr4*, C-X-C motif chemokine receptor 4; *dax1*, dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DEG, differentially expressed genes; *dmrt1*, doublesex and mab-3 related transcription factor 1; *DMY*, Y-specific DM-domain; dph, days post hatch; *egr2b*, early growth response 2b; *er*, estrogen receptor; *fam101b*, family with sequence similarity 101, member B; *fam192a*, family with sequence similarity 192, member A; *fam210b*, family with sequence similarity 210, member B; *fbox43*, F-box only protein 43; *fem1b*, fem-1 homolog B; *fgfr1a2*, fibroblast growth factor receptor 1-A-like; *foxk2*, forkhead box k2; *foxl2*, forkhead box L2; FSH, follicle stimulating hormone; *fshr*, follicle stimulating hormone receptor; *fstl3*, follistatin-like 3 (secreted glycoprotein); *fzd8*, frizzled class receptor 8; *gata4*, GATA binding protein 4; *gata6*, GATA binding protein 6; *gdf9*, growth differentiation factor 9; GnRH, gonadotropin-releasing hormone; GO, gene ontology; *gsdf*, gonadal somatic cell-derived factor; *hsd3b*, hydroxy-delta-5-steroid dehydrogenase, 3 beta; *hsd3b7*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 *hsd11b1* hydroxysteroid 11-beta dehydrogenase 1; *hsd11b2*, hydroxysteroid 11- beta dehydrogenase 2; *hsd17b2*, hydroxysteroid 17- beta dehydrogenase 2; *hsd20b2*, hydroxysteroid 20- beta dehydrogenase 2; *igf1ra*, insulin-like growth factor 1a receptor; *ipo4*, importin 4; KASS, KEGG, automatic annotation server; KEGG, Kyoto encyclopedia of genes and genomes; *kiss2*, kisspeptin2; *lhcgr*, luteinizing hormone/choriogonadotropin receptor; *mapk*, mitogen-activated protein kinase; *mis*, Mullerian inhibiting substance; *msl1b*, male-specific lethal 1 homolog b; NGS, next generation sequencing; *pcna*, proliferating cell nuclear antigen; *plzf*, promyelocytic leukemia zinc finger; *prlhr2a*, prolactin releasing hormone receptor 2a; PPA, protein phosphatase 2A; *pou5f1*, POU class 5 homeobox 1; qRT-PCR, quantitative real time reverse transcription PCR; RA, retinoic acid; RAR-RXR, retinoic acid receptor/retinoid x receptor heterodimers; *rspo1*, R-spondin 1; sdY, *sexually dimorphic on the Y chromosome*; *smad3b*, SMAD family member 3b; *sox9a*, SRY-box 9a; *SRY*, sex-determining region Y; SSR, simple sequence repeat; *StAR*, steriodogenic acute regulatory protein; *sycp1*, synaptonemal complex protein 1; *sycp3*, synaptonemal complex protein 3; *tac3a*, tachykinin 3a; *tcf3a*, transcription factor 3a; *TGF-β*, transforming growth factor-beta; *wnt*, wingless-type MMTV integration site family; *wt1*, Wilms tumor 1; *zar1*, zygote arrest 1; *zp2*, zona pellucida sperm-binding protein 2

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Role of Wnt signaling and zinc in common carp testicular function

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