Università di Catania
Dipartimento di Scienze
Biologiche, Geologiche e Ambientali

PhD Course in "Earth and Environmental Science"

Dottorato di Ricerca in "Scienze della Terra e dell'Ambiente"

XXXIV CICLO
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Titanium dioxide nanoparticles: toxicity and possible role as endocrine disruptors

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Ph. D. attended during 2018/2021

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ABSTRACT

In recent decades, developing countries have seen a reduction in male reproductive parameters, such as sperm quality and Leydig cell function. In 20% of infertile couples, the male factor is exclusively responsible of infertility. It is known that the increase in male infertility is due to exposure to xenobiotics with hormonal activity called Endocrine Disruptors (IE). They can affect the hormonal system of human and animals, thus compromising development, reproduction and health. Although the activity of "Classic Endocrine Disruptors" is well known, such as: pesticides, insecticides, persistent organic pollutants (POPs), perfluorinated chemicals (PFCs), polychlorinated biphenyls (PCBs), phthalates, bisphenol A (BPA) and heavy metals; it is necessary to include nanomaterials and in particular nanoparticles (NPs) among the exogenous substances present in the environment. The introduction and use of nanoscale materials is changing the our way of life, leading to a new revolution that can be called Nano Revolution. Nanoparticles find application in various sectors such as electronics, biomedicine, pharmaceuticals, cosmetics, food industry, the environmental sector; therefore their release into the environment can lead living beings, including humans, to exposure continuous and involuntary. Thanks to their extremely small size (1-100 nm), nanoparticles (NPs) are more absorbable by living organisms; consequently they can cross the circulatory system and then be distributed in various organs including the reproductive ones. Therefore, a possible endocrine activity by NPs cannot be excluded. Metal oxide nanoparticles (MONPs), are the most versatile category of nanoparticles that have multiple applications, and among these the Titanium Dioxide Nanoparticles (TiO_2-NPs) are used intensively.

The aim of my PhD thesis has been evaluated the possible toxic effect of titanium dioxide nanoparticles (TiO_2-NPs), as endocrine disruptors on the male reproductive system through *in vivo* and *in vitro* assays; in addition their toxic effect on embryonic development was also evaluated.

Commercial TiO_2-NPs (P25, Degussa) were tested at concentrations of 1 mg/L, 2 mg/L and 4 mg/L on *Danio rerio* embryos by ZFET (Zebrafish Embryo Toxicity Test) and on male adults using a 30-day chronic toxicity test.

At the end of the exposure, biomarker of oxidative stress such as Poly ADP-Ribose Polymerase-1 (PARP-1) and Heat Shock Protein-70 (Hsp70) were evaluated on zebrafish larvae, as well as the Sex Hormone Binding Globulin (SHBG) and the Protimosin-α (PTMA) were evaluated as biomarker that suggest the action of TiO_2-NP as endocrine disruptors. Rates of coagulated and hatched eggs, and changes in larvae phenotype, such as larva length, were evaluated throughout the exposure period; finally, heart rate was also assessed using the DanioScope™ software. On adult male gonads, the effect of TiO_2-NPs was evaluated through histomorphological changes and the expression of Sex Hormone Binding Globulin (SHBG) and P540 aromatase (P540) biomarkers by immunofluorescence assays; the expression of the SHBG, SRD5A2, SOD2 and GPX4B genes was also evaluated by qRT-PCR. Finally, the effect of TiO_2-NPs was evaluated on human spermatozoa through *in vitro* assays. TiO_2-NPs concentrations were tested at 50ppm, 100ppm, 250ppm and 500ppm, because these could be concentrations that coat a drug capsule. In fact, there is no regulation for the amount of TiO_2-NPs used as an additive in drugs. Swim-up by pellets was used to incubate the spermatozoa with the TiO_2-NPs solutions, for 1 hour exposure. At the end of the incubation the following sperm parameters were evaluated: motility, viability, DNA fragmentation by Halosperm and TUNNEL assay, intracellular evaluation of ROS by MiOXSYS and evaluation of biomarker Heat Shock Protein-70 (Hsp70), Sex Hormone Binding Globulin (SHBG) and Metallothionein (MT). Furthermore, the localization of TiO_2-NPs on spermatozoa was evaluated by Scanning Electron Microscopy.

The results obtained showed that the TiO_2-NPs did not interfere with the embryonic development of *Danio rerio*, the embryos exposed to all TiO_2-NPs concentrations (1mg/L, 2mg/L and 4mg/L) completed the development of the embryo and their hatching rate was higher than controls ($p < 0.05$). The exposed embryos did not show marked malformations; however, there was a greater decrease in body length and an increase in heart rate (BPM) in the 4mg/L group compared to the control and other exposed groups ($p < 0.05$). The biomarker evaluated showed that TiO_2-NPs induced oxidative

stress as demonstrated by the positivity to the biomarkers PARP-1 and Hsp70. Furthermore, a positivity to the SHBG biomarker was observed at the level of the larva's head, with a higher intensity for the 4mg/L concentration; instead the biomarker PTMA showed a positivity in the whole body of the larva. In the male gonads, the TiO_2-NPs caused an alteration of the morphological/structural organization of the gonad with evident detachment between the seminiferous epithelium and the basement membrane on which it physiologically rests. Disorganization of the testicular tubules was also observed for the concentration of 4 mg/L. The structural investigation using transmission electron microscopy also highlighted the presence of vacuoles and the necrosis of the Sertoli cells. The immunofluorescence investigation showed a positivity for the SHBG biomarker especially at the Leydig cell level and the same was observed for the P540 biomarker. The results of qRT-PCR showed an increase in the gene expression of the genes involved in oxidative stress and also of the gene responsible for the conversion of testosterone to dihydrotestosterone. Since Leydig cells are mainly involved in this activity, an increase in gene activity can be explained by the ability of TiO_2-NPs to act as endocrine disruptors and therefore with androgenic activity. For spermatozoa, no decrease in sperm motility and viability was observed, instead fragmentation of spermatic DNA was increased due to the oxidizing activity of TiO_2-NP as demonstrated by the increase in the ORP value for the concentration of 500 ppm (ORP: 206.7 mV) obtained from MiOXSYS, and the positivity for the biomarker Hsp70. A positivity in the neck of the spermatozoa was also observed for the SHBG biomarker.

Overall, our investigation confirms that TiO_2-NPs have the ability to induce oxidative stress both during embryonic development and at the level of the male gonad and human spermatozoa. This inevitably leads to an alteration of the ROS content with consequences on normal cellular functioning. In male gonads, the increase in ROS affects normal spermatogenesis, while in sperm cells it causes DNA damage with failure in fertilization. Furthermore, the androgen-like activity of TiO_2-NPs cannot be excluded. Therefore, the results obtained can contribute to increasing knowledge on the toxicity of TiO_2-NPs, whose data in the literature focus mainly on embryonic development and little on the reproductive system; furthermore, these results can be translated to man so as to increase knowledge on male infertility

Keywords: nanoparticles, endocrine disruptors, infertility, *Danio rerio*, human sperm cell, embryonic development, male organs.

Negli ultimi decenni, i paesi in via di sviluppo hanno registrato una riduzione dei parametri riproduttivi maschili, come la qualità del seme e la funzione delle cellule di Leydig. Nel 20% delle coppie infertili il fattore maschile è la principale causa di infertilità. È noto che l'incremento dell'infertilità maschile sia dovuta all'esposizione a xenobiotici con attività ormonale chiamati interferenti endocrini (IE). Essi possono influenzare il sistema ormonale dell'uomo e degli animali, compromettendo così lo sviluppo, la riproduzione e la salute. Sebbene sia ben nota l'attività di "interferenti endocrini classici" quali: pesticidi, insetticidi, inquinanti organici persistenti (POP), sostanze chimiche perfluorurate (PFC), policlorobifenili (PCB), ftalati, bisfenolo A (BPA) e metalli pesanti; è necessario includere tra le sostanze esogene presenti nell'ambiente anche i nanomateriali e in particolare le nanoparticelle (NP). L'introduzione e l'uso di materiali su scala nanometrica, sta cambiando il nostro modo di vivere, portando ad una nuova rivoluzione che può essere definita "Nano Revolution". Le nanoparticelle vengono applicati in vari settori come l'elettronica, la biomedicina, i prodotti farmaceutici, i cosmetici e l'industria alimentare quindi un loro rilascio a livello ambientale può determinare un'esposizione continua e involontaria per gli esseri viventi, uomo compreso. Le NP grazie alle loro dimensioni estremamente ridotte (1-100 nm) sono molto più assorbibili dagli organismi viventi; di conseguenza possono attraversare il sistema circolatorio e quindi essere distribuiti in vari organi compresi quelli riproduttivi. Non è quindi da escludere una possibile attività endocrina da parte delle NPs. Le nanoparticelle di ossido di metallo (MONPs), sono la categoria di nanoparticelle più versatili che trovano diverse applicazioni, e tra esse sono intensamente utilizzate le nanoparticelle di biossido di titanio (TiO_2-NPs).

Lo scopo della mia tesi è stato valutare il possibile effetto tossico delle nanoparticelle di biossido di titanio (TiO_2-NPs), in qualità di interferenti endocrini sul sistema riproduttivo maschile tramite esperimenti *in vivo* ad *in vitro*; inoltre, è stato valutato anche il loro effetto tossico sullo sviluppo embrionale. TiO_2-NPs commerciali (P25, Degussa) sono state testate alle concentrazioni di 1mg/L, 2mg/L e 4 mg/L sugli embrioni di *Danio rerio* tramite ZFET (test di tossicità acuta su embrioni di zebrafish) e sugli adulti maschi tramite un test di tossicità cronica della durata di 30 giorni. Nelle larve di zebrafish al termine dell'esposizione sono stati valutati biomarker dello stress ossidativo quali Poly ADP-Ribose Polymerase-1 (PARP-1) e Heat Shock Protein-70 (Hsp70), e i biomarker Sex hormone-binding globulin (SHBG), Prothymosin-α (PTMA) che suggeriscono l'azione delle TiO_2-NPs come interferenti endocrini. Per tutto il periodo dell'esposizione sono stati valutati i tassi di uova coagulate e schiuse, e cambiamenti nel fenotipo delle larve come la lunghezza della larva; infine, è stata valutata anche la frequenza cardiaca tramite il software DanioScope. Negli adulti maschi, è stato esaminato l'effetto delle TiO_2-NPs sulle gonade quindi sono state valutate tutte le alterazioni isto-morfologiche della gonade e tramite indagini d'immunofluorescenza sono stati valutati i biomarker Sex hormone-binding globulin (SHBG) e P540 Aromatase (P540), inoltre tramite qRT-PCR è stata valutata l'espressione dei geni SHBG, SRD5A2, SOD2 and GPX4B. Infine, l'effetto delle TiO_2-NPs è stato valutato sulle cellule spermatiche umane tramite esperimenti *in vitro.* Sono state testate le concentrazioni di TiO_2-NPs pari a 50 ppm, 100ppm, 250 ppm e 500 ppm che corrispondono a quelle che potrebbero rivestire una capsula di farmaco, dato che non esiste una regolamentazione per la quantità di TiO_2-NPs usata come additivo nei farmaci. È stato utilizzato uno swim-up da pellet per incubare le cellule spermatiche con le soluzioni di TiO_2-NPs, per un tempo totale di 1 ora. Al termine dell'incubazioni sono stati valutati i seguenti parametri spermatici: motilità, vitalità, frammentazione del DNA tramite Halosperm e saggio TUNNEL, valutazione intracellulare dei ROS tramite MiOXSYS e valutazione dei biomarker Heat Shock Protein-70 (Hsp70), Sex hormone-binding globulin (SHBG) e Metallothionein (MT). Infine, la localizzazione delle TiO_2-NPs sulle cellule spermatiche è stata valutata tramite microscopia elettronica a scansione.

I risultati ottenuti hanno dimostrato che le TiO_2-NPs non alterano lo sviluppo embrionale di *Danio rerio*, gli embrioni esposti a tutte le concentrazioni di TiO_2-NPs (1mg/L, 2mg/L and 4 mg/L) completavano lo sviluppo embrione, e il loro tasso di schiusa era maggiore rispetto ai controllo ($p< 0,05$). Gli embrioni esposti non mostravano malformazioni spiccate; tuttavia, è stata evidenziata una diminuzione nella lunghezza del corpo e un aumento delle frequenza cardiaca (BPM) maggiore nel

gruppo 4mg/L rispetto al controllo e agli altri gruppi esposti ($p< 0,05$). I biomarker valutati hanno dimostrato che le TiO_2-NPs inducevano stress ossidativo come dimostrato dalla positività ai biomarker PARP-1, Hsp70. Inoltre, una positività al biomarker SHBG è stata osservata a livello della testa della larva, con un'intensità maggiore per la concentrazione 4mg/L; invece, il biomarker PTMA evidenziava una positività in tutto il corpo della larva. Nelle gonadi degli adulti, le TiO_2-NPs hanno causato un'alterazione nell'organizzazione morfologica/strutturale della gonade con evidente distacco tra l'epitelio seminifero e la membrana basale su cui fisiologicamente poggia. Per la concentrazione 4mg/L è stato anche osservata una disorganizzazione dei tubuli testicolare. L'indagine strutturale tramite microscopia elettronica a trasmissione ha inoltre evidenziato la presenza di vacuoli e la necrosi delle cellule del Sertoli. L'indagine d'immunofluorescenza ha mostrato una positività per il biomarker SHBG soprattutto a livello delle cellule del Leydig e lo stesso è stato osservato per il biomarker P540. I risultati delle qRT-PCR hanno mostrato un aumento dell'espressione genica dei geni coinvolti nello stress ossidativo e anche del gene responsabile della conversione del testosterone in diidrotestosterone. Poichè le celle del Leydig sono principalmente convolte in questa attività, un aumento dell'attività genica si può spiegare con la capacità delle TiO_2-NPs di agire come interferenti endocrini e quindi con attività androgenica. A livello delle cellule spermatiche, non è stato osservata una diminuzione delle motilità e vitalità spermatica, invece è incrementata la frammentazione del DNA spermatico dovuta all'attività ossidante delle TiO_2-NPs come dimostrato dall'aumento del valore di ORP, per la concentrazione di 500ppm (ORP: 206,7 mV), ottenuto tramite MiOXSYS e dalla positività osservata per le Hsp70. Una positività a livello del collo dello spermatozoo è stata osservata anche per il biomarker SHBG.
Complessivamente, la nostra indagine conferma che TiO_2-NPs hanno la capacità di indurre stress ossidativo sia durante lo sviluppo embrionale, sia a livello della gonade maschile e delle cellule spermatiche umane. Ciò comporta inevitabilmente un'alterazione del contenuto di ROS con conseguenze sul normale funzionamento cellulare. Nelle gonade maschile, l'aumento di ROS ha effetti sulla normale spermatogenesi, invece nelle cellule spermatiche è causa di danno al DNA con insuccesso nella fecondazione. Inoltre, non è da escludere l'attività simil-androgenica delle TiO_2-NPs. Dunque, i risultati ottenuti possono contribuire ad aumentare le conoscenze sulla tossicità delle TiO_2-NPs, i cui dati presenti in letteratura si concentrano principalmente sullo sviluppo embrionale e poco sul sistema riproduttivo; inoltre, tali risultati possono essere traslasti sull'uomo così da incrementare le conoscenze sull'infertilità maschile.

Parole chiavi: nanoparticelle, interferenti endocrini, infertilità, *Danio rerio*, spermatozoi umani, sviluppo embrionale, organi maschili

1. Nanotechnology

In recent years nanotechnology has become one of the most important and exciting forefront fields in Physics, Chemistry, Engineering and Biology (Poole and Owens, 2003). Their products, nanoparticles (NPs) and nanostructured materials (NSMs), have become increasingly important and attracted increase investments form both governments and industries around the world (Wang, 2018). Therefore, the Nanotechnology is progressively entered in the everyday life conquered an increased importance in many fields (Chaurasia, 2017).

The simplest definition of nanotechnology is "technology on the nanoscale", it is able to operate on the nanoscale to manipulated and prepared easily materials at the dimensions between approximately 1 and 100 nm (Ju-Nam and Lead, 2008). At this level, single atoms and molecules are employed to form functional devices and structures that have every atom in the proper place (Kaehler, 1994). These materials, exhibit novel and significantly improved physical, chemical, and biological properties, phenomena, and processes due to their nanoscale size (NNI, 2000), for this reason the nanotechnology is also a term encompassing the science, engineering, and applications of nanoscale materials in fundamentally new and useful ways (Wang, 2018).

According to the International Organization for Standardization (ISO), Nanomaterials (NMs) are "material with any external nanoscale dimension or having internal nanoscale surface structure" (ISO/TS, 2015); whereas Nanoparticles (NPs), are isolated solid-phase objects with a size of 1-100 nm (Christian et al., 2008; Shukla, 2016). Nanomaterials and nanoparticles are different concepts and terms. Indeed, nanomaterials are materials composed of nanoparticles. Materials with nanometric dimensions have properties and behaviors significantly different from those of single atoms or bulk materials (Rao and Cheetham, 2001). This happens because particles which are smaller than the characteristic lengths associated with particular phenomena often display unusual physical, chemical, and biological properties which depends on their nanoscale sizes (Poole and Owens, 2003; Wang, 2018). For example, the electronic structure, conductivity, reactivity, melting temperature, and mechanical properties have all been observed to change when particles become smaller than a critical size. The dependence of the behavior on the particle sizes can allow one to engineer their properties (Poole and Owens, 2003). Therefore a valid definition of Nanotechnology is "the careful and controlled manipulation, precision placement, modeling, measurement, and production of materials at the nanoscale in order to make matters, systems, and devices by fundamentally novel properties and functions" (Abad, 2005).

Even if, the word nanotechnology, with their more definitions could be relatively new, the existence of functional devices and structures of nanometer dimensions is not new and in fact such structures have existed on Earth as long as life itself.

2. Nanoparticles

Nanoparticles have always existed, all aquatic and terrestrial environmental systems contain small particles, which cover the size range from 1 nanometer to several micrometers (Baalousha et al., 2011). Typically, the natural nanoparticles (NNPs) are generated by various physical, chemical, and biological processes, such as (bio)chemical weathering of minerals, photo-oxidation, redox and precipitation reactions, (bio)mineralization, physical fragmentation, gas-solid nucleation in the atmosphere, etc. (Simonet and Valcárcel, 2009; Sharma et al., 2015), moreover they can a result of human-related activity or processes (e.g., combustion), due to the life cycle of products containing nanoparticles or accidental releases, for example mining, tillage, and demolition/construction are source of dust generation. In any case, these natural nanoparticles (NNPs) move through different compartments (biosphere, lithosphere, atmosphere and hydrosphere) within global biogeochemical cycle (Lespes et al., 2020). However, human activities disturbed their natural cycle by affecting their emission and release, altering the environmental processes involving nanoparticles and introducing anthropogenic nanoparticles. Anthropogenic nanoparticles, are mostly engineered nanoparticles, they are the result of nanotechnology. Nanoparticles (NPs) are particles that are synthetized with extremely small size, at the nanometer scale, with one dimension in the range of 1–100 nm (10^{-9} m) (Lövestam et al., 2010; ASTM, 2012; Khan et al., 2019). Thanks their nanosize, nanoparticles (NPs) have properties and functions that differ from those with a larger scale (McNeil, 2005). NPs can have a variety of shapes including nanorods, spherical, cubical, and other possible shapes. Nanomaterials can be classified based on the number of dimensions of the material, which are outside the nanoscale (larger than100 nm) range. Accordingly, in zero-dimensional (0D) nanomaterials all the dimensions are measured within the nanoscale (no dimensions are larger than 100 nm). Most commonly, 0D nanomaterials are nanoparticles. In one-dimensional nanomaterials (1D), one dimension is outside the nanoscale. This class includes nanotubes, nanorods and nanowires. In two-dimensional nanomaterials (2D), two dimensions are outside the nanoscale. This class exhibits plate-like shapes and includes graphene, nanofilms, nanolayers, and nanocoatings. Three-dimensional nanomaterials (3D) are materials that are not confined to the nanoscale in any dimension. This class can contain bulk powders, dispersions of nanoparticles, bundles of nanowires, and nanotubes as well as multi-nanolayers (Astete and Sabliov, 2006; Ghosh Chaudhuri and Paria, 2012; Falchi et al., 2018). (Figure 1).

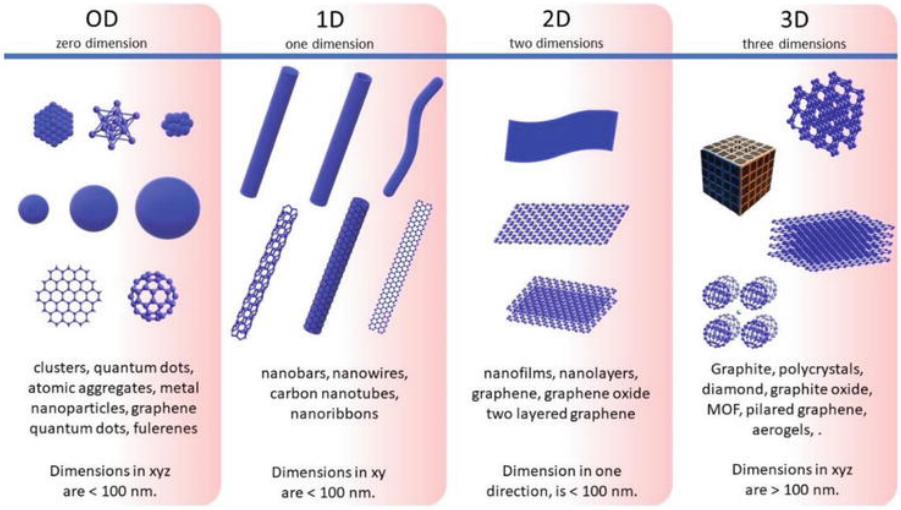

Fig.1: Different types of dimension of nanomaterials

Their classification is need because many types of nanoparticles (NPs) and nanostructured materials (NSMs) have been reported and many other varieties are predicted to appear in the future (Jeevanandam et al., 2018). They are very fast growing particles in the industry thanks their immense importance due to their diverse utilities. It is reported that there are more than 1.814 products including textiles, antibiotics, sport and food items in which nanosized particles are used and this number of products is rapidly increasing (Vance et al., 2015). Nanoparticles are extensively used in industrial and biomedical sectors (Vance et al., 2015), they have application in pharmaceuticals (like drug delivery system), cosmetics (they are used as sunscreens or additive), medicine, engineering, biology, biotechnology, agriculture, food industry (like additive), electronics, wastewater treatments (Penn et al., 2003; Liu, 2006; Kisin et al., 2007; Robertson et al., 2010). Thus, it can be observed that nanotechnology impacted our daily life with its tremendous contributions in different fields, and it is becoming increasingly harder to keep track of where nanotech is not. We are using nanoparticles in our daily lives and not even realizing it.

2.1 Classification of nanoparticles

Although all nanoparticles share the same features as: small size, high surface-to-volume ratio, chemically alterable physical properties, easy surface functionalization and different physical properties from bulk material, they can be categorize into different groups (Vaseem et al., 2010; Fard et al., 2015; Reverberi et al., 2016). Nanoparticles can be categorized according to: their origin and synthesis process, materials properties, dimensions and morphology (Ijaz et al., 2020). As mentioned above, nanoparticles can have a natural origin, be produced accidentally by human activity or voluntarily thanks to nanotechnology (Jeevanandam et al., 2018). The manufactured of

nanotechnology are the engineered nanoparticles (NPs) in which the atoms rearrangement give them new properties. Physical, chemical and biological (green synthesis) methods can be used to synthesize the engineered nanoparticles (Chavali and Nikolova, 2019). Physical methods, also known as the top-down approach, produces nanoparticles by deconstructing larger materials with the use of lithographic tools, then bulk material is modified and shaped into to the desired shape and size, for this reason the art of lithography has been relentlessly improved to create patterns of smaller geometries with higher resolution; instead chemical methods, known as the bottom-up approach, constructs nanomaterials from basic building blocks like atoms or molecules. This method mimicking biological processes in which individual atoms are piled up one at a time, on the substrate to form molecules. These molecules, thanks physical and chemical forces operative at the nanoscale, arrange themselves on the desired shape to produce the required nanostructures. Of particular note are techniques such as sol-gel synthesis, vapour deposition, atomic layer deposition, molecular self-assembly, and many others (Yu et al., 2013; Khanna, 2016a, 2016b). The biological (green synthesis) method, is an environmentally friendly method without use of dangerous organic solvents and inorganic salts, which are commonly used in physical and chemical methods, for this reason this method is commonly used to synthesized NPs that have biomedical applications (Ahmad et al., 2003, 2019; Shankar et al., 2004; Ankamwar et al., 2005; Huang et al., 2007; Das et al., 2017). Organisms such as simple prokaryotic bacterial cells or complex eukaryotes are used (Korbekandi et al., 2009). Unfortunately, this green approach doesn't allow to control the size, shape, and yield of NPs (Stankic et al., 2016). Based on their composition, the engineered nanoparticles are subdivided into: carbon-based nanoparticles, inorganic and organic nanoparticles. The carbon-based nanoparticles are made completely of carbon (e.g., fullerenes, graphene, carbon nanotubes), inorganic nanoparticles include metal-based nanoparticles (MNPs) that are made purely from metal precursors (e.g., Al, Cd, Co, Au Ag, Zn), metal oxides nanoparticles (MONPs) that are synthesized to modify the properties of their respective metal based NPs (e.g., Fe_2O_3, Al_2O_3, ZnO), ceramic NPs that are nonmetallic solids (e.g., HA, ZrO_2, SiO_2) and semiconductor NPs which have properties between metals and nonmetals (e.g., ZnS, CdS) (Ealia and Saravanakumar, 2017; Ijaz et al., 2020). Finally, the organic nanoparticles which include Polymeric NPs (e.g., PEG, PLGA, PLA) and lipid-based NPs (e.g., liposomes, niosomes) (Moreno-Vega et al., 2012). According to morphology, the nanoparticles can have a variety of shapes and geometries including spheres, ellipsoids, cylinders, sheets, cubes, spikes, and rods which considerably affect the toxicity (Cormode et al., 2014; Jeevanandam et al., 2018). Overall, the unique physical and chemical properties of NPs depend on the different way in which they are produced, and at the same time make NPs more stable and soluble than their corresponding

unengineered homologues, for this reason NPs will able to interact with different biological systems (Stankic et al., 2016).

2.2 Applications

Nanoparticles, thanks to their nanostructure-dependent properties have found commercial or medical applications. They are used in a variety of areas including advanced materials, electronics, magnetics and optoelectronics, biomedicine, food industry, pharmaceuticals, cosmetics, energy, and catalytic and environmental detection and monitoring (Penn et al., 2003; Liu, 2006). Inorganic nanoparticles of simple or complex nature, are used to develop novel nanodevices which can be used in numerous physical, biological, biomedical and pharmaceutical applications (Martis et al., 2012; Nikalje, 2015; Loureiro et al., 2016). In medicine, they are used for their ability to deliver drug in the optimum dosage range, often resulting in increased therapeutic efficiency of the drugs, weakened side effects and improved patient compliance (Alexis et al., 2008). Semiconductor and metallic NPs have immense potential for cancer diagnosis and therapy on account of their surface plasmon resonance (SPR) enhanced light scattering and absorption. Au NPs efficiently convert the strong absorbed light into localized heat which can be exploited for the selective laser photo thermal therapy of cancer (Prashant et al., 2007). Beside this, the antineoplastic effect of NPs is also effectively employed to inhibit the tumor growth. The multihydroxylated [Gd@C82(OH)22]n NPs showed antineoplastic activity with good efficiency and lower toxicity (Chen et al., 2005). Also silver nanoparticles (AgNPs) are more studied for cancer therapies, exploiting their unique properties to enhance potential therapeutic efficacy (Ong et al., 2013). While the magnetic properties of iron oxide have been used for therapeutic and diagnostic purposes, such as contrast agents for magnetic resonance imaging and ultrasound techniques (Ramos et al., 2017). Often, nanoparticles are applied to biomedical implants (implants, dental prostheses, medical devices) to improve their biocompatibility, strength, conductivity and durability (Bakand and Hayes, 2016). Studies have reported that ZnO nanostructures may successfully promote the growth, proliferation and differentiation of several cell lines, leading to the formation of new living tissue useful for organ repair. In particular, osteogenesis and angiogenesis have been effectively demonstrated in numerous cases (Laurenti and Cauda, 2017). Nanoparticles exhibit remarkable antimicrobial activity and are often referred to as “nanoantibiotics” (Wang et al., 2017a). Especially, metal or metal oxides nanoparticles have the greatest antimicrobial activity against pathogens, in fact they are included in many commercial products in order to minimize bacterial growth and infections; these products include sunscreens and cosmetics, skin lotions, disinfectants, clothing (Dizaj et al., 2014; Khan et al., 2016). Ag, silver oxide (Ag_2O), titanium dioxide (TiO_2), silicon (Si), copper oxide (CuO), zinc oxide (ZnO), Au, calcium oxide (CaO) and magnesium oxide (MgO) were identified to exhibit antimicrobial activity (Akhavan et al., 2011,

Pant et al., 2013, Qu et al., 2016) and *in vitro* studies revealed that metal nanoparticles inhibited several microbial species. The antimicrobial characteristics of inorganic NPs can be used to synthesize active food packaging materials and to extend the shelf-life of foods. Packaging with nanocomposites containing these nanoparticles offers advantages, such as reduction in the usage of preservatives and higher rate of reactions to inhibit the microbial growth (Hoseinnejad et al., 2018). Moreover, nanoparticles can be direct incorporated into foods, for example food additive such as titanium dioxide (E171), iron oxides and hydroxides (E172), silver (E174), and gold (E175) are highly used as colorants while silicon dioxide (E551) is generally used as anticaking in ultra-processed foodstuff highly used in the Western diets (Medina-Reyes et al., 2020). In the environmental field, nanoparticles have a high impact on agriculture and the environment. The aim of nanoparticles in agriculture is to reduce the amount of spread chemicals, minimize nutrient losses in fertilization and increased yield through pest and nutrient management. Nanofertilizers and nanopesticides are used to trail products and nutrients levels to increase the productivity without decontamination of soils, waters, and protect against several insect pest and microbial diseases (Bindraban et al., 2015; Liu and Lal, 2015; Hao et al., 2017; Prasad et al., 2017). Several studies have evaluated the environmental impact and ecological effects of nanoparticles (NPs), including their toxicological effects on plants, soil and microorganisms (Hao et al., 2016; Mukherjee et al., 2016; Rui et al., 2016). Servin and Colleagues (2015) observed that engineered nanomaterials can suppress plant diseases, increase crop yields and play vital roles as fertilizers and pesticides, while Ditta and Arshad (2016) have shown that nanomaterials can provide plants with more nutrients than conventional fertilizers. Overall, the nanoparticles have the ability to improve seed germination, growth and plant protection, and they provide innovative solutions for the treatment and protection of water and soil, thus increasing the production and quality of food (Iavicoli et al., 2017). Photodegradation by NPs is also very common practice and many nanomaterials are utilized for this purpose. Photodegradation reaction is facilitated by high surface area of NPs due to their very small size (<10 nm) (Rogozea et al., 2017). Nanoparticles are commonly used in the treatment of wastewater (Chiavola et al., 2016, 2017; Vilardi et al., 2018); they are efficient for removing organic and inorganic pollutants, heavy metals from wastewater and killing microorganisms through environmentally friendly (Stoller et al., 2016; de Mendonca et al., 2014), in addition, nanoparticles are expected to offer high performance rates with reasonable and cost-effective wastewater treatment solutions compared to large infrastructure (Qu et al., 2013). In electronics field, nanoparticles are used to improve energy efficiency, thus they are incorporated in photovoltaic panels, moreover their facile manipulation and reversible assembly allow to incorporate them in electric, electronic devices

(O'Brien et al., 2001). Finally the nanoparticles are present in commercial products such as paints, varnishes and clothing thanks their antibacterial activity (Foldbjerg et al., 2015).

2.3 Physicochemical properties of nanoparticles and toxicity relationship

Distinctive properties such as superior catalytic, optical, magnetic, mechanical, electrical properties, and high adsorption are associated with nanoparticles (Ferrari, 2005; Pattan and Kaul 2014). Material at nanoscale, often no display the same reactivity as the bulk compound. For example, even a traditionally inert bulk compound, such as gold, may elicit a biological response when it is introduced as a nanomaterial (Goodman et al., 2004). In particular, the fundamental components of nanoparticles are their surface and interface. Smaller particles occupy less volume, and the surface area increases, as consequence greater proportion of atoms/molecules are found at the surface compared to those inside (Cassee et al., 2002; Warheit, 2004; Yang and Watts, 2005). Thus, nanoparticles have a much larger surface area per unit mass compared with larger particles. The increase in the surface-to-volume ratio results in the increase of the particle surface energy which may improve their reactivity potential compared to conventional formulations of the same materia (Cunningham et al., 2002; Oberdörster et al., 2005a). For it, the nanoparticles are used in the treatment of wastewater (Chiavola et al., 2016; Vilardi et al., 2018), thanks their strong adsorption reactivity and capacity (Mauter et al., 2018). Decomposition of pollutant is achieved by oxidation because the nanoparticles are able to generate and use a hydroxyl free radical as a powerful oxidant to reduce the effects of pollutants (Forgacs et al., 2004). Moreover, surface coatings can be utilized to alter surface properties of nanoparticles to prevent aggregation or agglomeration with different particle-types, and/or serve to passivate the particle type to migrate the effects of ultraviolet radiation-induced reactive oxidants (Oberdörster et al., 2005a), while the conjugation with antibodies, ligands, and drugs functionalizes their surface to raise their potential applications in biotechnology, drug and gene delivery, magnetic separation and imaging (Yang et al., 2018; Liu et al., 2019; Wang et al., 2019). Unfortunately, their nano size facilitate the penetration to different live tissues and enables them possible interaction with the same sized organs like cells, proteins, and antibodies also the nanoparticles can accumulate in organs and tissues as a foreign body (Nemmar et al., 2002; Nel et al., 2006). As result, in presence of two NPs with the same mass, the smaller NPs have a larger specific surface area and thus provide a more available area to cellular interactions with nucleic acids, proteins, fatty acids, and carbohydrates. Thanks to their surface area, NPs can be absorbed by the surface of cell organelles (Huang et al., 2017). Interaction with DNA, proteins and mitochondria can lead to reactive oxide species (ROS) formation, which affect the physiological functions of cells. Thus, DNA damage, lysosomal hydrolases, ROS generation, mitochondrial dysfunction, apoptosis, cell membrane damage, cytoplasm impairment, alterations in ATP, and permeability of cell membrane, accumulation of NPs

in Golgi and variations in proteins are attributed to NPs interaction (Yu et al., 2020). Also, shape and size of the nanoparticles has been shown to have a distinct effect on the biological activity. The round-shaped NPs are more susceptible to endocytosis than NPs with fiber and tube geometry (Champion and Mitragotri, 2006). Also, plate-like and needle-like NPs induce physical damages to cells and live tissues by direct contact causing necrosis (Zhao et al., 2013). Based on size, NPs with smaller than 5 nm diameter generally can defeat cell entrance barriers and they are able to pass through cell membranes by translocation, while the larger NPs get into the cells by phagocytosis and other possible transportation mechanisms. *In vivo* experiments have established, that large NPs can be easily recognized by the immune system and prevents their entrance to the body (De Jong et al., 2008). The chemical composition, solubility, ionic charges and crystal structures also must be considered with full attention. Inorganic NPs with the same physical condition, but distinct chemical composition have shown different toxicological behaviors, because the toxicity depends on release of metallic ions into the cell. For example, metallic NPs with toxic nature are As, Pb, Cd, Hg and Ag since they can damage the cells (Roane et al., 2009). As well, metals like Fe and Zn useful from the biological aspect, become harmful at high concentration and cause toxicity reactions. The release of metal ions into the cytoplasmic environment directly dependent on the NPs' dissolution rate (Khan, 2020). Horie and Colleagues (2009) reported that NiO-NPs have more activity compared to NiO fine particles because, the NPs can release higher amounts of Ni^{2+} in the medium while fine particles do not have this capability. The NPs have higher solubility rate than the bulk materials, only for less than 100 nm size. Surface charge is a key determinant of nanoparticle's action on cellular processes (Beddoes et al., 2015). Schaeublin and Colleagues (2011), have shown that charged Au-NPs (1.5 nm) displayed toxicity as low as 10 μg $ml^{(-1)}$ whereas the neutral at 25 μg $ml^{(-1)}$. Furthermore, there was significant mitochondrial stress (decreases in MMP and intracellular Ca^{2+} levels) following exposure to the charged Au-NPs, but not the neutral Au-NPs (Schaeublin et al., 2011). It is known that the movement of particles in a fluid cause a net surface charge generation which can be defined by zeta potential. It is explained as the potential variation among the mobile dispersion medium and the stationary layer of the dispersion medium that is in attachment with the dispersed particle (Lu and Gao, 2010). It was proved that the physical interaction between cellular membrane and NPs is mainly governed by surface charge of NPs. Positively charged coated Ag NPs are more toxic than that of the negatively charged NPs (El Badawy et al., 2011), similar results were observed to ZnO NPs (Kim et al., 2014a). The positively charged particles are more toxic and the variance surface charge determines the cellular uptake. Instead, NPs with lower zeta potential values are prone to stick to each other to create aggregates or agglomerates. These agglomerates have various forms, from dendritic structure to chain or spherical structures. To maintain the characteristics of nanoparticles, they are often stabilized with

coatings or derivative surface to prevent agglomeration. The properties of nanoparticles can be significantly altered by surface modification and the distribution of nanoparticles in the body strongly depends upon the surface characteristics. In this regard, the body distribution and the effects of nanoparticles on the biological systems significantly change when, they are coating with different types and concentrations of surfactants to prevent their aggregation or agglomeration (Araujo et al., 1999; Kirchner et al., 2005).

Considering the adverse effects of nanoparticles, it has been necessary to look at the safety concerns of NPs then a new branch of toxicology was introduced. It is entitled "Nanotoxicology". It deals with the nanomaterials toxicity to define their effect on the environment and on humans (Pacheco et al., 2007; Bakand and Hayes, 2016). Nanotechnology and nanotoxicology are thus considered to be two sides of the same coin, as the same nanosize which offers plenty of beneficial effects may also pose unwanted adverse effects (Tirumala et al., 2021).

3. Metal oxide NPs (MONPs)

Among the several types of NPs, metal oxide NPs (MONPs) stand out as the category of versatile materials in fact they are used in several applications including cosmetics (Waghmode et al., 2019), detergents, agricultural systems, environment (Kanchi and Ahmed, 2018), antibacterial agents (Naskar and Kim, 2021), paints, textiles (Vigneshwaran et al., 2010), drug and medicine industry (Klębowski et al., 2018). In this regard, they are frequently utilized and improved in order to intensify their functions for biomedical applications, as anticancer, antidiabetic, antimicrobial purposes, drug delivery, imaging and also they have application in reproductive medicine (Sengupta et al., 2014). Although, they have great potential as adsorbents to environmental pollutants. For example, they have a high removal capacity and heavy metals selectivity. Metals oxide NPs (MONPs) are made of purely metal precursors, which often are present in human tissue and they are define metals essential because improve the organism functionality (Das et al., 2016; Zoroddu et al., 2019). For this reason, MONPs are typically classified as biocompatible since most of their inorganic materials will be more easily accepted by the organism. However, in high concentrations, these physiologically compatible metals have toxic effects on cells and can even cause cell death (Taylor et al., 2012). Due to their small size and large surface area, MONPs show enhanced colloidal stability and, therefore they are able to easily enter the pulmonary system and they can be adsorb through endothelial cells, the blood-brain barrier and also the blood–testis barrier (BTB) (Singla et al., 2016; Rizvi and Saleh, 2018). BTB, also known as the Sertoli cell epithelial barriers, is one of the tightest blood–tissue barriers in the mammalian body (Setchell, 2008; Mital et al., 2011; Pelletier, 2011; Franca et al., 2012; Mruk and Cheng, 2015; Stanton, 2016). It has multiple functions in the testis, as support spermatogenesis or protect the testis and then the spermatogenesis process, which occurs within them, from xenobiotic-induced toxicity

(Mruk et al., 2011; Cheng and Mruk, 2012). However, BTB is highly vulnerable to exogenous materials, such as NPs (Yoshikawa et al., 2018; Zhou et al., 2019). Accordingly, the reproductive toxicity of nanoparticles is a particularly important issue (Ema et al., 2010; Greco et al., 2015) since exposure of humans and other organisms to nanoparticles is increasing exponentially.

Metal oxide-based nanomaterials include manganese oxides, nanosized iron oxides, titanium oxides, cerium oxides, ZnOs, magnesium oxides, aluminum oxides, and zirconium oxides. In particular, titanium dioxide (TiO_2) is one of the most widely produced engineered nanomaterials and as consumption grows the chance of population exposure to TiO_2 nanoparticle increases (Naseem and Durrani, 2021).

3.1 Titanium dioxide nanoparticles

Titanium dioxide with formula TiO_2, also known as titanium (IV) oxide, titanic acid anhydride, titania, titanic anhydride, or Ti white, is the naturally occurring oxide of Ti, because Ti does not exist in the metallic state in nature and it has great affinity for oxygen and other elements. Titanium (Ti) is the ninth most abundant element in the earth's crust and it is widely distributed with average concentration of 4400 mg/kg in the earth's crust. The most common oxidation state of Ti is +4, but +3 and +2 states also exist, thus its affinity for oxygen and chlorine produce TiO_2 and $TiCl_4$, that are the compounds most widely used in industry (Zhang et al., 2016a). TiO_2 appear as a white powder noncombustible and odorless with a molecular weight of 79.9 g/mol, boiling point of 2972°C, melting point of 1843°C, and relative density of 4.26 g/cm^3 at 25°C.

TiO_2 is used as either pigment or photocatalyst. As a pigment for its brightness and opacifying strength (hiding power), while as a photocatalyst thanks its ability to both absorb and scatter the UV light (thanks to its high refractive index). TiO_2 is resistant to chemical attack and displays excellent thermal stability. For this reason, it is an irreplaceable ingredient in the production of paints, surface coatings, plastics, and paper (Akakuru et al., 2020). TiO_2 exists in three naturally crystal structures: anatase (3.2 eV) with octahedral crystals, rutile (3.0 eV) with prism shape and brookite (3.2 eV) with orthorhombic crystals (Siroha et al., 2018). Rutile is the most thermally stable polymorph, as both brookite and anatase are transformed into rutile when exposed to a temperature above 800 °C (Musial et al., 2020). All crystal forms of TiO_2 offer photoactive properties, but anatase being more chemically reactive (Sayes et al., 2006; Warheit et al., 2007). It was found that anatase has in TiO_2 electron structures higher band gaps than rutile and brookite (Zhang et al., 2017; Čaplovičová et al., 2012), consequently anatase is the most photoactive form. Traditionally, TiO_2 FPs (fine particles) have been considered as poorly soluble and low toxicity particles (Castleman and Ziem, 1994; Olin, 2000). However, studies have shown that their toxicity increased at high concentrations; rats developed lung tumors after two years of exposure to high concentrations of fine TiO_2 particles (Lee et al., 1985;

Kreyling et al., 2019). Therefore, TiO_2 was classified as a Group 2B carcinogen (possibly carcinogenic to human) by Agency for Research on Cancer (IARC, 2006). In recent years, thanks to the nanotechnology TiO_2 nanoparticles (TiO_2-NPs) have been produced to replace the TiO_2-FPs. As mentioned, the nanometric size level give to NPs versatile size-dependent and special properties such as catalytic, electrochemical, optical, magnetic features as well as increased surface to volume ratios which in turn make them the unique materials for modern applications. Manufacturing of the TiO_2-NPs, has expanded the range of TiO_2 utility. Titanium dioxide nanoparticles (TiO_2-NPs), are among the engineered metal oxide nanoparticles more manufactured in the world (Piccinno et al., 2012; Sharma et al., 2019). Annually about 4 million tons of TiO_2 are produced globally, and about 3000 tons of that process in the nano-scale form (Xu et al., 2016). Degussa P25 are widely used commercial TiO_2 nanoparticles (NPs) with an anatase to rutile phase ratio of 4:1. TiO_2 NPs have unique characteristics of a very high refractive index, whiteness, and opacity, efficiency, increased chemical stability, and minimum cost (Rajh et al., 2014), thus they are used in different sectors (Hou et al., 2019). Specifically, TiO_2-NPs are used in a wide variety of products such as food colorants (under E code number E171), nutritional supplements, personal care products (cosmetics, sunscreens), toothpaste (Weir et al., 2012; Wu and Hicks, 2020), paint (Larue et al., 2014). Moreover its photocatalysis property is considered today an efficient methodology in the area of wastewater treatment (Scuderi et al., 2014; Zimbone et al., 2018) for this reason TiO_2-NPs are used to remove pollutants from wasterwater (Tan et al., 2018). In agriculture sector, TiO_2-NPs are used for different purposes such as nano-pesticides and nano-fertilizers to introduce sustainable agricultural practices (Sastry et al., 2010). Finally, in medicine TiO_2 was tested as a new effective drug carrier (for example, as TiO_2 nanotubes) (Wang et al., 2016a) or in skin tissue engineering and wound dressing (Sastry et al., 2010; Gogos et al., 2012; Hou et al., 2019).

WASTEWATER TREATMENTS

The increasing world population and urban industrialization have caused an increase of type and amount of pollutants released in the environment, in particular water resources are become the many target of different pollutants, as pesticides, dyes, heavy metal ions, organic compounds (Ali and Aboul-Enein, 2004; Chiesa et al., 2016).

Titanium dioxide nanoparticles (TiO_2-NPs) allow to remove these pollutants thanks its photocatalytic activity, which is recently emerged as a totally-green technology for environmental applications (Guillard et al., 2003; Di Mauro et al., 2016). All kinds of contaminants, such as polycyclic aromatic hydrocarbons (Guo et al., 2015), chlorinated organic compounds (Ohsaka et al., 2008), dyes (Lee et al., 2008), pesticides (Kim et al., 2016), phenols (Nguyen et al., 2016), cyanide (Kim et al., 2016), arsenic (Moon et al., 2014) and heavy metals (Chen et al., 2016) can be degrade by TiO_2

nanoparticles, because they are little selective; moreover the photocatalytic properties of TiO_2 nanoparticles can kill a wide array of microorganisms, such as gram-positive and gram-negative bacteria as well as viruses, algae, fungi, and protozoa (Foster et al., 2011). Nowday, TiO_2 is the most exceptional photocatalyst, which main advantages are its endless lifetime and the capacity to remain unchanged during the degradation process of microorganisms and organic compounds (Liou and Chang, 2012). Photocatalysis is based on the interaction between semiconductor materials and "free" light from the sun, and not produce any harmful by-products (Chun et al., 2009). The photocatalytic disinfection efficiency is attributed to the oxidative damage mainly induced by reactive oxygen species (ROS), like $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ (Haider et al., 2017). These reactive oxygen species are produced on the surface of TiO_2 when illuminated by photons with energy greater than its band gap, so electron will excited from valance band to the conduction band, thus creating an electron-hole pair. With holes (h+) and hydroxyl radicals ($OH^{\bullet}$)generated in the valence band, and electrons and superoxide anions ($O_2^{\bullet-}$) generated in the conduction band, irradiated TiO_2 photocatalysts can decompose and mineralize organic compounds by a series of oxidation reactions leading to carbon dioxide and water molecules (Verdier et al., 2014) (Figure 2).

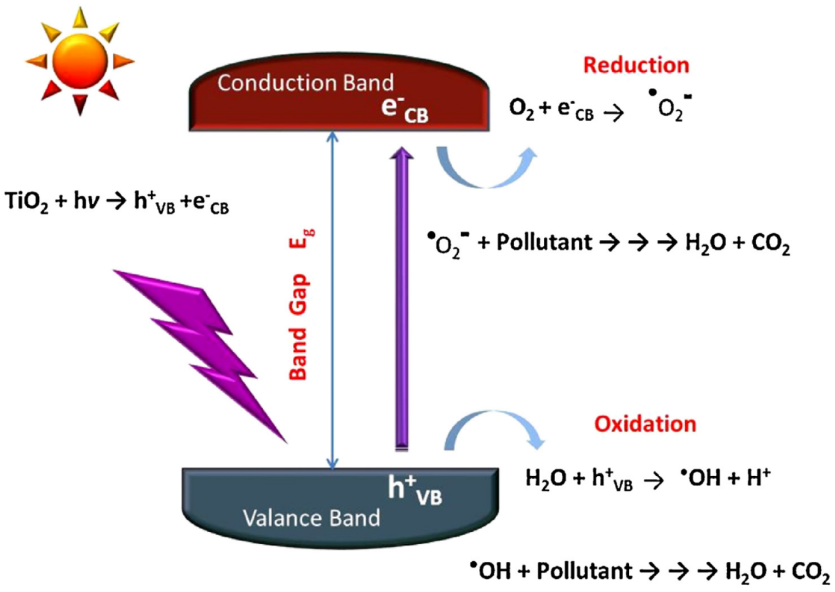

Fig.2: Illustration of photocatalytic disinfection by TiO_2 (Banerjee *et al.*, 2015)

Some have attributed this increased catalytic activity to TiO_2-NPs to their high surface area, while others attribute it to TiO_2-NPs being predominantly anatase rather than rutile (Sayes et al., 2006; Warheit et al., 2007). It has been suggested that TiO_2 anatase has a greater toxic potential than TiO_2 rutile (Xue at al., 2010; Petkovic et al., 2011) because as the anatase crystal structure increases, production of reactive oxygen species increases, too (Çeşmeli and Biray Avci, 2019). The rutile

titanium dioxide is considered as chemically inert, but when the particles become smaller, the surface area will increase and therefore the rutile titanium dioxide particles can become harmful according to the studies. Also, the modifications that are done on the surface of nanoparticles cause changes in the activity of titanium dioxide particles (Shi et al., 2013). Study *in vitr*o and *in vivo* have shown that the shape, higher dose, crystalline structure, and phases have the potential to cause toxicity.

ADDITIVE

TiO_2 as food additive, is referred to as E171 in Europe and INS171 in North America. It is adopted as an additive for development of plastic packaging (EFSA, 2019) and it is widely added to foods including cheeses and sauces, skimmed milk, ice-creams and pastries, as well as in sugar confectionary where it constitutes the coating of sweets and chewing-gum to achieve a white appearance or to make colourants used in food products look brighter and more appealing (Lomer et al., 2000; Weir et al. 2012; Chen et al., 2013; Peters et al., 2014; Periasamy et al., 2015; Dudefoi et al., 2017a). Titanium dioxide as food additive must be labelled according to the provision of Regulation (EU) No. 1169/2011 (European Commission, 2011). If present also in the form of engineered nanomaterials, according to article 18(3) of this Regulation, this shall be clearly indicated in the list of ingredients. In this case, the name of the ingredient shall be followed by the word 'nano' in brackets. The use of TiO_2-NPs is generally not permitted due to their different behavior and reactivity versus the bulk form, moreover the NP being potentially carcinogenic to humans (Group 2B, IARC, 2010) if ingested (Bettini et al., 2017) or inhaled (Tassinari et al., 2014). Titanium dioxide used as food additive E171 in food products undergoes no surface treatment and is not coated (EFSA, 2021). Although the use of particles with a size of below 100 nm (nano) would seem to be of no use to enhance the appearance of food, studies showed that a fraction of particles in the nano-range can be present (Faust et al., 2014; Yang et al., 2014; Athinarayanan et al., 2015; Dudefoi et al., 2017a). Recently scientific evidence on May 2021, about safety assessment of E 171 have shown that it could has genotoxicity effect, because it has the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations (EFSA, 2021). TiO_2-NPs, are also used in cosmetics as a white pigment, an opacifier and a physical UV filter. It has been used in makeup, sunbathing, hair, skin and oral care cosmetics. However, various scientific and regulatory bodies have considered titanium dioxide as a potential human carcinogen after inhalation exposure. Following the recommendation of the IARC of 2019, the European Commission adopted the classification of titanium dioxide as "Carcinogen category 2" (suspected of causing cancer, by inhalation; in powder form containing 1% or more of particles with aerodynamic diameter $\leq$ 10 μm). It should be remembered that this classification is based only on the inhalation route of exposure, and that no carcinogenic effects were found for oral and dermal exposure. In addition, carcinogenicity has been shown in animals (rats) but

has not been confirmed by epidemiological studies in humans (SCCS, 2020). In pharmaceutical industry, TiO_2 performs a number of important functions because it can use as pigment, to add whiteness or accentuate the boldness of other colours, then TiO_2 helps tablets stand out for both medical professionals and patients; as coatings TiO_2 is an essential component to preserve the safety, efficacy and quality of the active pharmaceutical ingredient, and to provide shelf-life stability. TiO_2 offers protection for photosensitive ingredients, which could be damaged by visible light and also ingredients that may be vulnerable to Ultraviolet (UV) light degradation (Carretero and Pozo, 2009). Finally in the packaging, TiO_2 is utilize to its ability to scatter light and absorb UV rays, for this reason it is routinely incorporated in the packaging of medicines to maintain shelf life and prevent any premature degradation from moisture, heat or light.

Even if the product is labelled as containing E171, no information is usually given about the quantity, particle size and particle structure. There is no regulation for the maximum concentration of TiO_2-NPs additive in drug, and information on their toxicity and distribution upon oral exposure is very limited. Could be an increase exposure to TiO_2 during a high consume of drug. Then, this could lead at more bioaccumulation of TiO_2 into the body and at negative consequence for the health.

3.2 Impact of titanium dioxide nanoparticles

The technology based industry realized that nanoparticles brought with their potential opportunities such as increase energy efficiency and clean up industrial contaminants (Wilson, 2018). However, the increasing production and use of manufactured nanoparticles (NPs) have inevitably led to their release into the aquatic environment, thereby posing a threat to aquatic organisms and also for human (Klaine et al., 2008; Handy et al., 2008). Human exposure to TiO2-NPs may occur during both manufacturing and use. As mentioned, TiO2-NPs are common in consumer products for dermal application, toothpaste, food colorants and nutritional supplements then oral exposure may occur during use of such products. TiO2-NPs can enter the body through skin contact, breathing, diet and other ways to induce inflammation (Gangwal et al., 2011; Weir et al., 2012; Zhao et al., 2020). It occurs because, the nanoscale size of TiO2 increase the surface-to-volume ratio making them more reactive in a cell, then TiO2 NPs increase their ability to produce reactive oxygen species (ROS) (Hong et al., 2015a). In addition, is generally known that an increase in surface area accelerates the dissolution processes as a result higher dissolution rates and smaller size of particles enhance their absorption through membranes, which leads to their deposition within tissues and organs. Nanoparticles can pass through biological membranes (Brooking et al., 2001; Wang et al., 2008) and affect the physiology of any cell in the body. Many in vivo and in vitro studies have revealed that TiO_2-NPs could cause inflammatory reaction (Hong et al., 2016a), oxidative DNA damage (Meena et al., 2015) and serious damage to the liver, kidneys, lungs, myocardium (Li et al., 2017; Liu et al., 2019), intestine, causing preneoplastic lesions (Bettini et al., 2017; Dudefoi et al., 2017b); moreover TiO_2-NPs can pass the blood-brain barrier, inducing brain injury (Song et al., 2015) and testicular blood barrier inducing effect on testis and then on male reproductive health. Several studies in rodent and mice, have shown the toxicity effect of TiO_2 on male reproductive system. In mice, oral exposed to TiO_2-NP (300 mg/kg for 35 days), result in oxidative stress increased that cause histological changes in testicular tissue, low sperm production and abnormal sperm morphology, and testicular and serume testosterone decreased (Khorsandi et al., 2017). Intragastrical administration of TiO_2-NPs (0, 10, 50, and 100 mg/kg^{-1} body) in mice have caused a reduced of the germ cell number, spherospermia, interstitial glands vacuole, malalignment, and vacuolization of spermatogenic cells in mice testes, moreover accumulation of reactive oxygen species have been observed (Song et al., 2017). Structural and functional sperm defects and DNA damage via oxidative stress were observed in mouse with administered intraperitoneally (Smith et al., 2015), while the intragastric administration of 2.5, 5, and 10mg/kg of TiO_2-NPs for 90 day results in testicular lesions, sperm malformations, and alterations in serum sex hormone levels (Gao et al., 2013). A target of TiO_2-NPs are also Sertoli cells, an decreasing viability with fragmentation of chromatin, mitochondrial and

endoplasmic reticulum swelling was observed *in vitro* experiment (Hong et al., 2016b), while apoptosi of Sertoli cells and consequently necrosis of seminiferous tubules with accumulations of TiO_2 nanoparticles were highlighted *in vivo* studies (Gao et al., 2013). TiO_2-NPs can effect on the puberty, the oral administered of TiO_2-NPs daily to male mice from 28th postnatal day (PND 28) to PND 70 can influence the levels of serum testosterone (T) through changes in both the synthesis and translation of T. Furthermore, the decreased serum T synthesis might contribute to the reduced spermatogenesis in mice exposed to TiO_2-NPs (Jia et al., 2014). TiO_2-NPs can change stereological and morphometrical parameters of the seminiferous tubules and reduce the number of Leydig cells and testosterone concentration in a treatments daily for 35 days: 75, 150 and 300 mg/kg TiO_2-NPs respectively (Khorsandi et al., 2016). On the other hand, lower concentrations of TiO_2 (0.1, 1, 2 and 10 mg/kg) do not lead to an accumulation of titanium dioxide at the testicular level, but a reduction in progressive motility and in the number of spermatozoa has been observed. The sex hormones taken into consideration (LH, FSH, GnRH, T), no variation was observed (Miura et al., 2017). Also spermatogenesis disturbances with thinning, disorganization of layers, and detachment of sperm cells from the basement membrane are induced by TiO_2 (Sharafutdinova et al., 2018).

These studies have associated cyto- and geno-toxicity of TiO_2-NPs with their photocatalytic activity (Smijs and Pavel, 2011; Sharma et al., 2019). The oxidative stress plays crucial role, because hydroxyl radical, hydrogen peroxide, and superoxide anion radical, constitute a group of reactive oxygen species, which may impair the cell function (Brezová et al., 2005; Smijs and Pavel, 2011; Sharma et al., 2019). It have been reported that TiO_2NPs reduce egg production in zebrafish (Wang et al., 2011a) and affect the development, cause fetal malformations and even death in mice (Philbrook et al., 2011). Previous studies showed that some toxic chemicals, heavy metals, pesticides, and radiation can lead to infertility (Jungwirth et al., 2012) likewise environmental residues of TiO_2-NPs have potential reproductive toxicity. For this reason, nanoparticle's applications have raised concerns about their biological effects. TiO_2-NPs are xenobiotic substances whose biological consequences must be predicted following their exposure.

4. Xenobiotics: what means?

Chemical substances foreign to animal life that includes for examples plant constituents, drugs, pesticides, cosmetics, flavorings, fragrances, food additives, industrial chemicals and environmental pollutants are define with the term Xenobiotic. Humans are exposed about to 1-3 million xenobiotics in their lifetimes (Idle and Gonzalez, 2007), moreover xenobiotic compound may persevere from months to years in the environment then it make them potential hazards both on ecosystem and human beings (Embrandiri et al., 2016; Dinka, 2018). The toxicity of any xenobiotic is related to the bioaccumulated chemical residue in the organism (Maenpaa, 2007), often the bioavailabilities of these substances are dependent upon the characteristics of the organism, the chemical, and the environment. Aquatic ecosystem are at greatest risk, from pollutants because all chemicals, whether initially released on land or directly into rivers will eventually find themselves in the rivers and oceans as the final repository. In addition, in aquatic environments biological and physicochemical factors are highly variable to affect the bioavailability of contaminants (Maenpaa, 2007). For example hydrophobic pollutants which are eventually stored in sediments become hazardous on exposure to benthic organisms. Any exposure to the sediments contaminated by xenobiotics possibly affects the lower trophic levels. It may also result in biomagnification or more serious toxic effects at higher trophic levels (Landrum and Robbins 1990; Streit, 1992; Lee, 2018). Subsequently, these chemicals substance pose risks to human and animal health. Many xenobiotics are biologically active and can lead to a serious adverse health effects (Dinka, 2018).

5. Endocrin disruptors

Several of these chemicals can have harmful effects on the body's endocrine (hormone) system, according EFSA (2010) any substance that has the ability to interact with one or more elements of an endocrine system i.e. exhibited endocrine activity, falls into the category of "endocrine active substances" also called "endocrine disrupting chemicals" (EDCs). They comprise compounds from different classes and are widely distributed in aquatic environments. EDCs exert agonistic and antagonistic effects at and after hormone receptor binding and interfere with steroid synthesis and elimination (Linderoth et al., 2006), resulting in alterations in development, reproduction, physiological homeostasis, and health of vertebrates (Nagahama and Yamashita, 2008; Segner, 2009). The endocrine system is a complex integrative network of glands, hormones and receptors. It involves the brain and associated organs and tissues of the body. These include the pituitary, thyroid, and adrenal glands and the male and female reproductive systems, all of which release hormones into the bloodstream (Keith, 1998). Through the hormones, endocrine system regulates such critical biological functions as metabolism, development, reproduction, and behavior for this reason it plays a central role in all vertebrates. In order to fulfil these functions, the endocrine system uses cycles and negative feedback loops, regulating the secretion of almost all hormones. The cycles of secretion of chemical messengers, whose duration can range from hours to months, maintain physiological and homeostatic control (Kortenkamp et al., 2011). The body's normal endocrine functioning involves very small changes in hormone levels, however exposure to exogenous substances that exhibit endocrine activity (i.e. EASs) may stimulate modulation in these feedback systems and small changes of level hormones can cause significant developmental effects or in general biological effects. If this modulation and its effects are continuous, for example these substances exhibit agonist or antagonist activity (or both), then they bind to the body's endocrine receptors to activate, block, or alter natural hormone synthesis and degradation (Schug et al., 2016), this effect is not considered endocrine modulation and hence adverse. Whereas if the effects are temporary and within the homeostatic capacity of the endocrine system of the exposed organism, the effect of the substance might be considered endocrine modulation, then non-adverse.

Therefore, it has been necessary to regulate chemicals on grounds of their toxicological properties on the endocrine system, because even low amounts of chemicals can alter the body's sensitive systems and lead to health problems. Thus, these chemicals are called Endocrine Disruptors (EDCs) (Kumar et al., 2020). The first definition of endocrine disruptor (EDCs) given by the U.S. Environmental Protection Agency (EPA) (Kavlock et al., 1996) during a workshop, defined it as: "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction,

development and/or behavior". However, this definition is ambiguous in not differentiating adequately between compensatory/homeostatic changes and those that lead to adverse health effects. The Weybridge definition (EU, 1998) of an endocrine disruptor makes explicit reference to adversity, then difine it as: "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function". Subsequently a new definition by WHO/IPCS (WHO/IPCS, 2002), have defined endocrine disruptor as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". In this definition, the word "conseguently" suggest the cause-effect linkage between endocrine activity and an adverse health effect; moreover the added of "(sub)populations" may make the definition more directly applicable to ecotoxicology. A survey presented in the SAAED Final report showed that most EU Member States acknowledge the WHO/IPCS definition, and this definition is extensively discussed in SAAED (Kortenkamp et al., 2011). Recently, looking at the potential of endocrine disruptor to affect the balance of normal hormonal functions and the endocrine system in animals (Keith, 1998) a simplest definition has been proposed by Endocrine Society (Zoeller et al., 2012): endocrine disruptor is "an exogenous (non-natural) chemical, or a mixture of chemicals, that interferes with any aspect of hormone action". Zoeller and Colleagues (2012) underlined that the ability of a chemical to interfere with hormone action, is a reliable predictor for adverse outcomes. In their view, uncertainty in the relation between the endocrine activity and the manifestation of an adverse consequence relate to the dose, duration and timing of exposure (Zoeller et al., 2012). For this reason, some actions may be termed endocrine disrupting, while others may be simply localized toxic effects on the reproductive system as the most sensitive component of the animals physiology (Kime, 1999).

5.1 Classification of Endocrine Disruptors

Endocrine Disruptors (EDCs), are highly heterogeneous (Blumberg, 2009; Grun, 2010) and can be classified according different ways. The first list of suspected EDCs was published in scientific literature in 1993 by Colborn (Colborn et al., 1993), followed by popular book for the layperson "Our stolen future"(Colborn et al., 1996). This book was instrumental in public awareness of the need to find out more, because it proposed that chemical pollution is threatening the intelligence, fertility, and survival of the human race. Generally, there are two main categories of Endocrine Disruptors (EDc) (Diamanti-Kandarakis et., 2009):

-**natural chemicals** food in human and animal food (e.g. phytoestrogen, including genistein and coumestrol);

-**synthetic chemicals** used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin), and pharmaceutical agents [diethylstilbestrol (DES)].

A classification of EDc, based on different observation, categorized EDc into three groups, that are commonly used by population: **(1)** ***pesticides***: are used to kill unwanted organisms in crops, public areas, homes and gardens, and parasites in medicine. However, many first generation pesticides have been found to be harmful to the environment. Some of them can persist in soils and aquatic sediments, bioconcentrate in the tissues of invertebrates and vertebrates, move up trophic chains, and affect top predators. Human are exposed to pesticides due to their occupations or through dietary and environmental exposure (water, soil, air) (Kolpin, et al., 2000; Mnif et al., 2011). About 105 substances of pesticides can be listed. Of these, 46% are insecticides, 21% herbicides and 31% fungicides; some of them were withdrawn from general use many years ago but are still found in the environment (ex. DDT and atrazine in several countries). Pesticides are active at low concentrations in food daily consuming by adult population and in agricultural commodities consumed in large quantities especially by infants and children (Matisova and Hrouzková, 2012). It is known that organochlorine pesticides (OC) affect the reproductive function (Mnif et al., 2011), moreover much of the damage caused by pesticides appears to be during gametogenesis and the early development of the fetus (Sultan, 2001; Skakkebaek, 2002; Hardell et al., 2006; Sharpe, 2006). **(2) Chemicals in products:** several evidence have showed the presence of numerous EDC families (mainly phthalates, bisphenols, parabens, and benzophenones) in cosmetic products and personal care products (PCPs). For example, the phthalates are used in >90% of the 77 feminine products like pads, panty liners, tampons, and wipes (Gao and Kannan, 2020). Moreover, they are also found in hair care products, perfumes, skin tones, deodorants, and creams; but also, products for dental hygiene and rinse-off

products (including body wash, shampoos, hair conditioners, face cleaners, and shaving gels) (Gao and Kannan, 2020). Therefore, we can come in contact with them through the use of these product used daily. In most of the cases, we are not aware of these facts since EDS are not always included in their chemical compound list. Also, heavy metals such as lead, cadmium and mercury, may impact endocrine function in addition to their other modes of toxicity (Meeker, 2012). Lead is used in lead-acid batteries, paints, jewellery, children's products and in many other products. The children might pick up these products and put them into their mouth, for this reason they are the potential candidates of lead poisoning because the amount they ingest per unit body weight is obviously higher. The Children, do not have a fully developed blood-brain barrier, then neurological effects are always higher in children than to adults (Gore et al., 2014). **(3) Food contact materials:** are the many containers used to store foods or used to lining canned foods. Bisphenol-A (BPA) is a widely studied EDC. It is a plasticizer used in the manufacturing of polycarbonate plastics, epoxy resins and thermal paper (Klecka et al., 2009). Polycarbonate is a clear, rigid plastic that has been used for water bottles, and other items, while epoxy resins are found in the lining of many canned foods (Meeker, 2012). Much of the exposure to BPA comes from drinking water, while it has been banned in infant formula bottles in all of Europe due its toxicity. However, many plastic food and drinking containers still contain BPA then under various conditions it can leach out of containers. Foods or beverages are a source for human exposure (Rogers et al., 2013). However, because over 85,000 manufactured chemicals may be EDc, a short list of representative EDCs and their used in our daily life is provide in Table 1 (Dodson et al., 2012; Gore et al., 2014).

Common EDCs used in our daily life	Uses
DDT, chlorpyrifos, atrazine, 2,4-dichlorophenoxyacetic acid, glyphosate	Pesticides
Lead, phthalates, cadmium	Children's products
BPA, phthalates, phenol	Food contact materials
Brominate flame retardants, PCBs	Electronics and building materials
Phthalates	Personal care products, medical tubing
Triclosan	Antibacterials
Perfluorochemicals	Textiles, clothing
Parabens, phthalates, glycol ethers, fragrances, cyclosiloxanes	Cosmetics, personal care products, cleaners
Tributylin	Antifoulants used to paint the bottom of the ship
Nonylphenol (alkylphenols)	Surfactants-certain kinds of detergents used for removing oil and their metabolites
Ethinyl estradiol (Synthetic steroid)	Contraceptive

Tab.1: Common EDCs used daily

It is quite clear that the processes by which EDCs enter the body can be diverse (through diet, air, skin, and water) because they are ubiquitous. At the same time EDCs are distributed in the environment due to their widespread use, then we are not exposed only to a single chemical but to multiple chemicals at the same time (Björvang and Damdimopoulou, 2020). This mixture exposure can lead to combinatory effects of chemicals called cocktail effects. As chemicals are usually assessed individually, the hazards and risks could be underestimated because possible additive ($1 + 1 = 2$), synergistic ($1 + 1 > 2$), or antagonistic ($1 + 1 < 2$) properties are not accounted for (Taylor et al., 2016). EDCs can be divided into "persistent EDCs" and "non-persistent EDCs", according their degree of liposolubility, namely their resistance to physical, chemical, and biological degradation. Persistent EDCs are persistent, bioaccumulative and toxic chemicals. Low biodegradability, volatility, bioaccumulation in the trophic chain, and biomagnification are its most outstanding characteristics (WHO UNEP, 2001; Arrebola et al., 2014). Due their liposolubility, they accumulate to fatty tissues and for humans, the largest source of exposure is diet (Björvang and Damdimopoulou, 2020). They biomagnify through the food chain and have been found globally, even in regions where they have never been used (Lallas, 2001). Furthermore, they can be transmitted to the offspring through the mother during pregnancy and lactation (Botella et al., 2004). Many of this Persistent EDCs present in the environment originate in municipal wastewater, because they are not eliminated by conventional water treatment methods, and, through the urban water cycle they can enter ground and surface waters (Encarnação et al., 2019). On the other hand, non-persistent EDCs are less liposoluble, and therefore, they are prone to be metabolized and excreted rapidly (Frederiksen et al., 2007; Søeborg et al., 2014). They have relatively short half-lives in the human body and do not accumulate significantly, but have also been reported impact on human reproduction and development (Diamanti-Kandarakis et al., 2009).

5.2 Mechanism of action

Endocrine disruptors (EDCs), have multiple method of action to exert their effects on endocrine system (Mendes, 2002). They are able to mimic or block hormones, consequently endocrine's normal functions are disrupted because many disrupters can alter normal hormone levels, inhibit or stimulate the production of hormones, or change the way hormones travel through the body. Therefore, it occurs a weaker or stronger than normal response at inappropriate times compared to natural body's hormones (LaFleur and Schug, 2011). EDCs can interfere by blocking/activation the nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors (Diamanti-Kandarakis et al., 2009; Cookman and Belcher, 2014). In these case, EDCs are capable of mimicking natural hormones then they interact directly with hormone receptors, thereby preventing the action of normal hormones. EDCs are

antagonists of natural hormones (Kelce et al., 1995). However, some studies have shown that in addition, EDCs are able of acting through non steroid receptors, transcriptional coactivators, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine but also reproductive systems (Diamanti-Kandarakis et al., 2009; Diamanti-Kandarakis et al., 2010). It known that the well-working endocrine system functioning is the proper hormone-receptor binding at the appropriate level and time. Once a receptor and a hormone bind, the receptor carries out the hormone's instructions by either leading to alteration of the cell's existing proteins or alteration of gene expression. Both of these actions can create reactions throughout the body. Endocrine system diseases and disorders occur when one or more of the system's components are not working well. As it was discussed by Mnif and Colleagues (2011), changes in hormone levels affect developing organisms more than adults and can result in abnormalities in reproduction, growth, development and can disorder the immune system. EDCs are structurally similar to many hormones and function at extremely low concentrations. They may also exert non-traditional dose-responses due to the complicated dynamics of hormone receptor occupancy and saturation. Thus low doses may have more impact on a target tissue than higher doses, and the effects may be entirely different (Schug et al., 2011; Colborn, 2012). Consequently, it not known a safe dose of EDCs because beyond their act at low concentrations they act in combination with endogenous hormones. Moreover, individuals are exposed to more type of EDc at the same moment, then it is difficult to predict their effect due the possible synergistic, additive, or antagonistic actions between chemical residues (the cocktail effect). In general terms, is difficult to establish a threshold level of no effect. Moreover, the relation of exposure dose to EDCs with adverse effects is not linear (Kortenkamp et al., 2007). The response does not always increase in the same proportion as the exposure dose (Soto et al., 1992) and in certain individual, is great difficulty to determinate the association between exposure and negative result because the exposure to EDCs can have consequences in subsequent generations (Anway and Skinner, 2006; Skinner and Guerrero-Bosagna, 2009). An increasing number of studies have linked exposure to EDCs with epigenetic changes in humans or in general to their genomic involvement (Perera and Herbstman, 2011; Collotta et al., 2013). An unexposed individual may show epigenetic changes due to (1) altered ovum or sperm after EDC exposure or (2) *in utero* exposure to EDCs. The modifications involve changes in DNA methylation and histone acetylation. For this reason, the effects of exposure can occur after long latency periods.

However, it is know that exposure to EDCs cause different consequences depending on age and gender. For example, adverse effects are observed during periods of special vulnerability of the individual's development like pregnancy, lactation and puberty. EDCs can also be transferred from

mother to child through trans-placental route as well as through breast milk. Adults require higher concentrations for EDCs to cause toxicity and their effects only last as long as the EDC is present. Low dose exposure during development can result in that lasts long after the EDC is gone from the body (Schug et al., 2011). The term "the fetal basis of adult disease", or FeBAD used by field of endocrine disruption, describe the interactions between the developing organism and the environment that determine the propensity of that individual to develop disease in their adult life. This concept has been extended beyond the fetal period to include the early postnatal developmental period when organs continue to undergo substantial development (Diamanti-Kandarakis et al., 2009). More evidences have shown that such exposures may increase the susceptibility of the child to several non-communicable diseases in their adult life. In general terms, EDCs can potentially target various hormone systems, but primarily they involve the reproductive system, puberty, embryonic development and sex differentiation in fetal life (Kumar et al., 2020).

The implications on male and female reproductive system are several. However, for both us the chronic conditions such as metabolic syndrome and its components (obesity, insulin resistance, hypertension, or dyslipidemia), neurobehavioral development disorders, and poor thyroid function are also on the list of possible effects of EDC. In case of women, effects are evident on female reproductive hormones and their receptors through estrogenic, anti-estrogenic, androgenic and anti-androgenic mechanisms. EDCs affect the menstrual cycle, alter fertility and oogenesis, in addition they are implicated in diseases such as polycystic ovary syndrome (PCOS) and endometriosis (Kumar et al., 2020). Other evidences, have shown that *in utero* exposure to some EDCs has been linked to increased risk for breast cancer or endometriosis (Benagiano and Brosens, 2014; Cohn et al., 2015).

Whereas for men, EDC exposure is suspected to cause alterations in the development of the genitourinary system including cryptorchidism, testicular cancer, and infertility (Fernández et al., 2004; Olea and Fernandez, 2007). Human studies indicate that these chemicals affect pubertal growth, semen quality and also reproductive hormone production (Petrelli and Mantovani, 2002). Such trends seem to confirm the hypothesis of a "testicular dysgenesis syndrome" associated with the prenatal exposure to endocrine stressors (Sharpe, 2003), because EDCs act on male reproductive system by disrupting fetal endocrine balance due to their action on steroid hormone receptors (SHRs) or interference with the synthesis, kinetics or metabolism natural hormones (World Health Organization and United Nations Environment Program, 2013).

Moreover reactive oxygen species (ROS), generated during metabolism of these chemicals, cause damage on sperm functions. It has been shown that ROS produce damage to the plasma membrane and also on sperm DNA. This is possible effect that leading to infertility (Bansal and Bilaspuri, 2011; Sidorkiewicz et al., 2017).

6. Effect of Endocrine Disruptors (EDCs)

Various environmental analyses have reported that the EDCs are persistent in the environment, therefore the interest and concern related to EDCs among researchers has increased. It was expressed a great concern related to increasing levels of EDCs found in the environment, because they have shown adverse effects on the wildlife and humans when present also at levels under (Jobling and Charles, 2003). It is necessary for the researchers focus more on toxic effect of EDCs and its prevention techniques.

Concerns regarding exposure to these EDCs are primarily due to (1) adverse effects observed in certain wildlife, fish, and ecosystems; (2) the increased incidence of certain endocrine-related human diseases; (3) endocrine disruption resulting from exposure to certain environmental chemicals observed in laboratory experimental animals (4) moreover they generate possible adverse effects on reproductive organ morphological and functional development and, ultimately, on fertility (Damstra, 2002; Dinsdale and Ward, 2010). Humans through scientific and technological developments, have used excessively the resources creating a disturbance to the natural ecosystem (Sikandar et al., 2013). Ecosystems have been contaminated by the huge amount of EDCs released from industrial processes, that caused a widespread contamination. Consequently, both human and wildlife are exposed to these compounds (Letcher et al., 2010; Embrandiri et al., 2016). As mentioned, the different EDCs present in the environment include especially anthropogenic contaminants as pesticides, industrial chemicals, including personal care goods, and industrial chemicals by-products, drugs. Due to this variety, is not possible to define a "typical" EDCs, accordingly each of it or mixture must be carefully evaluated. The mixture of ED that leaches into the soil and waterbodies (e.g. pesticides, contraceptive pills and other chemicals from urban and agricultural waste) accumulates in the environment, wildlife populations and in animals higher up on the food chain (Diamanti-Kandarakis et al., 2009; Letcher et al., 2010). Global transport of EDCs occurs mainly through long range atmospheric and ocean water routes making their presence ubiquitous even in remote regions like the Arctic (Lohmann et al., 2007). Most of the EDCs in the atmosphere are present in gaseous phase, while a few sorb onto suspended and few sorb to particles due to their semi-volatile nature. They are transported by dry and wet gaseous vapor deposition, volatilization, sorption, dissolution, sedimentation, resuspension and erosion in the environment (Schneider et al., 2013). EDCs that accumulated in soil and sediment are potentially volatilized back to the atmosphere when levels in the air are reduced (Lohmann et al., 2007). EDCs in water partition into particles and dissolved phases that deposit to bottom sediments or taken up by aquatic biota. From the sediment, EDCs are transported back to the water column via diffusion or resuspension (Lohmann et al., 2007). During these environmental cycling, certain EDCs enter the food chain and bioaccumulate in tissues through inhalation and ingestion. For example, the

chronic exposure to EDCs by human is done through inhalation and skin contact (Stahlhut et al., 2009), however the major route of human exposure is ingestion of food (e.g. meat, fish, dairy products and vegetables), as well as plain water and other beverages. ED-contaminated food and water may contain environmental pollutants such as pesticide residues (Schilirò et al., 2011) and heavy metals (Iavicoli et al., 2009), in addiction to processing aids and anabolic steroids used in food production. Disruption of the endocrine system can lead to a variety of adverse health effects, for example very subtle effects on the endocrine system can result in changes in growth, development, reproduction, and behavior. Exposure during fixed time frames in development when programming of the endocrine system is occurring may result in permanent changes; whereas, exposure during "nonprogramming" time periods may not result in any significant or detectable effect (Damstra, 2002). Although there is no consensus on their regulation, EDCs are addressed in various cases in EU law, such as the Water Framework Directive, Registration, Evaluation and Authorization of Chemicals (REACH), Plant Protection Products Regulation (PPPR) and Cosmetic Regulation. In the last 20 years, a large number of research papers have been published, which address various aspects of endocrine disrupting chemicals (EDCs), including environmental occurrence, ecological effects and consequences of human exposure (Encarnação et al., 2019). There are mainly scientific evidences for chemical products that are recognized as potential endocrine disruptors. These include plasticisers as phthalates and bisphenol A, flame retardants, water disinfection byproducts, industrial chemicals including alkylphenols, perfluoroalkyl and polyfluoroalkyl substances (PFAS), metals and dioxins, air pollutants such as polycyclic aromatic hydrocarbons and pesticides. In addition an increase of data about *in vitro* and *in vitro* studies on NPs, support the notion that different types of nanoparticles are capable of altering the normal and physiological activity of the endocrine system (Iavicoli et al., 2013). However, more investigations must be considered to assess their mechanisms of action and identify areas in which further investigation is needed in order to obtain a deeper understanding of the role of nanoparticles as endocrine disruptors.

6.1 Effect in Wildlife

Exposure to certain EDCs have contributed to adverse effects in some wildlife populations including mammals, birds, reptiles, mollusks and fish (Ankley and Giesy, 1998). In particular, it was observed an interference in sex determination, sex reversal, mortality, neurodisorders, hormonal activation and cell proliferation (Annamalai and Namasivayam, 2015). Critical periods of development may include *in utero* or *in ovo* exposures, exposure at different stages of lifecycle, or exposure at different stages of the reproductive cycle. It is evident that in mammals, birds, and other classes of vertebrates, developmental stages appear to be among the most vulnerable periods in their life cycle (Berg et al., 1999; Eroschenko et al., 2002). As such, there are effects on developmental processes that have impact immediately on the viability of the young animal as well as lifelong impact on individual fitness, in particular when EDCs mimic and/or interfere with gonadal steroids whose organizational action on nervous system and behavior is well characterized. On the other hand, EDCs has a long-term effects that impact on adult function. In birds, a primary route of exposure to EDCs occurs via maternal deposition of lipophilic compounds into the yolk with increasing concentrations in the yolk according to the availability of the compound from the maternal circulation (Lin et al., 2004). In addition, maternally deposited steroids from the hen into the egg have the potential of inducing altered behavioral responses in exposed individuals (Adkins-Regan et al., 1995; French et al., 2001; Carere and Balthazart, 2007; Hahn et al., 2015). Moreover, in birds it was observed eggshell thinning and altered gonadal development, resulting in severe population declines (Giesy et al., 1994). Another example are male alligators exposed *in ovo* (as embryos) to various pesticides which subsequently exhibited significantly reduced plasma testosterone concentrations, aberrant testicular morphology, and small penis size while females exhibited ovarian abnormalities associated with reduced fertility and high embryonic mortality (Guillette et al., 1994; Guillette and Edwards, 2008). In fish, there are extensive evidence that EDCs affect reproductive endocrine function and contribute to alteration in reproductive development, because they have estrogenic, androgenic, anti-androgenic, and antithyroid actions (Guillette and Edwards, 2008). Different studies reported that effects of endocrine disruptors on fish are: the inappropriate production of the blood protein vitellogenin (VTG; the female-specific and estrogen-dependent egg yolk protein precursor) in male and juvenile fish, inhibited ovarian or testicular development, abnormal blood steroid concentrations, intersexuality and/or masculinization or feminization of the internal or external genitalia, impaired reproductive output, precocious male and/or female maturation, increased ovarian atresia (in female fish), reduced spawning success, reduced hatching success and/or larval survival, altered growth and development (thyroid hormone-like effects) and alterations in early development (altered rate orpattern) (Kosai et al., 2011). In marine gastropods, it was observed a masculinization due to

tributyltin (TBT) that resulted in worldwide declines of gastropods (Matthiessen and Gibbs, 1998) also a population declines affected some marine mammals (e.g., Baltic seals) as consequence of reproductive and immune function impaired (Ross et al., 1996).

6.2 Reproductive Effects

EDCs have associated with adverse reproductive outcomes in male and female. The vertebrate neuroendocrine system is a clear example of the evolutionary homology principle, as that its development and organization is substantially conserved and similar across the various classes. Then, an exam all relevant data, come from experimental animal and also observations in wildlife will provide a warning to human health (Myers et al., 2004; Hamlin and Giulietta, 2010). During embryonic development, organogenesis and tissue differentiation proceed through a series of tightly regulated and temporally coordinated events at the cellular, biochemical, and molecular levels, ultimately resulting in a functional, mature structure. However, exogenous environmental chemicals namely EDCs alter this process and deflect the developmental trajectory, often leading to lifelong phenotypic changes such as increased endocrine disease propensity. The targets of endocrine glands typically exhibit heightened sensitivity to hormones during specific developmentally critical windows. During these periods, hormonal signals cause changes to cells at the molecular (often gene expression and/or epigenetic) level and dictate or modify structural and functional organization of the tissues. Molecular changes in response to EDCs often precede morphological consequences, sometimes by weeks, years, or decades (depending upon life span), and experimental studies showing gene or protein expression changes in response to EDCs may be sentinels for disease propensity later in life (Collman, 2011; Gore et al., 2015). However, particular concern is the difficulty of linking prenatal, postnatal, and childhood exposure to later functioning in adult life (Damstra, 2002). However, particular concern is the difficulty of linking prenatal, postnatal, and childhood exposure to later functioning in adult life (Damstra, 2002). Accordingly the WHO (2013), released The State of the Science of Endocrine Disrupting Chemicals, to express the concerns to public health due to EDCs. Some EDCs, at environmentally relevant doses, bind to hormone receptors and act either as agonists or antagonists, thus enhancing, dampening, or blocking the action of hormones. They also alter the number of hormone receptors in different cell types and the concentration of circulating hormones (Martinez-Arguelles et al., 2014; Xu et al., 2014a). Animal data has shown that EDCs with estrogenic and anti-androgenic activity, during critical periods of development have alter reproductive tract development (Astolfi and Zonta, 1999). As consequences, concerns are alteration of function and structure of male and female reproductive organs, as reported by several researches. Male and female reproductive disorders, can be caused by exposure of EDCs during intrauterine

development and in adulthood. Strong evidences gained from laboratory studies showing that EDCs causing disrupt of ovarian function, spermatogenesis, and fertility outcomes.

FEMALE REPRODUCTIVE HEALTH

The adverse effects of EDCs on ovary, uterus, vagina, anterior pituitary, and/or production of steroid can lead to reproductive disorders such as early puberty, infertility, abnormal cyclicity, premature ovarian failure/menopause, endometriosis, fibroids, and adverse pregnancy outcomes (Schug et al., 2011; Meeker, 2012; Uzumcu et al., 2012; Caserta et al., 2014). Increasing exposure to EDCs compounds has been suggested as a possible factor accounting for the anticipating onset of human puberty (Herman-Giddens et al., 1997; Aksglaede et al., 2009). In physiology condition, puberty is regulated by the activation of the hypothalamic-pituitary-gonadal (HPG) axis and HPA axis through sexual hormones (Shpakov et al., 2018). In this regard, a dysregulation of these complex system profoundly affect pubertal development. EDCs, may mimic naturally occurring estrogens and androgens in the body or they may potentially cause overstimulation of hormonal pathways. In addition, EDCs might bind to receptor within a cell and block the functions of endogenous hormones, acting as antiestrogens and antiandrogens (Rasier et al., 2006; Caserta et al., 2008). Although in humans, it is difficult to demonstrate exposure to low doses of chemicals starting in prenatal life or mainly in early life, the Expert Panel and Endocrine Society Scientific have reviewed the literature on human studies and then they have confirmed the associations between EDCs exposure and puberty timing (Buck Louis et al., 2008; Diamanti-Kandarakis et al., 2009).

Particularly a delayed puberty for rat female have been caused by exposure to DBPs and chlorotriazine simazine (herbicide) (Zorrilla et al., 2010; Narotsky et al., 2013). Whereas, a dysregulation of hypothalamic–pituitary–gonadal (HPG) axis with advanced puberty onset, increased levels of serum luteinizing hormone and estradiol have been showed by Du et Collegues (2019) in female rats exposed to PFOA or PFOS (Du et al., 2019). A similar effect was observed for exposure to (2-ethylhexyl) phthalate (DEHP), a high molecular weight phthalate. It has determined also, a disruption of estrous cyclicity in all three generations of female (Rattan et al., 2018). Animal studies showed an ovarian function altered by exposure to EDCs. Bodensteiner andColleagues (2004) have showed an reduce of the number of primordial follicles and total healthy follicles in prepubertal rabbits, that they were exposed daily to dibromoacetic acid (a DBPs) from gestation day 15 throughout life (Bodensteiner et al., 2004). In mice, iodoacetic acid inhibited antral follicle growth and reduced estradiol production by ovarian follicles *in vitro* (Jeong et al., 2016). *In vitro* investigation by Gonsioroski and Colleagues (2019) analyzed the gene expression and sex steroid hormone levels of mouse ovarian follicles. They showed that iodoacetic acid dysregulated the expression of apoptotic factors, cell cycle regulators, steroidogenic factors, and estrogen receptors,

subsequently disrupting cell proliferation and steroidogenesis. In mice, Chen et Colleagues (2017a) showed that maternal exposure to PFOA inhibited corpus luteum function, decreased levels of serum progesterone, decreased the ovarian expression of Star, Cyp11a1, and Hsd3b1, increased the ovarian expression of tumor protein (p53) and Bax, and reduced the expression of Bcl-2 in the ovary, leading to embryo resorption, reduced fetal growth, and reduced postnatal survival (Chen et al., 2017a). Furthermore, PFOA exposure induced apoptosis and necrosis in mouse oocytes, which is likely related to reactive oxygen species (ROS) generation and gap junction intercellular communication disruption between the oocyte and the granulosa cells (López-Arellano et al., 2018). Prenatal BPA exposure inhibited germ cell nest breakdown in ovaries of the F1 generation in mice, decreased the numbers of primordial, primary, preantral, and total healthy follicle numbers at post-natal day 21, and decreased estradiol levels in female rats dosed for 1 year, suggesting that BPA targets the ovary (Berger et al., 2015; Patel et al., 2017). Exposure of BPA, also initiated an excessive premature activation of primordial follicles in mouse mature ovaries via the phosphatase and tensin homolog/phosphatidylinositol-3-kinase/ protein kinase B (PTEN/PI3K/AKT) signaling pathway by downregulating phosphatase and tensin homolog (PTEN) expression *in vivo*. An prematurely activating primordial follicles and altering levels of sex-steroid hormones is caused by exposure to bisphenol A (BPA) and phthalates (Berger et al., 2015; Patel et al., 2017). 2-etilesil-ftalato (DEHP) exposure also accelerated folliculogenesis in adult mice orally exposed to DEHP as well as its metabolite MEHP. Following DEHP exposure, the mice had decreased primordial follicle numbers and increased primary, preantral, or antral follicle numbers compared to non-exposed mice (Moyer and Hixon, 2012; Hannon et al., 2014); moreover DEHP, had significantly caused a decrease of germ cells as well as accelerated folliculogenesis compared to control (Rattan et al., 2018). Pesticides causes a dysregulated estrous cycles with extended periods of diestrous (Cooper et al., 2007; Rollerová et al., 2011). Furthermore, adult females exposed to imidacloprid had decreased ovarian weight, increased FSH, and decreased LH and progesterone levels in serum, increased lactoperoxidase activity in the ovary, and decreased antioxidant capabilities in the ovary (Kapoor et al., 2011). Others unpleasant effects include delayed vaginal opening, reduced ovary, uterine (Laws et al., 2000; Cooper et al., 2007) caused by pesticides; instead in female mice PFOA exposure caused a delayed or absence of vaginal opening (Zhao et al., 2012). Phthalate exposure is associated with increased resorptions and decreased pregnancy, implantations, and fetal weights of offspring (Kaul et al., 1982; Agarwal et al.,1989). Other studies showed that BPA exposure affected implantation and the establishment of pregnancy in mice and rats, caused intra-uterine growth restriction in mouse fetuses. It also, altered steroid hormone signaling in mouse uteri, and impaired the number of nerve fibers in the wall of the porcine uterus (Li et al., 2016; Martínez-Peña, et al., 2017; Rytel, 2018;

Müller et al., 2018; Neff et al., 2019); in addition, *in utero* exposure to BPA results altered mammary gland development and morphology with increased epithelial volume, and the altered ductal morphology of mammary glands in mice (Paulose et al., 2014; Hindman et al., 2017). Exposure to several NPs have led to cytotoxic effects on ovarian structural cells, impairing oogenesis and follicle maturation, and altering normal sex hormone levels. *In vitro* study on ovary cells (CHO), epithelial-like ovarian constituent cells have shown the ability of TiO_2-NPs to be internalized by CHO-K1 and to induce a dose-dependent decrease in cell viability following both acute (Uchino et al., 2002; Zhu et al., 2009a; Di Virgilio et al., 2010) and subacute exposure (Wang et al., 2011b, 2011c). This effect was associated to the increased ROS concentrations induced by nanoparticles (Uchino et al., 2002). Similar observation was found in Aluminium oxide NPs exposure (Di Virgilio et al., 2010). Overall, the cytotoxicity in ovarian cells by metal-based NPs may produce an effect through induction of oxidative stress, while carbon-based NPs induce the activation of MAPKs cellular signaling/over-expression of COX-2 (Jiang et al., 2010). The genotoxic effects have included frequency of sister chromatid exchange, micronuclei and DNA strand breaks (Zhu et al. 2009a; Di Virgilio et al., 2010). Alteration in oocyte maturation and fertilization and their subsequent embryonic development was finding by exposure to TiO_2-NPs and Quantum Dots (QDs) that are indicate as endocrine disruptors. Dose-dependent decrease in oocyte maturation rate, reduced fertilization, impairment of cell proliferation and dose-dependent enhanced blastocyst apoptotic rate were observed on oocytes by *in vitro* studies on TiO_2-NPs (12.5–50 μg/mL) and on Cadmium selenium core QDs (CdSe-core-QDs) at 0–500 nM (Hou et al., 2009; Xu et al., 2012). Modification of oogenesis and follicle maturation, has also been observed in several *in vivo* studies. *Danio rerio* exposed to 0.1 and 1.0 mg/L TiO_2-NPs has reported a distribution of follicular developmental stages toward immature statuses and reduction the expression of gene coding for growth factors implicated as paracrine stimuli in oocyte maturation (Wang et al., 2011a). NPs are able to alter physiological sex hormone levels in the female, Au-NPs and QDs alter estrogenic hormonal levels. The exposure of rat ovarian granulosa cells to 2.85×10^{10} NPs/mL of metal-based NPs induced a greater output of estradiol after 1–5 h of treatment, while a significant decrease was observed after 24 h. *In vivo* studies showed that the serum levels of estradiol significantly increased, while progesterone, FSH, LH and testosterone levels diminished in female CD-1 (ICR) mice sub-chronically treated with TiO_2-NPs (Gao et al., 2012). However, it is important underline that the effects reported in these studies most dependent on the physicochemical characteristics of the NPs investigated. Evaluation of this variable must be necessary to define their act as endocrine disruptor (Iavicoli et al., 2013).

MALE REPRODUCTIVE HEALTH

Evidence suggest that increasing exposure to endocrine-disrupting chemicals (EDCs)has significant adverse effects on male reproductive health and on the decline of fertility (Meeker, 2010; Marques-Pinto and Carvalho, 2013; Jeng, 2014; Hauser et al., 2015). Male reproductive decline may result from a combination of morphological, functional and molecular alterations in the reproductive organs. The testes, are the male organs responsible for the production of spermatozoa and for the synthesis and secretion of male sex hormones. In Vertebrates during the embryogenesis, the differentiation of the testis start from a bipotential gonad. Thanks to different genetic or environmental signals, this bipotential gonad can be induce into testis or ovary (Trukhina et al., 2013). Mammals have an XX:XY sex chromosome system and the Y chromosome-linked SRY gene acts as the master sex determinant, directing testis formation (Sinclair et al., 1990). SRY functions as a genetic switch that directs the bipotential gonadal primordium towards testis morphogenesis. Therefore individuals lacking SRY, such as XX genetic females or rare XY cases in which SRY is mutated or deleted, develop ovaries (Lovell-Badge and Robertson, 1990; Hawkins et al., 1992). However, SRY is absent in non-mammals and in the various classes of Vertebrate the development of testis can follow different pathway because involve different genis or environmental signals (Gubbay et al., 1990; Smith et al., 2009; Ioannidis et al., 2020; Ye and Chen 2020). Nevertheless, in all Vertebrate the testis has an intertubular (interstitial) and a tubular compartment. The tubular compartment (seminiferous tubules) is delimited by a basement membrane and myoid cells, and has the germinal epithelium inside. The germinal epithelium containing spermatogenic cells and Sertoli cells, nutritional cells that have the heads of maturing sperm embedded in them. An active epithelium may exhibit all stages of developing sperm. The lumen, or tubule cavity, contains the tails of many sperm (the heads of which are embedded in Sertoli cells), free sperm, and fluid that is probably resorbed. The intertubular compartment (testicular stroma), which fills the spaces between seminiferous tubules, consists mainly of connective tissue, blood and lymphatic vessels and contains Leydig cells (Kent, 2018). Leydig cells, are the mainly source of testosterone that is stimulated by luteinizing hormone (LH), which is produced by the anterior pituitary and acts via receptors on the surface of the Leydig cells. The testosterone is important to stimulate the spermatogenesis process (Utiger, 2018) and it is highly regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Sperm development and quality is under multiple levels of regulation, therefore be disrupted at many points (Sharma et al., 2020). EDCs having an impact at the endocrine system level besides reproductive outcomes and basic seminal parameters (Zamkowska et al., 2018). Evidences on animal model for chemical products that are recognized as potential endocrine disruptors (namely water disinfection byproducts, perfluoroalkyl and polyfluoroalkyl substances, bisphenol A, phthalates, pesticides)

suggest their harmful action on morphology and function of the male reproductive system. Bisphenol A (BPA), disrupts the HPG axis in mice, rats, and zebrafish (Molina et al., 2018). Male rats treat with pesticides (Atrazina) showed decreased serum levels of testosterone and inhibin-B, and increased serum levels of FSH and LH (Song et al., 2014a); also prenatal exposure to Di-(2-ethylhexyl) phthalate (DEHP) has induced decreased circulating testosterone concentrations (Barakat et al., 2018). In male rats, during puberty, the perfluorooctane sulfonate (PFOS) cause delayed Leydig cell maturation because it low serum testosterone levels without altering luteinizing hormone and follicle-stimulating hormone levels (Li et al., 2018); instead prenatal exposure to PFOS decrease sperm count and serum testosterone concentration in male rat offspring (Ma et al., 2015). In general prenatal exposure to EDCs determine testicular anomalies later in life, which includes reduced semen volume and quality, increased incidence of cryptorchidism and hypospadias and increased incidence of testicular cancer (Sharpe and Skakkebaek, 1993). Moreover, it is known that EDCs have adverse effects on the structure of testis and consequently on its function. Histopathologic changes in the testis and epididymis have been observed in male rats exposed to dibromoacetic acid, a water disinfection byproducts (DBPs). It have caused atrophy of the seminiferorus tubules and also the formation of a large atypical residual bodies that are the result of the impaired degradative function in Sertoli cells. Additionally, the exposure caused the retention of spermatids, fusion of mature spermatids, deformed sperm heads and vesiculation of the acrosomes of late spermatids (Villanueva et al., 2004; Melnick et al., 2006). Irregular and disordered arrangement of seminiferous epithelium, decreased numbers of spermatozoa, increased numbers of abnormal spermatozoa, decreased levels of total antioxidant capacity were observed in mice after exposure to pesticides (Mathias et al., 2012; Song et al., 2014a, 2019). Others studies in mice have shown that BPA exposure impacted on testicular seminiferous tubules with formation of morphologically multinucleated giant cells (Takao et al., 1999), disruption of the blood-testis barrier (BTB) (Su et al., 2011); moreover, it caused a decrease of sperm motility, decreased sperm membrane integrity, decreased sperm count and in general impaired sperm function (Vilela et al., 2013; Dobrzyńska et al., 2014; Wang et al., 2016). EDCs may contribute to increased DNA damage in sperm cells because some of them may induce oxidative stress and decrease the cellular levels of GSH and protein-sulfhydryl groups (Duty et al., 2003; Meeker et al., 2008; Tiwari and Vanage, 2013).

Also nanoparticles can interfere on male reproductive system at different level, several studies have shown that nanoparticles are able to modify the testicular structure, impair spermatogenesis and alter the biosynthetic and catabolic pathways of testosterone (Li et al., 2009a, 2009b, 2012; Takeda et al., 2009; Yoshida et al., 2009, 2010; Bai et al., 2010a; Noori et al., 2011); moreover they cause toxicity

effect on spermatozoa as sperm DNA damage, alteration on concentration, motility and acrosome reactions (Guo et al., 2009; Takeda et al., 2009; Wiwanitkit et al., 2009).

Althoug if is well-known that exposure to EDCs affect male reproductive function at multiple levels, and the male infertility is investigate in numerous animal and human studies (Sweeney et al., 2015), more complete investigations will be needed because the details of these impacts are not adequately studied or understood (Embrandiri, 2016).

EFFECT ON EMBRYO DEVELOPMENT

In non-human animals of laboratory, the toxicological effects have been studied also from embryo development to birth. For example, studies have shown that the exposure to water disinfection byproducts in drinking water can cause cardiac anomalies in developing rat and porcine embryos (Andrews et al., 2004; Pagé-Larivière et al., 2016). Moreover they were associated with neural tube defects, cardiovascular defects, cleft defects, as well as chromosomal abnormalities; and also with stillbirths (Dodds and King, 2001; Rivera-Núñez et al., 2018). In rat embryos, the primary effects were dysmorphogenesis, heart defects, and to a lesser extent, prosencephalic, visceral arch, and eye defects. An higher toxicology effects appeared for embryos exposed to the combination of DPBs than single compounds, it suggest that the developmental toxicity of these DBPs was additive (Andrews et al., 2004). Developmental effects in zebrafish embryos, are investigated by Teixidó et Collegues (2015), who investigated 10 water disinfection by-products (DBPs). DPBs exposure has been associated with adverse developmental effects, especially a significant reductions in the tail length and increases in malformation rates (Teixidó et al., 2015). Wang (2018) reported that halobenzoquinones, an emerging class of DBPs that have been detected in drinking water and swimming pool water, induce reactive oxygen species (ROS) generation and inhibited the antioxidative response of cells in developing zebrafish, resulting in death, physical malformations, oxidative DNA damage, and apoptosis (Wang, 2018). For the nanoparticles, studies have shown that is necessary to take in account that not only chemical composition and physical parameters affect the final toxicity of nanoparticles, but also the application and routes inside organisms differ among diverse species and significantly influence the possible toxicity of NPs. In particular, chicken embryos seem to be more resistant to NP treatment. The injection into the air sac or albumen can lead agglomeration of NPs in albumen or bind into albumen protein, than the penetration into the embryo could be much lower. Platinum nanoparticles (NP-Pt), did not affect the growth and development of the embryos but induce apoptosis and decrease the number of proliferating cells in the brain tissue (Prasek et al., 2013), while zebrafish embryos floating directly surrounded by egg water with dispersed NP. Smaller NP could passively diffuse through the chorion and a greater number of larger NP was found to be incorporated in deformed embryos (Lee et al., 2012a). Single Ag NPs passively

entered the embryos through their chorionic pores via random Brownian diffusion and stay inside the embryos throughout their entire development (120 h), as consequence they have effects on embryonic development (Lee et al., 2012a).

7. Male infertility

In recent years, many developed countries are experiencing dramatic increases in the number of couples facing infertility. The declining birth rate, is today the most serious social problems among the industrialized countries (Skakkebaek et al., 2016; Sumner et al., 2019). Social and environmental factors are regarded the main causes. Social factor bring the woman to have the first pregnancy at older age, but on the other hand the environmental factors as pollution by chemicals substances play significant role in reproductive disease causation or progression, or may later higher the susceptibility to these over a life time (Hart and Tadros, 2019). As mentioned, the reproductive system is highly sensible to molecules with endocrine disruption that mimic or disrupt steroid hormone actions. In particular, male reproductive function has attracted the increasing attention due to the several biological problems observed and although the male reproductive health appears as a multifactorial event, because it can be impair by interaction of genetic syndromes, occupational exposures or drug treatments, the exposure to environmental chemicals such as EDCs, during intrauterine development and in adulthood, can be a potential cause of male reproductive disorders (Petrelli and Mantovani, 2002; Street et al., 2018). Increasing evidences prove that male fertility is in deterioration and the endocrine-disrupting chemicals are associated to the etiopathogenesis of male disease (Street et al., 2018). Daily human exposure to these chemicals, as they are found ubiquitously in the environment and in everyday objects, have adverse effects on testes, resulting in testicular damage at structural and consequently functional level as shown by experimental studies (Nordkap et al., 2012; Sweeney et al., 2015). Direct effects on the proliferation and differentiation of the seminiferous epithelium, but also mechanisms affecting the control of spermatogenesis were observed. Sperm motility, together with concentration and morphology, is considered one of the most important steps in the evaluation of male partner in infertile couples (Čipak et al., 2009). Infertility is defined as failure to conceive after 12 or more months of regular unprotected intercourse (Practice Committee of the American Society for Reproductive Medicine, 2008). It became the most problem of our times and 15% of couples fail to conceive after a year of attempts. In the 20% of infertile couples, male factor is the main cause of infertility (Thonneau et al., 1991; Hull et al., 1985). A decline in semen quality was observed in the last 40 years (Centola et al., 2016; Virtanen et al., 2017), in particular have been reported a significant decrease in total sperm count, motility, viability and normal shape, resulting in a reduction in the chances to procreate (Carlsen et al., 1992). The causes of this decline have been associated to the exposure to environmental chemicals (EDCs), during intrauterine development and

in adulthood as highlighted in numerous animal and human studies (Nordkap et al., 2012; Sweeney et al., 2015). Spermatogenesis is a complex process with a multiple levels of regulation, therefore EDCs can disrupt it at many points. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which stimulates luteinizing hormone (LH), and follicle-stimulating hormone (FSH) release from anterior pituitary gland. These hormones act on the testis to regulate the spermatogenesis process, which depends also on dynamic interactions between the Sertoli cells and the germ cells (de França et al. 1993; Boekelheide et al. 2000) and between Leydig cells and germ cells.

Leydig cells are stimulates by LH to produce testosterone in the testicular via second messengers (Bliss et al., 2010; Singh et al., 2017; Darbre, 2021) Testosterone (T) is essential for spermatogenesis process. Also, Sertoli cells are target of LH; after its binding Sertoli cells are induce to produce androgen-binding protein (Bliss et al., 2010; Darbre, 2021). The surface of Sertoli cells also has receptors for FSH, and once FSH has bound, it works simultaneously with the testosterone released from the Leydig cells to promote the proliferation of spermatogonia (Bliss et al., 2010). The release of testosterone creates a negative feedback loop by inhibiting the secretion of LH from the anterior pituitary (Feher, 2012). Sertoli cells provide physical support to developing germ cells (Cheng and Mruk, 2002) and secrete hormonal and nutritive factors that act with a paracrine-signaling mechanisms between these cells, moreover they stablish among themselves which divide the tubular area into adluminal and basal compartments as well as form the basis of the blood–testis barrier that protects the maturing germ cells (Saunders, 2003). Sertoli cells also regulate apoptosis in the seminiferous epithelium (D'Abrizio et al., 2004). Adverse effects have been reported on alterations of concentrations of the hormones described (Hanaoka et al., 2002; Inyang et al., 2003; Kumar et al., 2009; Meeker et al., 2009a, 2009b, 2010; Han et al., 2010; Mendiola et al., 2010; Lassen et al., 2014; Den Hond et al., 2015). Phthalates were associated with increased LH, decreased testosterone, and increased estradiol production by Leydig cells, as well as increased Leydig cell numbers (Sharma et al., 2020); triclosan (TCS) reduces the production of Testosterone in Leydig cells and disturbs the function of major steroidogenic enzymes (Kumar et al., 2008; Forgacs et al., 2012); pesticides cause a significant decrease in the levels of serum LH, FSH, cholesterol, pregnenolone and T compared in male rats (Kumar et al., 2009; Geng et al., 2015). Benzo[a]pyrene (B[a]p) decrease serum testosterone and increase apoptotic germ cells in a dose-dependent manner (Chung et al., 2011), while the concentrations of Cadmio (Cd) were positively correlated with FSH, T, E2, LH and inhibin B and negatively correlated with prolactin (Jurasović et al., 2004; Akinloye et al., 2006). Additionally, Yang and Colleagues (2019) showed that levels of GnRH and LH were significantly higher in occupationally manganese (Mn)-exposed group compared with the non-exposed men. It has been reported that also NPs can induce a negative impact on the anterior pituitary gland and hypothalamus

and then interfere with the levels of secreted hormones, resulting in the reduction of FSH and LH secretion and a consequent further decline in the T level. In male rats, sub-dermal exposure of Ag NPs for 7 and 28 days have caused alteration in testosterone (T), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels (Olugbodi et al., 2020); Ni NPs exposure at different doses (5, 15, 45 mg/kg BW) decreased the value of FSH and T, thus indicating the occurrence of testicular injury (Kong et al., 2014) similarly, oral administration of Al_2O_3 NPs and ZnO NPs at doses of 70 and 100 mg/kg BW/day for 75 days in Wistar male albino rats induced, respectively, reduction in TSH and T levels, and increases in LH and FSH levels (Yousef et al., 2019). While, in male mouse the intravenous administration of Ag NPs at low dose (1 mg/kg/dose) significantly increased testosterone level as well as gold nanoparticles (Au NPs) (25, 50, and 100 ppm) in male rats (Zhang et al., 2015a). Overall, the decrease of FSH worse the testicular damage, while the T level reflects the extent of spermatogenic cell decline as well as the degree of altered spermatogenesis.

However, adverse effect can be observed on Sertoli and Leydig cells. Chemicals could direct effect on Leydig cells and thus change the amount of testosterone synthesize (Ariyaratne et al., 2003, Ramos-Trevino et al., 2018), or they could affect Sertoli intercellular junctions by either reducing the amount or inducing aberrant intracellular localization of junctional proteins as reported by chlorinated insecticides, dinitrobenzene, cadmium chloride, cisplatin or bisphenol A (Fiorini et al., 2004). Chemical substance can also increase spermatocyte apoptosis via Sertoli cell damage (Jiang et al., 2016) or up-regulation of apoptotic proteins (Zhang et al., 2016b). Some studies suggest that EDCs such as BPA inhibit ATP production (Hulak et al., 2013), perhaps by disrupting mitochondria, impairing sperm motility (Rahman et al., 2017), moreover it also can cause apoptosis, DNA damage, disruption of intercommunication among cells, mitochondrial damage, disruption of tight junctions, arrest of proliferation (Adegoke et al., 2020). In addition, a direct acute effect of chemicals on sperm is linked to their presence in semen. Several studies have shown these presence in a range of species, including the human (La Rocca et al., 2015; Wang et al., 2015; Levine et al., 2017). As consequence have been observed alteration in count, morphology and motility sperm, but also another quality parameter of ejaculate as DNA fragmentation (Rex et al., 2017) was observed. Changes in these aspects threaten male reproductive health, which is commonly associated with infertility (Isiah et al., 2011). In some areas, young males demonstrate a suboptimal quality and number of spermatozoa (Andersen et al., 2000; Huang et al., 2017). Sperm count and motility are the first parameters evaluated (WHO, 2010; Wang and Swerdloff, 2014). The first global decline of mean sperm concentration was reported by Carlsen and Colleagues (1992) which have denoted a decrease from 113 million/mL in 1938 to 66 million/mL in 1991 (Carlsen et al., 1992). A meta-analyses, including a systematic review and meta-regression analysis by Levine and Colleagues (2017), has supported

this. A decline in sperm concentration globally at a rate of 0.70 million/mL/year from 1973 to 2011 was observed (Rodprasert et al., 2021). Current data supports the association between BPA exposure and lower semen concentration, lower total sperm count, lower sperm motility (Li et al., 2011) and similar association was observed for phthalate metabolite (Pant et al., 2008, 2011, 2014a,; Jurewicz et al., 2013; Kranvogl et al., 2014; Thurston et al., 2016; Chang et al., 2017; Chen et al., 2017b) and organochlorine pesticides (Ayotte et al., 2001; De Jager et al., 2006; Aneck-Hahn et al., 2007; Pant et al., 2014b; Lin et al., 2021) that can also cause abnormal morphology on sperm (Aneck-Hahn et al., 2007). *In vivo* studies on rats, have shown that high doses of TCS (50 and 200 mg/kg) decrease daily sperm production and at the same time increase the percentage of sperm abnormalities on tail and head sperm (Lan et al., 2015); also, in human the urinary TCS concentrations have been association between to these poor semen quality parameters (Zhu et al., 2016). Current evidence have shown that metal oxide nanoparticle affect sperm motility, in particular gold and silver nanoparticle reduce the motility in dose-dependent manner (Moretti et al., 2013; Nazar et al., 2016) and similar effect was observed to ZnO (Afifi et al., 2015). Moreover, they increase the abnormal sperm morphology (Thakkar et al., 2010; Nazar et al., 2016). Finally, the main sperm damage is DNA damage because sperm DNA integrity is essential for the correct transmission of genetic information (Agarwal and Tamer, 2003). The origin of human sperm DNA damage involves certain mechanisms such as: (1) alterations in chromatin modeling during the process of spermiogenesis, (2) apoptosis, and (3) oxidative stress (Aitken and De Iuliis, 2010). In rats, BPA exposure was associated with a significant increase in sperm DNA damage (Tiwari and Vanage, 2013) as well as such heavy metals (e.g., Hg), PCBs and insecticides (Duty et al., 2003; Arabi, 2005; Rignell-Hydbo et al., 2005; Hauser et al., 2007; Rignell-Hydbom et al., 2007; Meeker et al., 2008; Zhou et al., 2016). In human, urinary concentrations of phthalate metabolites were associated with sperm DNA damage (Duty et al., 2003; Hauser et al., 2007). To evaluate the DNA damage by nanoparticles on human spermatozoa, *in vitro* studies have been performed. Significant DNA damage have been inducted by metal oxide nanoparticles like cerium dioxide nanoparticles (CeO_2) at very low concentrations (0.01 $mg \cdot L^{-1}$) (Préaubert et al., 2018) and AgNPs at 200 μg ml−1 and 400 μg ml have increased the DNA damage after 60 min of exposure (Wang et al., 2017b). *In vitro* studies on animals spermatozoa are in accord with these results (Pawar and Kaul, 2014; Yoisungnern et al., 2015). The xenobiotic substances, as chemicals or nanoparticles, are preventable risk factor, thus an accurate identification and characterization is the first step to understand the reproductive hazards.

8. *Danio rerio*: a versatile organism model

Danio rerio, or commonly known as zebrafish, is a is a small teleost fish, belonging to the Cyprinidae family that live in tropical water of Asia (Engeszer et al., 2007). It became an established Vertebrate model compared to rodents mice and chickens. In general, fishes are good for *in vivo* investigation to study the potential toxicity resulting from environmental pollutants, like chemical compounds or nanomaterial, and drug (Westerfield, 1995; Dooley and Zon, 2000; Sipes et al., 2011; Appiani et al., 2014); the advantages are the easy waterborne exposure and the low cost to maintain. Zebrafish require relatively inexpensive housing, making them very cost-effective, and are small in size, reducing housing requirements as well as the quantity of agent required for testing (Westerfield, 1995; Dooley and Zon, 2000); in addition, zebrafish has high homology with humans, and its development of the central nervous system, cardiovascular system, visual system and internal organs has much in common with the human counterparts (Bai and Tang, 2020). About 70% of human genes possess at least one ortholog in *D. rerio*, which thus represents an optimal model for the study, also through the use of transgenic organisms (Carney and Mosimann, 2018), of genes whose mutations are associated with appearance of a specific disease (Bradford et al., 2017) and of the genes that govern the processes responsible for mitigating the toxic effects, or affected by the mutagenic action, of xenobiotics (Hill et al., 2005). The usage of zebrafish in scientific research play a significant role in the (eco) toxicological studies, but also in translational medicine for the research of the molecular causes of numerous pathologies; *D. rerio* has been proposed as a model for the study of pathologies of the blood (Gore et al., 2018), of the renal system (Morales and Wingert, 2017), of the cardiovascular system (Wilkinson and van Eeden, 2014), of the immune system (García-Moreno et al., 2019) the nervous system (Calvo-Ochoa and Byrd-Jacobs, 2019) and development biology (Broughton et al., 2001; Golling et al., 2002; Hortopan et al., 2010). The developmental processes underlying embryogenesis and organogenesis are highly conserved between fish and tetrapods (Mindnich and Adamski, 2009) and zebrafish shares further physiological aspects with its terrestrial counterparts, such as the endocrine system including hormones, receptors, and signaling cascades, which are highly similar (Mindnich and Adamski, 2009; Busby et al., 2010; Löhr and Hammerschmidt, 2011) and reproductive system. Zebrafish display similar anatomy of germ cell organs to that in humans (Van den Hurk and Resink, 1992; Siegfried and Nüsslein-Volhard, 2008) in male and female reproductive system. Male zebrafish have paired testes with tubule organizations. Within each tubule, the walls are lined by Sertoli cells and they function mainly to support testes morphogenesis and spermatogenesis while Leydig cells detected in the interstitial spaces act as primary testosterone producer (Van den Hurk and Resink, 1992; Siegfried and Nüsslein-Volhard, 2008). Another special feature of zebrafish is the presence of spermatogenic cyst which consists of a group of Sertoli cells

enveloping germ cells that develop synchronously (Schulz et al., 2015). Reproductive science or fertility is became the most popular filed in medical research, as mentioned exposure to harmful chemicals and unfavorable environmental conditions are some of suggested underlying pathogenic mechanisms in infertility (Snijder et al., 2012; Al-Griw et al., 2015). In this context, zebrafish is a well model for the evaluation of reproduction and development and can be used for studying the biodistribution and potential toxicity of endocrine disruptors (Sun et al., 2013). Researchers have study and understand the system of reproductive regulation in a more comprehensive way, thanks the close degree of similarity between zebrafish and human. Zebrafish is a promising model in assessing reproductive complications owing to its developmental and physiological advantages (Lee et al., 2012b; Hou et al., 2014; Zhang et al., 2015b; Bassi et al., 2016). First, zebrafish exhibit a high fecundity rate, with a single female able to produce around 200–300 eggs can be produced from a single mating every 5–7 days (Westerfield, 1995; Ribas and Piferrer, 2014). They develop rapidly, with the basic zebrafish body plan well-established by 24 hours post fertilization (hpf); the embryogenesis is complete by 72 hpf and most organs fully developed by 96 hpf. Adult hood is reaching in around 3 months (Stainier and Fishman, 1994).

Rapid embryonic development along with transparent embryos facilitate the observation of morphogenetic changes and organogenesis in real time (Kimmel et al., 1995; Fadool and Dowling, 2008). Therefore, they offer a variety of screening schemes that target develop and reproductive toxicity caused by xenobiotic (Bai and Tang, 2020). Embryonic development ex uterus eliminates the confounding factors associated with any maternal toxicity and also facilitates exposure to the substances to be tested. Zebrafish is increasingly used in studies concerning endocrine disrupting chemicals (EDCs) (Scholz and Mayer, 2008). Most endocrine receptors are prenatally expressed, then the study of hormones's action in prenatal prenatal period can be observed using zebrafish (Busby et al., 2010); moreover, zebrafish has a tremendous variety of steroid biosynthetic pathways exist, whit some steroid hormones that play a similar role compared to mammals. Thus, zebrafish are beneficial for assessing the effects, targets, and mechanisms of action of EDCs (Segner, 2009; Lyche et al., 2010). Zebrafish embryos have been used to assess the toxicological effect of classic endocrine disruptors (Ortiz-Villanueva et al., 2018) to understand the effects of estrogens on organ formation and function (Sun et al., 2010; Gorelick and Halpern, 2011; Namdaran et al., 2012; Padilla et al., 2012; Hao et al., 2013; Carroll et al., 2014; Gorelick et al., 2014; Kinch et al., 2015; xRomano and Gorelick, 2014; Truong et al., 2014; Bouwmeester et al., 2016; Tal et al., 2016). Xenobiotics substances could have an impact also on the rapid cell divisions and consequently morphogenesis (He et al., 2014; Nishimura et al., 2016), particularly sensitive to perturbation are the eye, brain, heart, notochord and fin. In the same way, zebrafish is a valuable model species for characterizing the

toxicity of NP (von Westernhagen, 1988) thanks the possible to study various physiological functions and biological processes, such as malformation (including pericardial oedema and bent notochords) and oxidative stress caused by nanoparticles. As mentioned, oxidative stress can cause reproductive toxicity in animals, which can lead to infertility during the extreme conditions (Chen et al., 2016), then the adult male zebrafish can be used to create the adverse conditions in which humans are exposed. The toxicological test, in this way help to improve the knowledges about the cause of infertility. *Danio rerio* represents a good compromise between carrying out *in vitro* studies and the use of higher organisms (Horzmann and Freeman, 2018).

9. Aim of the research

Over the past 50 years, exposure to environmental chemicals such as endocrine disrupting chemicals (EDCs) have become a significant public health concern. Evidences have suggested their consequences in the development of disorders about immune and neurological systems or progression of other important diseases such as diabetes, obesity, breast, ovary, testicle and prostate cancer, endometriosis (Diamanti-Kandarakis et al., 2009; Iavicoli et al., 2009; De Coster and van Larebeke 2012). Moreover, the association between EDCs and reduction of the possibility of procreation is strong due to studies that have confirmed the decline in sperm quality, significant decrease in total sperm count, motility, viability and normal shape (Centola et al., 2016; Virtanen et al., 2017). EDCs play an important role in the onset of the aforementioned diseases by altering hormonal and homeostatic systems (Wormley et al., 2004; Rogers et al., 2013) and as mention above they can affect male reproductive function at multiple levels. Despite the studies carried out in recent years and the categorization of chemicals that are likely or suspected to be EDCs (Johnson and Harvey, 2002; Okkerman and van der Putte, 2002; Petersen et al., 2007), is still limited the current knowledge of EDCs. A possible explanation is that the researches have been limited on a few groups of chemical substances (disinfection byproducts, perfluoroalkyl and polyfluoroalkyl substances, bisphenol A, phthalates, pesticides, pharmaceutical agents and heavy metals) consequently the data on a number of other xenobiotics that may act as EDCs are still scant and incomplete (Iavicoli et al., 2009). For instance, engineered nanoparticle (ENPs) must be included among chemical compounds to which the humans are exposed non-intentional (i.e., by inhalation, transdermal) and intentionally (i.e., by injection, food additives, ingredients and supplements containing NPs) (Yah, 2013) because they are included in different products of our daily life (Oberdörster al., 2005b). Especially, the reproductive toxicity is considered as an important issue to be investigated in overall toxicology (Yah, 2013), thereby the aim of my research has been investigate the possible action of engineered metal oxide nanoparticles (ENPs) as endocrine disruptor. Commercial Titanium dioxide nanoparticles (Degussa, P25) were used for our investigation Their toxic effects were examined: (1) on embryonic development, (2) on the male reproductive organs, and (3) on the male gametes. We performed three independent experiments, two *in vivo* and one *in vitro*. For the *in vivo* experiments *Danio rerio* as model organism was chosen, in particular the *D. rerio* embryos were used to carry out acute toxicity experiments to evaluate the alterations of embryonic development, while the adult males of *D. rerio*, with mature age, have been used to investigate alterations in the structure and function of the testis. Finally, *in vivo* experiments on human spermatozoa evaluated sperm quality parameters.

10. Materials and Methods

10.1 Experimental design

Toxicological experiments with *Danio rerio* and their embryos are the most common method for the screening of pollutant toxicants: pesticides, microplastics, antibiotics and nanoparticle (Chun et al., 2017; Sangabathuni et al., 2017; Da Silva et al., 2018; Eryilmaz et al., 2018). Thanks to the collaboration with Institute for Microelectronics and Microsystems of Catania (National Research Council, Italy) and the Fish Pathology and Experimental Centre of Sicily (CISS) of the Department of Veterinary Science (University of Messina), this research examines the toxicological effect of TiO_2 NPs on zebrafish development and its male reproductive system. First of all, it was defined the concentrations of the experimentations. According to the data in the literature and the suggestions by researchers of National Research Council we have chosen the following concentrations to tested: 1mg/L, 2 mg/L and 4mg/L of TiO_2-NPs (Kotil et al., 2017). Therefore, embryos zebrafish were used to acute TiO_2 NPs exposure experiments by Z-FET (zebrafish embryo toxicity test), whereas long-term TiO_2 NPs exposure experiment on adults zebrafish have been approved by the Italian Health Ministry (authorization n°1244/2015-PR). It was approved the use of 40 adult specimens. Both embryos and adults zebrafish were supplied by Fish Pathology and Experimental Centre of Sicily (CISS) of the Department of Veterinary Science (University of Messina). Subsequently, toxicological effect of TiO_2-NPs was investigated at cellular level by *in vitro* experiments on the human spermatozoa, which were supplied by donors fertile men of "Centro Biomedico ASTER", Catania (Italy).

10.2 Nanoparticles characterization

The titanium dioxide nanoparticle powders (Degussa, P25) supplied by CNR-IMM (Microelectronics and Microsystems of Catania-National Research Council, Italy), were purchased from Sigma Aldrich. According to manufacturer's information, the purity of the material was 99.5% with metal traces. The nanopowder crystalline phase was mixed: 86% anatase, 14% rutile and they had an average size of about 50 nm. SEM analyses were performed in plan-view, so to characterize the morphology of the nanoparticles. The analyses were performed by using a field emission Zeiss Supra 25 microscope, located at the CNR-IMM Catania (University) Unit. The nanoparticles are clearly agglomerated, as evidenced by the SEM images reported below (Figure. 3).

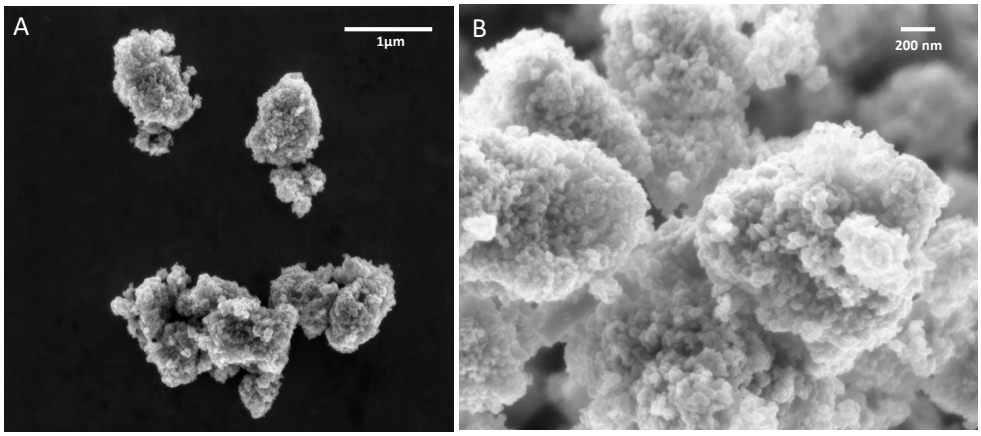

Fig.3: Representative scanning electron microscope (SEM) image of TiO_2-NP aggregates in the test solution. Magnification of 1µm **(A)** and 200 nm **(B)**.

10.3 *In vivo* experiments on zebrafish

10.3.1 Titanium dioxide NP dispersions and dosing to Zebrafish experiments

The dry TiO_2-NP powder was used to prepare the three different solutions: 1 mg/L, 2 mg/L and 4 mg/L. Briefly, each mass (1 mg, 2 mg, 4 mg) was dispersed in 1L of osmosis water (270 µS conductivity, 26-28°C, 6,9-7,5 pH, 6.00 ppm oxygen concentration) which is reconstitute with the addition of inorganic salts (Sea Salt, Red Sea Salt); it is optimal for the housing of zebrafish according to Nüsselin-Volhard and Dahm (2000) and also prevents fungi growth. Four cycles of sonication was carry out for each solution, in particular each cycle was 20 min with 10 min of break using a ultrasonic baths (FALC Labsonic LBS2) with a frequency of 40 kHz under extractor fan, in order to disrupt any possible aggregates (Pecoraro et al., 2017a).

10.3.2 Acute Toxicity Experiment of Zebrafish Embryo

The assay was conducted in accordance to the guidelines of Fish Embryo Acute Toxicity (FET) test with the zebrafish (*Danio rerio*) develop by Organization for Economic Cooperation and Development (OECD TG 236) (OECD, 2013; Sobanska et al., 2018). Zebrafish, were raised in a ZebTEC ActiveBlue Stand Alone system (Tecniplast) at Fish Pathology and Experimental Centre of Sicily (CISS) of the Department of Veterinary Science (University of Messina), which is accredited, since 2006, for use and, since 2010, for production of aquatic organisms for experimental research, and all procedures have performed following Directive 2010/63/EU. Zebrafish adults (males and females) were kept in bredding room in optimal condition as regarding photoperiod (light/dark cycle:14h/10h), quality water (27 ± 1◦C, pH 7.2 ± 0.3, 6.00 ppm dissolved oxygen content (DO)) and feeding. They are fed twice daily with *Artemia* nauplii (JBL Artemio Pur, BL GmbH & Co. KG, Germany).

Male and female fishes (ratio 2:1) were placed in a hatching tank which equipped with steel grids for the eggs to fall through to the bottom of the tank and avoid their predation by adults. They were left undisturbed overnight. On the next morning, spawning was triggered once the light was turned on and the eggs collection by Pasteur pipettes have started. Eggs were rinsed in aquarium water at 28°C and they have analyzed under stereomicroscope (≥30-fold magnification). The first cleave starts after about 15 min post fertilization and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers. At these stages, fertilized eggs can be clearly identified by the development of a blastula. The infertile eggs were discarded, whereas fertilized eggs undergoing cleavage and showing no obvious irregularities during cleavage (e.g., asymmetry, vesicle formation) or injuries of the chorion were selected. In order to ensure developmental synchronization at the beginning of exposure, the embryos at the blastula stage of about 3-3.5 hours post fertilization (hpf) were exposed to the three different solutions of TiO_2-NPs (1mg/L, 2mg/L and 4mg/L) and control solution (only water dilution). As recommended by OECD (2013) and according to protocol procedure by Pecoraro et Colleagues (2017b), 24 eggs were transferred into a 24-well multi-plates with one embryo per well. In each 24-well multi-plates, twenty embryos (one embryo per well) were exposed to TiO_2-NPs concentration to assess, while four embryos were exposed to dilution water because they were internal plate controls (negative control). Others multi-well plates have been done to: positive controls and negative controls. For the positive controls, 20 eggs (one embryo per well) were exposed to 3,4-dichloroaniline (DCA) at the concentration of 4mg/L in water; instead for the negative controls 20 eggs (one embryo per well) were exposed to water dilution. Multi-well plates were set up for the 1 mg/L, 2mg/L and 4 mg/L TiO_2-NPs solutions, in which the embryos (one per well) were exposed to 2 ml of work solution at 28°C. The same volume of 2 ml was used to negative's embryos (water

dilution) and the positive's controls (DCA solution). The maintained of 26 ± 1◦C in wells was ensured by a controlled of room temperature, moreover every 24 hours each work solutions were renewal in all wells (semi-static renewal) (OECD, 2013). Three replicates were performed for each concentration of TiO_2-NPs i.e. 1mg/L, 2mg/L, 4mg/L and also for controls. According the OEDC (TG 236, 2013) the test is valid if overall survival of embryos in the negative (dilution-water) control is ≥ 90% and the positive control (e.g., 4.0 mg/L 3,4-dichloroaniline for zebrafish) result in a minimum mortality of 30% until the end of the 96 hours exposure.

EVALUATION OF TOXICOLOGICAL ENDPOINTS

According to OEDC every 24 hours, eggs were observed in the well under a stereo microscope connected to a camera device. The exposure time was selected over to 96hpf, because even if the most organs of the embryos are well developed at 96 hpf, the larvae is formed after 120hpf (Kimmel et al., 1995; Giannaccini et al., 2014), this is useful for evaluate the expression of protein markers associated to the endocrine disruptors, and thus inducted by TiO_2-NPs. The test finished at 144 hours post fertilization. The acute toxicological endpoints analyzed in zebrafish were: coagulated embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat. According to OECD, these endpoints are useful for the determination of lethality from 24 to 96 hrs post fertilization. All endpoints were assessed and quantified as observed or not observed, because any positive outcome in one of these observations means that the zebrafish embryo is dead. All endpoints can occur after 24hrs of exposure, except to heartbeat which in normal zebrafish development is visible after 48 hours. In detail, OECD (2013) suggest that coagulated eggs are characterized to milky white color and dark color under the microscope, whereas the formation of somite is indicate by spontaneous movements (lateral contractions) of embryo because the right count of 20 somite after 24 hrs in normal zebrafish embryo development (26 ± 1°C) is hard.

Instead, the detachment of the tail from yolk is observed thanks to posterior elongation of the embryonic body. Heart beat was evaluated under a minimum magnification of 80x for at least one minute. The absence of heart beat suggest that zebrafish embryo is dead. Finally, hatching was evaluated in treatment and control groups on a daily basis starting from 48 hpf. For this reason, the number of coagulated eggs, hatched embryo, the number of dead embryos and the number of deformed embryos were recorded. In addition any other observation was recorded as further lethal or sublethal endpoints. For example thank to the use of DanioScope™ software (Noldus Information Technology bv, Wageningen, Netherlands) it was evaluated: heart beat, body length of larvae and malformations.

DANIOSCOPE ANALYSIS

Optical transparency of embryos zebrafish, allow excellent visualization of heart beating and blood flow. The heart is the first organ to form and is already good visible at 48hpf. For this reason the cardiology measurements, blood flow and also embryo activity, were recorded throught DanioScope™ software (Noldus Information Technology bv, Wageningen, Netherlands). This software works by analyzing videos to supply a quantitative data of each endpoints investigated. Daily, after the observation of acute endpoints the embryos were immobilized on dish of agarose and acclimated for at least 3min before to record videos. Video recordings from up to three fully clips of 5 min each were capture utilizing stereomicroscope E200 MV-R LED (NIKON) provides of camera CMOS (Nikon). Videos were recorded in color with a resolution of 1024 by 470 pixels at 60 frames per seconds (fps) and bit rate of 50 Mb/s, then saved in AVI format. They were analyzed by DanioScope™ software (Noldus Information Technology bv, Wageningen, Netherlands) to automatically determine: heart rate or inter-beat intervals, embryo activity and morphology measurements. For the detection of cardiological activity it was necessary select the heart area above the ventricle. The software apply a changing pixel intensity algorithm to detect changes in pixel density during ventricular contractions, because this changes are correlated directly with cardiac muscle contraction. Then it automatically calculates the number of beats per second (BPS) and beats per minute (BPM). Finally, morphology measurements as body length of larvae and size eyes were determinate using images uploaded on Danioscope. After calibration with stage micrometer scale, DanioScope™ has measured these parameters. All measurement were performed independent.

IMMUNOHISTOCHEMICAL ANALYSIS

In this context, an exposure until 144 hpf was indicated as optimal to evaluated marker of oxidative stress or which highlighted the actions of TiO_2-NPs as like endocrine disruptor. An immunohistochemical analysis was carried out to localize in whole larvae, marker of oxidative stress anti-P540 Aromatase (P540), Heat Shock Protein-70 (Hsp70), Poly ADP-Ribose Polymerase-1 (PARP-1)) and marker that suggested the action of TiO_2-NPs as like endocrine disruptor Sex hormone-binding globulin (SHBG) and Prothymosin-α (PTMA). Moreover, also metallothionein, a specific marker of exposure to TiO_2-NPs, was detected. The procedure has been based on immunofluorescence protocol's Pecoraro et Colleagues (2017b). At the end of exposure, all larvae (exposed and controls) were fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature. In summary, the primary antibodies raised in one species (e.g., mouse and rabbit): anti-rabbit-HSP70 (GeneTex, 1:1000), anti-rabbit-P540 Aromarase polyclonal (Creative Diagnostic®, 1:1000), anti-mouse-PARP-1 (Invitrogen, 1:1000), anti-rabbit-SHBG (GeneTex, 2 µg/ml), anti-mouse-PTMA (abcam, 1:100) and anti-mouse-MTs (GeneTex, 1:1000) were applied to each larvae placed on the slides for incubation overnight in a humid chamber at 4°C. However, before this the larvae were washed with Phosphate Buffered Saline (PBS) (pH 7.4, 0.1 M), permeabilized with PBS-Triton X-100 (for 15 min) to improve antibody penetration and then they were incubated with blocking solution of bovine serum albumin (BSA) for 20 min to blocked non-specific antibody binding. It was followed incubation with the primary antibodies. After the primary antibodies incubation was complete, larvae were washed twice (each time for 5 min) in PBS–Tween 20 to remove the excess of primary antibodies. The larvae were incubated with TRITC-conjugated anti-rabbit secondary antibodies (at a dilution of 1:1000) for cytochrome P540, HSP70, and SHBG, while with FITC-conjugated anti-mouse secondary antibodies (at a dilution of 1:1000) for PARP-1, PTMA and MT. Incubation was performed for 1 hour at 4°C in the dark. Finally, the secondary antibodies were remove through washed in PBS–Tween 20 (2 times for 5 min) at room temperature. The larvae were dehydrated in increasing alcohol solutions (70°, 80° and 95°) for 1 min each and air dried. Consequently, each larvae were mounted with DAPI (Abcam) and sealed with rubber cement to be examine under fluorescence microscope (NIKON ECLIPSE Ci fluorescence microscope). The images were capture with the NIKON DS-Qi2 camera connected to fluorescence microscope. TRITC-conjugated anti-rabbit secondary antibodies exhibited a red fluorescence, whereas FITC-conjugated anti-mouse secondary antibody exhibited a green fluorescence.

EVALUATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Intracellular ROS content was detected on larvae exposed to all concentrations of TiO_2-NPs, including controls. It was used 2,7 dichlorodihydrofluorescein diacetate (DCFH 2-DA) (Sigma-Aldrich), a fluorescent probe useful to measure the reactive oxygen species (ROS). After exposure was complete, all larvae were stained with ROS-detection solution as described by Mugoni and Colleagues (2014). Larvae collected were washed with Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) twice for 2 minutes each in a small tubes. ROS-detection solution (5µM DCFH 2-DA in HBSS) was added to each tube, which was incubate in the dark for 15 min at 28 °C to avoid light exposure. At the end of the incubation time, ROS-detection solution was removed and the larvae was washed twice (each time for 2 min) with HBSS. Larvae were placed on a glass slide, then the fluorescence was detected by fluorescence microscope (NIKON ECLIPSE Ci), equipped with camera NIKON DS-Qi2.

STATISTIC ANALYSIS

The coagulate, survival and hatching rate of the TiO_2-NPs exposed groups and the unexposed group were represented as the average percentage of the coagulate, survival and hatching rate from three replicates. Statistical analysis was carried out by one-way analysis of variance (ANOVA) test to comparison difference between groups, A $p < 0.05$ was considered to be a statistically significant difference. Moreover, Image J software was used to quantified the fluorescence intensity of equal area in treated and control groups.

10.3.3 Adult Zebrafish Exposure Experiment

In this study, one-two year-old zebrafish wildtypes, not consanguineous, with body weight about 0.5 g and average length of 3 cm were used. Zebrafish were raised in a fish room at the Fish Pathology and Experimental Centre of Sicily (CISS) of the Department of Veterinary Science (University of Messina). Prior to TiO_2-NPs treatment, fishes were acclimated to experimental conditions (26-28°C; light:dark/14h:10h; daily water change), including daily manipulation and nutrition. During acclimation, fishes were fed daily with "GEMMA micro 150 Skretting Zebrafish" commercial feed, the meals were two: morning between 9:00 a.m. and 10:00 a.m., afternoon between 16:00 p.m. and 18:00 p.m. Moreover, water parameters were monitored daily with multiparameter probe (HI9829 Aquaprobe, Hanna instruments, Padova, Italy) and showed the following values: temperature 27 ± 1.5 °C, pH 7-7.5, dissolved oxygen ≥ 6 ppm; furthermore the fishes were monitored twice a day (morning between 08:30 a.m. and 10:00 a.m., afternoon between 16:00 p.m. and 18:00 p.m.) to assess behaviors of distress, stress or discomfort. It is necessary ensuring the highest animal welfare as it established by the World Organization for Animal Health. After 30 days acclimation, the zebrafish were brought to the Fish Facility (Zeb Tech, Stand-Alone) and randomly divided into four groups of 10 fishes: three experimental groups (1mg/L, 2mg/L and 4mg/L TiO_2-NPs) and control group (without TiO_2-NPs only osmosis water). The tanks were equipped with aerator and fishes were subjected a semi-static exposure regime for 30 days (water change every 24h with a new solution of TiO_2-NPs). Parameter's water were monitored daily before and after the replacement of the solutions by multiparameter probe (HI9829 Aquaprobe, Hanna instruments, Padua, Italy). During experimentation the fishes were maintained as follows: photoperiod 10h dark/14h light with intensity equal to 250 lux, 6.9-7.5 pH, 26-28°C temperature and dissolved oxygen≥ 6.00 ppm; in addition they were fed with commercial feed "GEMMA micro 300 Skretting Zebrafish" twice a day before to change of water TiO_2-NPs solution in order to avoid adsorption of nanoparticles by food particles. After added the food, fishes were monitored for 10 minutes to verify that the food had been consumed, while after change of TiO_2-NPs solutions fishes' behavior was evaluated until one-hour in order to highlights change in swimming speed, respiration, loss of balance, bottom stationing and any possible abnormal behavior. Until the end of experimentation (30 days) the zebrafish were kept under the condition mentions and without sources of noise and/or vibrations. Complete the experimentation, the fishes were euthanized by anesthesia with a dose of 0.7 g/L tricaine methane sulfonate (MS-222) buffered then testis and gills tissues were dissected.

TiO_2-NPs ACCUMULATION

Through the Single Particle Inductively Coupled Plasma-Mass Spectrometer (spICP-MS), was evaluated the concentration of TiO_2-NPs into organs of zebrafish. This new technique allows determination of particle number-based concentration, the number of particles, size and size distribution. Prior to analysis, 100 mg of each organ from treated groups, including the control, were accurately weighed into (15 mL) conical tubes, then they followed an alkaline digestion according to Gray and Colleagues (2013) method. Ultrasonic bath was used to break tissue and release the nanoparticles without altering them (30 min at 37 °C), then the samples were left to digest at room temperature to 24h. Next day, the digested solutions were diluted to 50 mL using high purity water 0.22µm filtered (Millipore, Bedford, MA, USA) to 1% tetramethylammonium hydroxide (TMAH) and 0.1% Triton X-100, useful to prevent particle aggregation. Titanium nanoparticle stock solution was prepared from a TiO_2-NPs standard (60 nm TiO_2 Nano Powder, rutile, 99.9%, AEM) purchased from Nanovision (Brugherio, MB, Italy), while Ti ion standard (1000 mg/L, CPAchem) was used for *sp*ICP-MS calibration of dissolved titanium.

All digested samples and calibration solutions were sonicated for 30 min before analysis to allow a homogeneous dispersion. TiO_2-NPs were analyzed using ICP-MS NexION® 350D (Perkin Elmer, Waltham, MA, USA) with the Syngistix Nano Application software (Perkin Elmer, Waltham, MA, USA. Thanks to this technique, data on total TiO_2 particles (Ps-Tot) and TiO_2 nanoparticles (NP <100 nm) were acquired (Grasso et al., 2020). LOD was 1.3×10^3 particles/mL, while LOQ was 2.5×10^3 particles/mL. Referring to the sample weight and digestion volume used, they resulted 2.6×10^5/g and 5.0×10^5/g, respectively. In addition, LOD in size (LODnm) was estimated 35 nm.

HISTOLOGICAL EXAMINATION

Histological examination was performed following our standard protocol. Briefly, the dissected testes, gills, tissues were fixed in newly made 4% formaldehyde (Bio-Optica) in phosphate buffered saline (Sigma Life Science) overnight at room temperature. After washing with phosphate buffered saline (PBS: 0.1 M, pH 7.4) three times of 10 min each one, all tissue were dehydrated in ascending alcohols series (35°, 50°, 70°, 95°, absolute ethanol) for 10min each one. After, they were clarified in xylene (1h at room temperature) and embedded in paraffin (VWR-Chemicals) 60°C with (Thermofisher Histostar) tissue processor. 5 µm thick histological sections were prepared using microtome (Reichert Jung 1150 Autocut), the histological sections were collected on microscope slide. At least 10 slides of each tissue were collected. The sections were deparaffinized in xylene and stained with Haematoxylin-Eosin (HE) (Bio-Optica). The observation was carried out using optical microscope (Set E200 Nikon) and the images were captured by digital camera (CMOS Nikon)

connecting to microscope. It was identified potential morphological alterations on structure of testis, and gills tissues.

IMMUNOHISTOCHEMICAL ANALYSIS

An immunohistochemical analysis was carried out on testis sections to detect SHBG protein and also the expression of P540. It was followed our standard protocol of immunohistochemical used for several experimentation on zebrafish (Pecoraro et al., 2017a), furthermore the same primary antibodies used for the immunohistochemical investigation on zebrafish larvae were used. Primary antibodies anti-rabbit-SHBG (GeneTex, 2 μg/ml) and anti-rabbit-P540 Aromatase polyclonal (Creative Diagnostic®, 1:1000). Prior to incubation with the primary antibodies, the sections were deparaffinized in xylene overnight at room temperature. After they were rehydrated in descending alcohols series (100°, 95°,70°, 50°,35°) until tap water for 2 min each, then treated with hydrogen peroxide solution (1:10 in Methanol) to disable endogenous peroxidases for 20 min and after blocked with bovine serum albumin (BSA at 1% in PBS) for 20 min in a humid chamber. BSA is used as a blocker in immunohistochemistry. It was followed incubation with our primary antibodies at 4°C overnight. At the end of primary antibodies incubation, the sections were rinsing with PBS (3 times) and incubated with TRITC-conjugated anti-rabbit secondary antibodies (1:1000) for 1h at 4°C in darkness. Finally, the sections were quickly dehydrate in increasing alcohol (70°, 80°, 95°, 100°), mounting with DAPI (Bioptical) and sealed with rubber cement. The fluorescence, was examined under fluorescence microscope (NIKON ECLIPSE Ci fluorescence microscope) connecting to NIKON DS-Qi2 camera. Images captured showed a red fluorescence due to TRITC-conjugated anti-rabbit secondary antibodies.

PROTOCOL FOR PREPATATION OF SEMITHIN SECTIONS

Semithin sections of testicular tissue were obtained according to the electron microscopy protocol. The testes of the treated and control groups were fixed with 2.5% glutaraldehyde (brand) for 90 min at + 4 °C. After the washes in PBS, the second fixation with osmium trethoxide followed (1%) for 45 min. Washing with PBS and dehydration in ascending alchool (35°-50°-70°-95°-100°), finally propylene oxide. Samples were treated with 1:1 propylene oxide/ resin mixture for 1h on a rotor, after wit 2:1 resin mixture/ propylene oxide overnight. Next day, samples were treated with 100% resin mixture for 30 min then embedded in embedding molds. The polymerizzation of resin was carry out at 60°C for 48hours. Semithin sections (0.85 μm) of testicles were cut using an ultra microtome (Leica Ultracut UCT). They were stained with toluidine blue (Merck) and evaluated under light microscopy (Nikon eclipse E200) connecting with CMOS camera. Semithin sections provide a high-resolution of the morphology section useful to appreciate the structural and morphology of the tissue exanimated.

ELECTRON MICROSCOPY STUDY

For transmission electron microscopy (TEM), the samples embedded in resin were cut to 0.085 µm thick using ultra microtome (Leica Ultracut UCT). Ultrathin sections were collected on 300 mesh copper-rhodium grids, then contrasted in uranyl acetate and lead citrate (Viscuso et al., 2015). Observations were made with Delong Instruments Schottky field emission LVEM25 Low-Voltage Electron Microscope, operating at 25 kV in bright-field TEM. TEM images were recorded by a Zyla Andor sCMOS Camera equipped with a 2560x2160 pixels sensor.

RNA EXTRACTION AND qRT-PCR

The real-time quantitative PCR (qRT-PCR) was used to evaluate the mRNA levels of marker genes in testis. SHBG, PTMA, SRD5A2, CFTR, SOD2 and GPX4B were selected for analysis, and also β-actin as used as an internal standard.

RNA was extracted with MagCore triXact RNA kit according the procedure of production. 2 mg of frozen testis for each experimental groups (1mg/L, 2 mg/L, 4mg/L and control) was transfer into a RNase-free microcentrifuge tube. It was added 400 µl RB Buffer (containing β-ME) and it was used a micropestle to grind the tissue for few minutes. The samples were incubate at room temperature for 5 min and then they were applied to the top of column (Filter Column Set) provided by kit. Centrifuged for 2 min at 13000 rpm. The supernatant was transferred to Sample Tube, then it and DNase I mixture (200µl) were placed into the correct well of T-Rack of the instrument (MagCore Nucleic Acid Extractor). The extraction of RNA in a final volume of 60µl was carried out about in 60min. The amount of RNA was quantified by Qubit 4 Fluorometer (Invitrogen). Reverse transcription was performed immediately with SuperScript III First-Strand Synthesis SuperMix (Invitrogen) kit, briefly 6µl of total RNA was transcribed by SuperScript III/RNaseOUT Enzyme Mix reverse transcriptase. Then cDNA was amplified by qRT-PCR (QuantStudio 1 Real-Time PCR System, ThermoFisher) using suitable primers listed in Table 2. and the following program: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 20 sec, 95°C for 15sec, 60°C for 1 min and 95°C for 15 sec. The relative mRNA expression level was calculated by valute of ΔCt, which is the result of the difference between the level of RNA sample and the β-actin.

Gene	Primers Sequences (5'-3')
SHBG	Forward: GTGCTTTCACTGCGTGATGGC Reverse: TCCCAGGGGGTGCTGAG
SRD5A2	Forward: GCGTACGGACGCTATGTGGA Reverse: GCCTGGCAAACTTTCCGTTG
SOD2	Forward: TGGCCAAGGGTGATGTGACAA Reverse: CACCGCCATTGGGTGACAGA
GPX4B	Forward: TGCAACCAGTTCGGAAAGCA Reverse: GAGCTGCGTCTCCGTTCACA
β-Actina	Forward: GCTGTTTTCCCCTCCATTGTT Reverse: TCCCATGCCAACCATCACT

Tab.2: Listed of Forward (F) and reverse (R) primers used in the gene expression evaluation by RT-qPCR

10.4 *In vitro* experiments on human spermatozoa

10.4.1 Titanium dioxide NP dispersions and dosing to human spermatozoa experiments

The same powders of titanium dioxide nanoparticles (TiO_2-NPs) were used to prepare the solutions for the *in vitro* experiments on human spermatozoa. For starts, the same concentrations of zebrafish experiment were used (1 mg/L, 2 mg/L and 4 mg/L). TiO_2-NPs thanks its photocatalytic activity are used for water purification by xenobiotics (Kanakaraju et al., 2014), for this reason TiO_2-NPs are released into the aquatic environment as a result, the human risk of exposure to nanoparticles is increasing (Karn et al., 2009). Furthermore, TiO_2-NPs are used as additive (E171 or INS171) in foods such as gum, candy and puddings, but also in pharmaceuticals products. In food products was investigated the potential hazardous effects of ingested NPs (Dudefoi et al., 2017b) and the whatever the kind of food, the addition of TiO_2 is limited to 1% of the overall food weight in the United States (USFDA, 2005), while in Europe it is authorized "at quantum satis" levels, which means that although no maximum use level is specified for this additive in Europe, it shall be used in accordance with the good manufacturing practices (GMPs), that is, at a level not higher than is necessary to achieve the intended technical effect (EFSA, 2016). Instead for pharmacological products, the addition of TiO_2-NPs as pigment is not always specify, neither there is an information about its concentration because at present there is no legal obligation to inform consumers. TiO_2-NPs is commonly added in pharmaceutical products such as gelatin capsules, tablet coatings and syrups (Dave, 2008). Medications with this excipient are for example: antibiotics, drug class for skeletal muscle relaxants, gamma-aminobutyric acid analogs, nonsteroidal anti-inflammatory drugs, agents for pulmonary hypertension, narcotic analgesics and impotence agents. For this reason men can be expose to TiO_2-NPs when intake these drug, especially if they overuse it for impotence disorder and they want

improve their ability of fertilization. Although there aren't suggestions about concentrations of TiO_2-NPs into this drugs, it have been investigated their toxic effects on human spermatozoa. Therefore, due to the lack of indications about its concentration in drugs, following concentrations: 50 ppm, 100 ppm, 250 ppm and 500 ppm were selected. Dudefoi and Colleagues (2017b), have shown that 100ppm and 250 ppm are found in the human intestine after sampling 1-2 pieces of gum or candy, then we have hypothesized that similar concentration could arrive to the testis, because nanoparticles via blood circulation can pass through the blood–testis barrier (BTB) and exert their toxic actions on spermatogenesis (Hussein et al., 2016), in addition they can cause changes in sperm morphology, decrease the number and viability of sperm (Gao et al., 2013; Song et al., 2017; Karimi et al., 2019). Additionally, to 100 ppm and 250 ppm, we have tested the 50 ppm and 500 ppm concentrations, which were respectively the half of 100 ppm and the twice of 250 ppm. All work solutions were prepared by dispersing the powders of TiO_2-NPs in Sperm Medium (Gems) with 4 cycle of sonication for 10 min and 5 min of break, using a probe sonicator (Bandelin Sonoplus). In this way was ensure homogeneous dispersion of titanium dioxide nanoparticles (Salou et al., 2020).

10.4.2 Exposure procedure

All the experiments were conducted under good laboratory practice conditions, according to WHO guidelines 2021 and Organization for Economic Co-operation and Development guideline (WHO, 2021; OECD, 2014). They were selected ejaculated with a good semen parameter, that were within the reference values of noormozoospermia according to WHO (2021) (Table 3).

Sperm parameters	
Volume (ml)	1,5 ml
pH	> 7,2
Sperm concentration (10^6 sperm/ml)	> 15 mil
Motility Progressive	> 32%
Motility Non Progressive	15%
Immobile	13 %
Vitality	> 58%

Tab.3: Reference values of noormozoospermic samples

The ejaculates were collected from healthy fertile men (between 20-39 years) by masturbation after 3–5 days of recommended abstinence. The subjects were recruited from center of P.M.A. (Procreazione Medicalmente Assistita) MEDI.SAN "Clinica del Mediterraneo di Ragusa" (RG), after their consent for inclusion of samples in research experiments according to guidelines established for

research on the human (General Assembly of the World Medical Association, 2014). The collected ejaculate was allowed to liquefy in an incubator at 37°C.

After liquefaction within 30 minutes of collection and no later than 60 minutes after collection, the examination of semen volume, pH, sperm concentration, viability, motility and morphology was started according to the WHO 2021 guidelines.. Each sample included in our study were aliquoted for allow a good washing. During the sperm washing process sperm is separated from the seminal fluid. Briefly, in each aliquots ($5x10^6$ sperm/ml) in ratio 1:1 was added Sperm Wash Medium (Giems), which is specifically designed for *in vitro* human sperm and embryo culture. Samples were centrifugated for 10 min at 1500rpm. The supernatants were discarded, then 500 µL of work solution's suspensions were carefully disposed on each pellet. Each pellet were incubated with eppendorf inclined of 45° and the cap open, to perform swim-up by pellet. Incubation was performed at 37°C in a cell culture incubator (Celis) with 5% CO_2. In a preliminary study, we used the following suspensions to treated sperm pellet: 1mg/L, 2mg/L and 4mg/L. They were the same concentrations used to the experiments *in vivo* on zebrafish. Later, as mentioned above, we assessed the 50 ppm, 100 ppm, 250 ppm and 500 ppm of NPs-TiO_2. In all experiments, an aliquot of control (sperm pellet incubated only with sperm medium) was included. Time exposure was 1 hour, after which we analyzed the supernantants containing motile-selected spermatozoa (Préaubert et al., 2018). All the experiments were done in triplicate and we analyzed 3 replicate slides from each experiment. Sperm parameters as motility, viability, DNA damage, intracellular ROS levels were evaluate at the end of exposure, moreover we investigated if the exposure to NPs-TiO_2 induced biomarker expression by spermatozoa to define the action of nanoparticles like endocrine disruptors.

SPERM MOTILITY

Sperm motility was assessed by observing under an optical microscope (Leica DMLB) 10 µl of each group's treated with TiO_2-NPs and control on a slide (WHO, 2021) to distinguish spermatozoa with progressive, non-progressive and immotile motility. We counted 100 spermatozoa for slides to define the percentage of progressive, non-progressive and immotile spermatozoa.

SPERM VITALITY

The vitality of sperms was observed by Eosin dye exclusion method. This method is based on the principle that damaged plasma membranes allow entry of stain (eosin) for this reason dead sperms will be red-stained, while live sperm will be unstained because their intact plasma membranes not allow entry of stain (WHO, 2021). For each experimental group, we mixed a drop of semen sample 10µl with 10µl of 0.5% Eosin Y solution (Bio Optica) and after 30 second we observed the preparations at ×400 under a light microscope (Set E200 NIKON). We counted 100 spermatozoa for slides to define the percentage of live (unstained) and dead (red-stained) spermatozoa.

ASSESSMENT OF DNA FRAGMENTATION

Sperm Chromatin Dispersion Test (SCD)

The SCD test is a non-automated and subjective method, that offers a practicable and reliable option (Grèze et al., 2019). This test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with non-fragmented DNA, following acid denaturation and removal of nuclear proteins (Fernández et al., 2003). The SCD test was performed using the Halosperm® kit based on the manufacturer's protocol (Halotech DNA, Spain). The Eppendorf tubes of low-melting point agarose (50 µL) provided in the kit were placed in a water bath at 90–100 °C for 5 min. Then 25 µL of each sperm sample was quickly added at the melted agarose, to careful mixed. The slides were pipetted with 25 µL of the cellular suspensions, immediately covered (22 × 22 mm coverslip), then held at 4 °C for 5 min. Once the gel formed with the spermatozoa embedded inside, the coverslips were gently removed and the Denaturation solution (DA-soltuion) provided in the kit was applied for 7min at room temperature. The slides were then placed in the Lysing solution (Triton X-100, Dithiothreitol) for 25min, and washed with distilled water for 5 min at room temperature (Grezè et al., 2019). After dehydration by successive increasing concentrations of ethanol (70, 90 and 95%), the slides were dried and readied for bright-field microscopy by staining Diff-Quik. The slides were stored in the dark at room temperature, then we observed the slides under light-microscopy with 1000X magnification.

TUNEL assay

The TUNEL assay is the most widely used *in situ* test for the study of sperm DNA fragmentation. It is based on the incorporation of modified deoxyuridine triphosphate (dUTPs) by the enzyme terminal deoxynucleotidyl transferase (TdT) at the 3'-OH end of fragmented DNA. These dUTPs are directly conjugated to a fluorescent dye (fluorescein-dUTP), then can be detected directly using fluorescence microscope. In this way, the breaks present in DNA are label and then can be quantify. The

Spermatozoa ($2x10^6$ sperm/ml) of each experimental groups were smeared on glass slides, fixed with 4% paraformaldehyde for 1 hour then permeabilized in Triton (0.25% in PBS) for 20 minutes at room temperature. The glass slides were washed twice with deionized water, then the assay TUNEl was performed according to the manufacturer's protocol. The Click-iT® Plus TUNEL kit (ThermoFisher Scientific) was used. At the end of protocol, the slides were observed under fluorescence microscope (NIKON ECLIPSE Ci fluorescence) and at least 200 spermatozoa were scored as recommended by WHO (WHO, 2021). Moreover, pictures were made through camera (NIKON DS-Qi2) connected to microscope.

EVALUATION OF INTRACELLULAR ROS LEVELS

Oxidative stress (OS) is one of the major causes of male infertility. It occurs when production of reactive oxygen species (ROS) outweighs the concentration of antioxidants in human semen (Agarwal and Bui, 2017). Evaluation of intracellular ROS levels was performed with help of DCF protocol. It was used the procedure of Santonostaso et Colleagues (2019). 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA) (Sigma Aldrech) at concentration of 130 µM was diluted in150 µL of each aliquot treated, to obtain the final concentration of 13 µM. It was following an incubation at 37 ° C for 30 minutes in the dark, and then the samples were washing with PBS and counterstained with DAPI solution for 5 minute. The spermatozoa were put on a glass slide and observed under fluorescence microscope (NIKON ECLIPSE Ci fluorescence). Intracellular ROS levels were quantified as the percentage of sperm cells exhibiting a response (green halo) on total spermatozoa. Additionally, thanks to the MiOXSYS System it was measured the oxidation-reduction potential (ORP) to detect the Oxidative stress (OS). It a way to evaluate directly the redox balance between ROS and antioxidants. We carried out the instructions of use, then we have used disposable test sensors, that are inserted into a galvanostatic analyzer of instrument. 30 µl of semen sample was applied onto the sample port of sensor, then we waiting for a few minutes to receive the result. The ORP value appears on the analyzer display screen as millivolts (mV), which parallels the degree of OS. A higher ORP corresponds to higher levels of oxidant activity (Kohen and Nyska, 2002). Value ORP was expressed as mean value for each experiment.

MARKER IDENTIFICATION ON SPERMATOZOA

To evaluate the possible toxicity of TiO_2-NPs on the sperm cells, Metallothioneins and Hsp70 biomarkers were determined after exposing to TiO_2-NPs and compared with the control. Moreover, it was investigated SHBG biomarker to highlight the probable action of TiO_2-NPs as endocrine disruptor (for details about antibodies see paragraph 10.3 Immunohistochemical analyses). Sperm samples diluted ($2x10^6$ cells/mL) in PBS were fixed in glutaraldehyde 1% (v/v) at room temperature

for 10 min. Cells were centrifugated at 2000 g for 10 min, and the supernatant was discarded. The pellet was resuspended in PBS for washing (2 time for 5 minutes), then forty microliters of cell suspension was placed onto polylysinated slides (Dako) and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min. Afterward, the cells were washed two times with PBS and covered with 5% blocking solution (BSA) for 20 min in a wet chamber. Slides were rewashed in PBS and incubated with the primary antibody anti-MT, anti-Hsp70 and anti-SHBG at 4 °C overnight in a wet chamber. At the end of incubation with primary antibodies, the slides were washed three times with PBS and incubated with the secondary antibody for 1 hour at 4 °C in a wet chamber. After rinsing in PBS two times, the samples were mounted with DAPI (Abcam) and sealed with rubber cement to preserve cell fluorescence. Cells were visualized with a Nikon ECLIPSE Ci fluorescence. All samples were processed in duplicate, and at least 150 spermatozoa were scored per slide.

SCANNING ELECTRON MICROSCOPY

To evaluate the presence of TiO_2-NPs on the surface of spermatozoa, we carried out scanning microscopy protocol. Samples were centrifugated at 2000 rpm for 3 min, and the pellet were collected to be fixed in glutaraldehyde (2,5%) for 1 hour at 4°C.

Fixed sperm pellets were then washed two times for 5 min each in PBS and then dehydrated in an increasing gradient of ethanol 35°-50°-70°-80°-95°-100° each for 2 minutes. Finally 1:1 (alchool 100°: hexamethyldisilazane, $C_6H_{19}NSi_2$, Merck) and only hexamethyldisilazane for 1 minutes. The pellet was put on SEM stubs, to be metallized. The observation was carried out with SEM–energy-dispersive Xray (EDX) analysis (Cambridge Stereoscan 360C Instruments, EDX, INCA, Oxford).

STATISTICAL ANALYSIS

Statistical analysis was carried out by one-way analysis of variance (ANOVA) test to comparison difference between groups. A $p < 0.05$ was considered to be a statistically significant difference.

11. Result and Discussion

11.1 Embryonic development of zebrafish

The toxicity of TiO_2-NPs on zebrafish embryos was defined by observing specific toxicological endpoints, as mentioned. Compared to unexposed embryos, the number of coagulated eggs was increased already at 24hpf for the concentration of 2 m/L of TiO_2-NPs, and then they remain unchanged. Figure 4, shows the rate of coagulated eggs at 24hpf and 48hpf, because it was similar until the end of experiment.

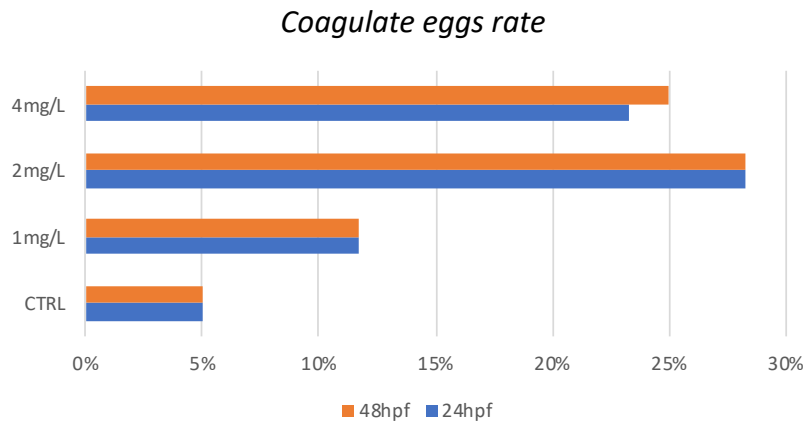

Fig.4: Coagulated eggs rate of embryos following aqueous exposure to TiO_2-NPs at 24hpf and 48hpf. The coagulated rates in all experimental groups were statistically higher than unexposed (analysis of variance, $p < 0.05$).

At 24hpf and all the exposure period, TiO_2-NPs- were evidently deposited on the bottom of the wells. As reported in other studies, the phenomenon of aggregation involve many NPs including TiO_2 (Drobne et al., 2009, Zhu et al., 2009b,Chen et al., 2011b) and the time of precipitation can be quickly, for example Zhu and Colleagues (2009b) showed that more than 80% ZnO-NPs, quickly sank after dosing and the aggregates almost entirely settled on the bottom of the wells after 48h. However, TiO_2-NPs also adhered on the surface of embryonic chorion with increasing concentrations of TiO_2-NPs, as shown in Figure 5.

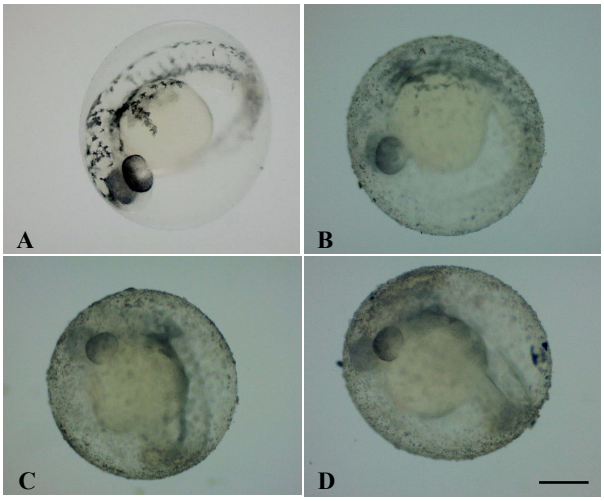

Fig.5: TiO_2-NPs on the surface of embryonic chorion, the increase of the aggregations is concentration (TiO_2-NPs) and time-dependent. **A)** Zebrafish embryo unexposed to TiO_2-NPs. **B)** Zebrafish embryo exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish embryo exposed to 2mg/L TiO_2- NPs. **D)** Zebrafish embryo exposed to 4mg/L TiO_2- NPs. Scale bar 410 µm

Although the accumulation of TiO_2-NPs being dependent on nanoparticles type and size, zebrafish chorion is a special biological structure that covers the embryo until hatching. It has a protective effect against pollutants, because it is known that an increase of embryo's sensitivity to exogenous compounds occur after chorion removal (van Pomeren et al., 2017; Liegertová et al., 2018; Vranic et al., 2019). Therefore, the chorion is similar a barrier which block the entry of various pollutants (Kristofco et al., 2018), but simultaneously thanks your pore the chorion allows the transport of necessary oxygen, salt ions, nutrients from the aquatic environment to the embryo and excretion of waste in the opposite direction (Rawson et al., 2001; Sun et al., 2010). Scanning-electron microscopy has shown that the pore has a diameters between 300 nm and 1 micron (0.5 µm), thus a variety of NPs adhering to the chorion can enter the embryo through these channels (Rawson et al., 2001; Fent et al., 2010). Pitt andColleagues (2018) have investigate the toxicological effects of NPs with small size that can penetrate the chorions, single Ag nanoparticles (5–46 nm and 11.3 ± 2.3 nm) enter the chorionic space of the embryo by Brownian diffusion (Lee et al., 2007; Lee et al., 2012b), while SWCNT agglomerates are too large to cross the canals (Cheng et al., 2007). NPs diffuse may be toxic to embryo development during the period of organogenesis (Cheng et al., 2007), however there is scant literature about the interaction of NPs with the chorion, and how this structure interacts and affects the absorption, accumulation and distribution of nanoparticles in the embryos (Pereira et al., 2019). Although it was evident the sedimentation of TiO_2-NPs during the experimentation, the embryos and larvae were constantly exposed to the TiO_2-NPs aggregates because the animals were mostly located on the bottom of the wells, also after the hatching when they can freely swim. The TiO_2-NPs formed an external white layer on the chorion, that not has affected the hatching of embryos. Hatching of the larvae began at 48hpf, in particular an high rate of hatching occurred for

the embryos exposed to TiO_2-NPs compared to control groups (Figure 6). Hatching rate was statistically different between exposed groups and unexposed group ($p< 0,005$).

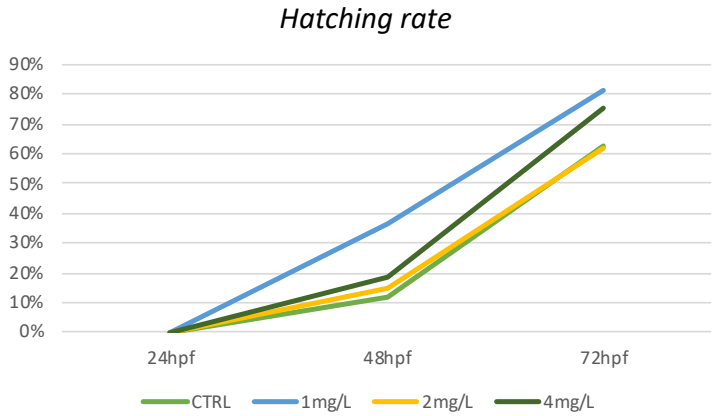

Fig.6: Hatching rate of embryo following aqueous exposure to TiO_2-NPs from 24hpf to 72hpf. The hatching rates in all treated groups were statistically higher than unexposed groups (analysis of variance, $p < 0.05$).

Several studies have shown that TiO_2-NPs could cause premature hatching in a dose-dependent manner (Clemente et al., 2014; Samaee et al., 2015), furthermore the exposure to TiO_2-NPs at the concentrations up to 500 mg/L (for 96h) did not affect hatching rate as showed by Zhu andColleagues (2008). After 96h of exposure to TiO_2-NPs, the survival of the hatched embryos for control was up above 90% as describe by Kimmel and Colleagues (1995), while the survival of exposed groups was below 90% but remained unchanged until the end of experimentation (Figure 7).

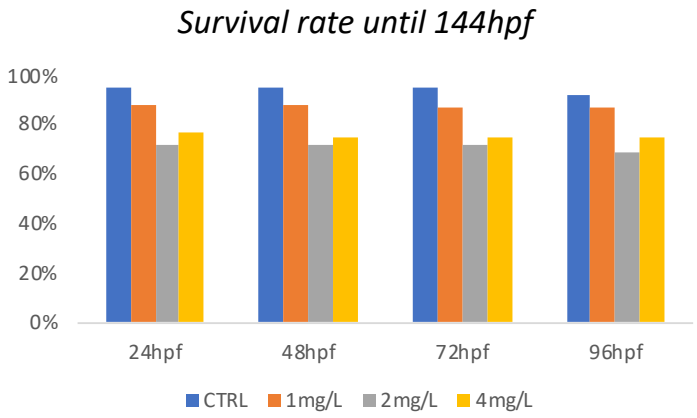

Fig.7: Survival rate of embryos following aqueous exposure to TiO_2-NPs until 144hpf. Untreated group has a statistically survival rate higher than exposed groups (analysis of variance, $p < 0.05$).

Not statistically significant increase in mortality rates for groups exposed to 2mg/L and 4mg/LNPs-TiO_2 relative to the control treatments were observed ($p <0,005$). The maximum mortality was 25% for the entire test period (144 hpf) for the 4mg/L concentration ($p <0,005$). TiO_2-NPs has low acute toxicity to fish survival (Federici et al., 2007) and TiO_2-NPs concentrations higher than our

experimental groups not affected survival rate in zebrafish (Chen et al., 2011). In addition, groups control showed normal development (Kimmel et al., 1995) as well as the exposed groups a low dose (1mg/L) of TiO_2-NPs (Wang et al., 2014a). All embryos have showed a complete development of head, notochorda, fin, pigmentation and the organs's heart and eyes. There were no morphological malformation compared to control group (Figure 8).

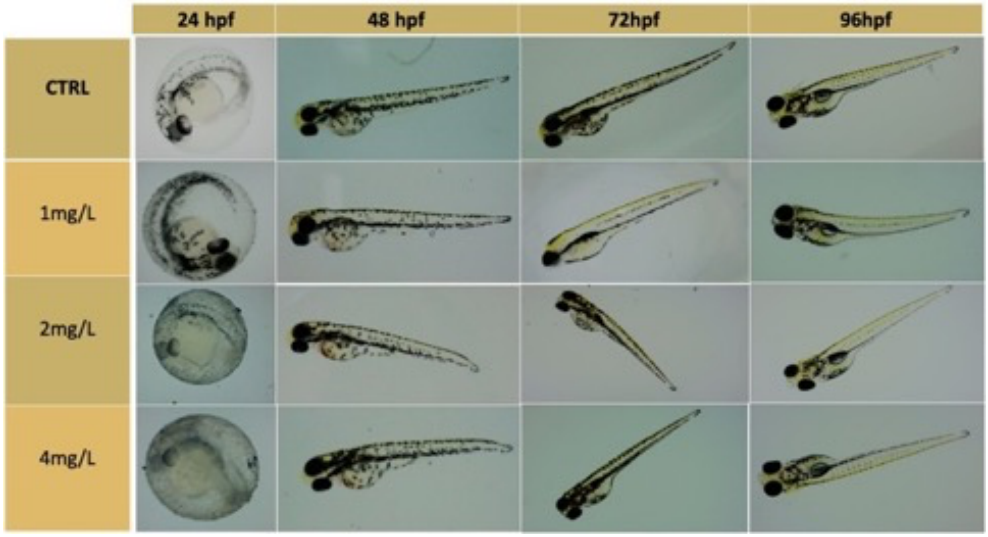

Fig.8: Phenotypes of larvae exposed to TiO_2-NPs and unexposed group from 24 to 96 hpf.

However, through the DanioScope software was showed that TiO_2-NPs affected the body length of larvae, because they exhibited reduced body length compared to the controls ($p<0,05$) at 96 hpf. The mean of body length in exposed group was 172 µm, while in unexposed group was 215 µm (Figure 9A). Our data are in accord to other studies of nanoparticles toxicity; change in the body length was observed at 1 mg ml^{-1} concentration for three different types of NM-TiO_2 by Faria and Collegues (2014), whereas Bai andColleagues (2010b) have shown that nano-ZnO (>25 mg/L) induced a more severe decrease in body length. Instead, the size eyes was the same in all experimental groups. Heart rate variability is of the utmost significance parameter for the study of cardiac function. In zebrafish, physiologically the heart rate is around 120-180 bpm, but its alteration is associate to cardiotoxicity (Gu et al., 2020; Wei et al., 2021). Thanks to the DanioScope software, it was highlighted an increase of heart rate in embryos exposed. The heart rate was measured trough the registration of beats per minute (BPM). An increase of BPM was observed already in the 1mg/L group and an higher BPM appeared in 4 mg/L groups as shown in figure 9B. Cardiotoxicity effects was also showed by exposure to other nanoparticles. Johnson and Collegues (2007) have highlighted that embryos treat with high concentration of copper (93, 327 and 464 gCu/L) had the fastest heart rates at 28 hpf suggesting that stress responses were induced in the embryos during the exposure. Silver-nanoparticle exposure have

caused development defects with abnormal cardiac morphology, pericardial edema and circulation defects (Asharani et al., 2011). Moreover, it was observed an accumulation of gold, silver, and platinum nanoparticles during development with serious threat to the organism (Chakraborty et al., 2016). Our data are in accordance to the literature. The exposure to TiO_2-NPs resulted in statistically significant increase of heart rates in embryos exposed ($p < 0.05$).

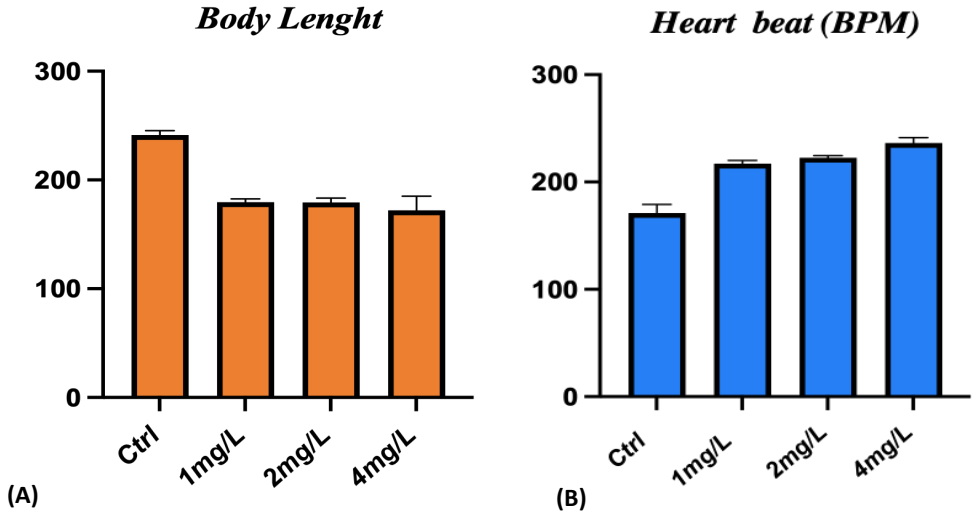

Fig.9: (A) Body length and **(B)** beats per minute (BPM) of larvae exposed to TiO2-NPs and unexposed indicated as mean with standard deviation.

After hatching, for our experiment already at 48 hpf, zebrafish embryos have lost the chorion, their protective barrier and they become susceptible to exposure to NPs during the larval period. Therefore, nanoparticle may cause serious biological impacts that will effect on adult zebrafish. For example, absorbed NPs can be transported to the gastrointestinal tract, heart, brain, yolk and liver (Chen et al., 2017c; Pitt et al., 2018), however bioaccumulation and distribution of NPs depend on the developmental stage of the zebrafish, as well as different biomarkers expressed by embryos under toxicological conditions. The immunohistochemical investigation confirm the susceptibly of TiO_2-NPs to induce the production of ROS (Federici et al., 2007) which enhance oxidative stress and oxidative damage to macromolecules such as proteins, DNA and lipids, finally leading to the damage of different cellular organelles (Sabatini et al., 2009; Ramsden et al., 2013; Xiong et al., 2011). Generally, ROS is the collective noun used to describe a number of reactive molecules and free radicals that are formed during normal mitochondrial oxidative metabolism (Ogrunc, 2014) and they are considered as the metabolic by-products of biological systems (Pizzino et al., 2017). The production of ROS is in dynamic equilibrium with the elimination of ROS under normal physiological conditions (Liu et al., 2014), however the ROS are generated in response to exogenous substances. Over production of ROS often occurs when the antioxidant defense system is not sufficient to

counteract excessive ROS (Ray et al., 2012). Therefore, the change of this indicator is an important pathway to highlight the toxicity of many pollutants. The production of ROS has long been regarded as a mechanism of nanoparticles induced toxicity (Xiong et al., 2011), it is associated to their small size and high specific surface area (Lovern and Klaper, 2006) that make NPs more reactive than their counterparts bulk (Carlson et al., 2008, Ispas et al., 2009, Mironava et al., 2010). Through DCFH2-DA assay, the probe DCFH 2-DA diffuses and crosses the cell membrane and it change into DCFH-2 by action of intracellular esterase. DCFH-2 is membrane-impermeable and reacts with intracellular ROS to give the fluorescent compound DCF measured by fluorescent microscopy (Chen et al., 2010). Fluorescence intensity of ROS staining was calculated using Image J. Compared with the control group, ROS content increased significantly in the groups exposed at higher concentration of TiO_2-NPs (4mg/L). Our results are in accordance to data in literature. Previous studies have shown that the exposure of zebrafish embryos to ZnO-NPs induced an excessive production of ROS which active the apoptosis pathway mediated by mitochondria and caspases (Zhao et al., 2016), also CuO-NPs induct the production of ROS in embryo larvae (Kumari et al., 2017), whereas for TiO_2-NPs was found that induced oxidative stress in the brains of rainbow trout (*Oncorhynchus mykiss*) (Federici et al., 2007). *In vivo* studies on zebrafish larvae have shown the citrate-functionalized TiO_2 nanoparticles induce production of ROS with an inverse relationship between particle size and toxicity, smaller citrate-functionalized TiO_2 nanoparticles (6nm) have generated higher levels of ROS (Kim et al., 2014b), moreover in a comparative study between TiO_2-NPs and AgNPs, it was observe that TiO_2 nanoparticles under simulated solar light (SSL) exposure induced more ROS than the Ag nanoparticles (George et al., 2014). The presence of ROS have induce the DNA damage, and then the Poly (ADP-ribose) polymerase-1 (PARP1) as shown by immunohistochemical analysis (Figure 10). A positivity was observed already at the lower concentration (1mg/L), and increased at the higher concentration (4mg/L) compared to the control.

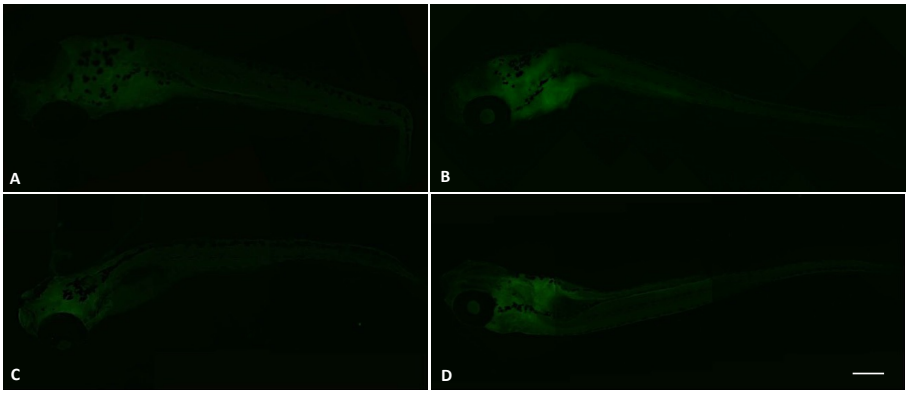

Fig.10: PARP-1 antibody-staining of the 144-hpf zebrafish larvae after exposure to TiO_2-NPs including untreated zebrafish larvae. **A)** Zebrafish embryo unexposed to TiO_2-NPs. **B)** Zebrafish embryo exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish embryo exposed to 2mg/L TiO_2- NPs. **D)** Zebrafish embryo exposed to 4mg/L TiO_2-NPs. Scale bar 420 µm

Poly (ADP-Ribose) Polymerase (PARP) is a group of proteins involved in DNA damage repair (Noordermeer and van Attikum, 2019), in particular PARP-1 is the most abundant and ubiquitous member of this superfamily of proteins. In mammals, PARP-1 controls a widely cellular processes, such as DNA repair, transcription, cell division, heat shock response etc. (Ko and Ren, 2012; Zaalishvili et al., 2012). PARP1 is known as a "guardian of genome integrity" due to its involvement in regulation of multiple DNA repair mechanisms (Malanga and Althaus, 2005; Vidaković et al., 2005). It is especially implicated in single strand break (SSB) repair (Cerbinskaite et al., 2012). PARP1 binds DNA and synthesizes Poly (ADP-Ribose) chains in a process called PARylation, as a result other proteins involved in SSB repair will be recruited and the damage will be repaired and cell viability will be maintained. Although PARP-1 activation is necessary for the elimination of DNA breaks, an overactivation of PARP-1 observed during irreversible DNA damage conditions may activate cellular death pathways (Andrabi et al., 2006). The role of PARP-1 has been extensively studied in higher vertebrates, while it is less explore in lower vertebrates including zebrafish. However, according to the recent results PARP inhibition in DMS treated embryos larvae dramatically increased both the mortality and the DNA damage level, thus like in mammalian the PARP-1 is involved in the repair of DNA damage induced by alkylating agents (De Murcia et al., 1997; Heacock et al., 2010; Kondo et al., 2010). Therefore, in the light of our results, the involvement of PARP-1 in the repair of DNA damage by nanoparticles cannot be excluded.

Moreover, oxidative stress by nanoparticles have induced the expression of Heat Shock Proteins 70 (HSP70) (Figure 11).

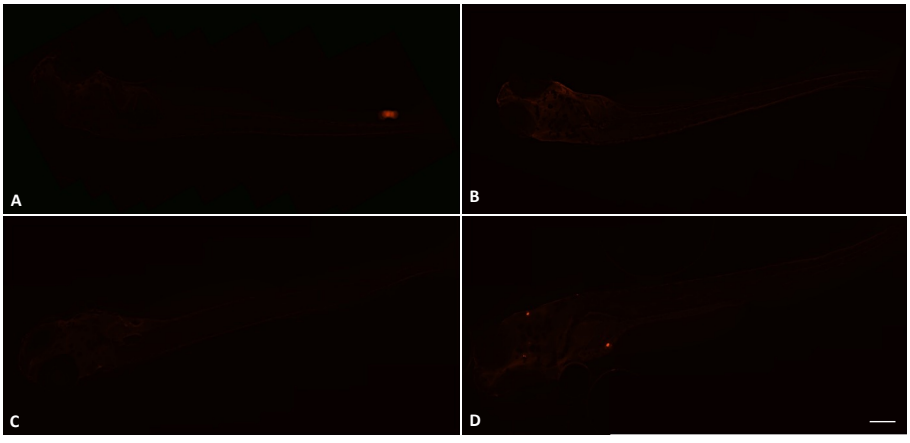

Fig.11: Hsp70 antibody-staining of the 144-hpf zebrafish larvae after exposure to TiO_2-NPs including untreated zebrafish larvae. **A)** Zebrafish embryo unexposed to TiO_2-NPs. **B)** Zebrafish embryo exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish embryo exposed to 2mg/L TiO_2-NPs. **D)** Zebrafish embryo exposed to 4mg/L TiO_2-NPs. Scale bar 420 µm

While a lower expression for cytochrome P540 was observed, in the head of the larvae. Heat Shock Proteins (HSP70) are a several families of highly conserved proteins whose expression is upregulated

in response to an environmental stressors, including heat shock, alcohol, heavy metals, and oxidative stress. They are associated with protein synthesis, folding and translocation, and assembly of larger protein complexes, all of which can be impaired by stress (Li et al., 2015), in particular Hsp70 is the major heat shock proteins, which expression increases in response to environmental and physiological stressors (Lindquist and Craig, 1988) and protect the cells against induction of cell death by a variety of stresses. In this regard, our results suggest that TiO_2-NPs are stressful stimuli for zebrafish embryos. Similar evidences are shown in investigation of Chitosan nanoparticles and ZnO (Hu et al., 2011), and on transition metals oxide (CuO, ZnO, NiO, and Co_3O_4) which have induced increased expression of the heat shock protein 70 (Lin et al., 2011). As regards metallothioneins (MTs), positivity was found in the expression of this biomarker in the whole body of the embryo with the exception of the end of the tail (Figure12).

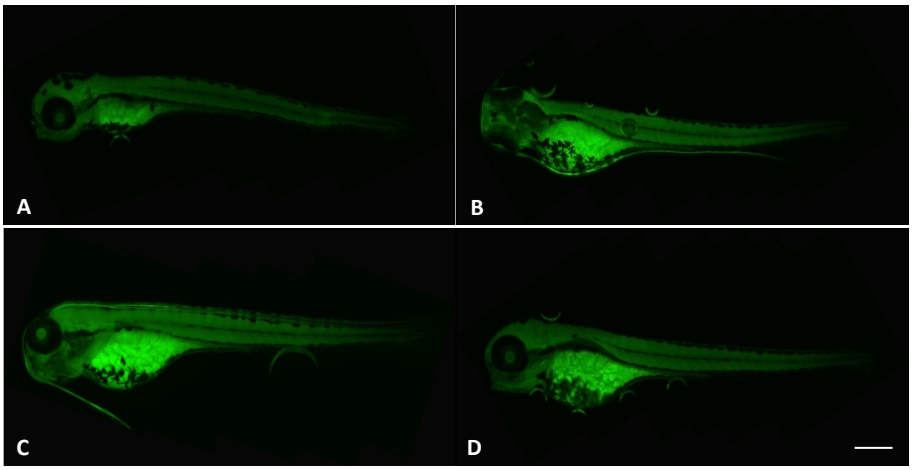

Fig.12: MT antibody-staining of the 144-hpf zebrafish larvae after exposure to TiO_2-NPs including untreated zebrafish larvae. **A)** Zebrafish embryo unexposed to TiO_2-NPs. **B)** Zebrafish embryo exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish embryo exposed to 2mg/L TiO_2-NPs. **D)** Zebrafish embryo exposed to 4mg/L TiO_2-NPs. Scale bar 420 µm

Metallothioneins (MT) are biomarkers widely used in environmental monitoring programs for aquatic creatures. They are involved in homeostasis, protection against heavy metals and oxidant damages, and metabolic regulation, sequestration and/or redox control (Dziegiel, 2004). Generally, the MT expression level is dose-dependent on heavy metals. However, the response of MT to metals is not positively correlated when the amount of metals overdose (Walker et al., 2014). In addition, metallothionein gene expression is regulated by oxidative stress (Glen, 1999) as consequence synthesis of MTs could be regarded as a step of oxidative stress, thus the organisms with the induction of MTs could tolerate oxidation stress better, because MTs have effects of removing hydroxyl ($OH^{\cdot}$) and super oxide (O_2^{-}) radicals (Amiard et al., 2006). These evidence, further highlights that TiO_2-NPs are oxidative stress inducers in zebrafish embryos; previous studies have shown the positivity of MTs in zebrafish embryos exposed to AuNPs (Brundo et al., 2016). However, at the light of our

results namely no death until the end of experimentation (144hpf), not malformations and expression of marker mentioned, it is evident that the larvae zebrafish with their nature detoxification pathways are able to resist at the presence of toxic substances and then they can tolerate the presence of metal concentrations. Expression of biomarker are confirmed by Image J software. A statistically significant difference ($p < 0,005$) at higher concentration of MTs biomarker in the larvae exposed compared to control was found. As regards, SHBG and PTMA a positivity of both was observed. In particular for SHBG, a positivity was observed on the head of embryo, with a higher expression for the 4mg/L TiO_2-NPs concentration compared to control (Figure 13).

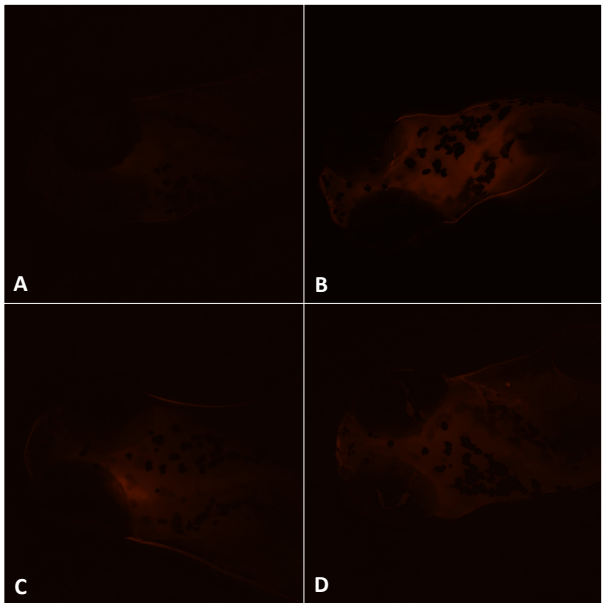

Fig.13: SHBG antibody-staining of the 144-hpf zebrafish larvae after exposure to TiO_2-NPs including untreated zebrafish larva. Fluorescence is visible on the head of larvae. **A)** Zebrafish larva unexposed to TiO_2-NPs. **B)** Zebrafish larva exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish larva exposed to 2mg/L TiO_2-NPs. **D)** Zebrafish larva exposed to 4mg/L TiO_2-NPs.

SHBG is a protein capable of binding steroid in the blood of fish and other Vertebrate species. It is well characterized in human (Lin et al., 2010; Hammond, 2011), but only one ortholog (shbg) has been so far identified in zebrafish being expressed in the digestive tract, liver, gills, pancreas, and testis (Miguel-Queralt and Hammond, 2008; Bobe et al., 2010). Besides to sexsteroid transportation, regulation, and action (Bobe et al., 2010), SHBG has shown affinity to synthetic steroids such as ethinylestradiolor gestagens (Miguel-Queralt et al., 2004; Miguel-Queralt and Hammond, 2008) and in addition has been reported its bind with phthalates and other environmental contaminants (Dechaud et al., 1999; Hodgert et al., 2000; Hong et al., 2015b). Phthalate plasticizers, bisphenol A, octylphenol and nonylphenol are able to bind with SHBG (Sheikh et al., 2016a, 2016b). In this regard, SHBG is a potential target for environmental compounds found into the body. As consequence increase the risk of potential disruption in the steroid homeostasis. Human SHBG is synthesized in liver (Hammond, 2011; Laurent and Vanderschueren, 2014) and it transports sex steroids, mainly DHT,

testosterone, and estradiol in blood and regulates their metabolic clearance and availability to the target tissues (Anderson, 1974; Hammond, 2011). Dysfunctions and abnormal plasma concentrations of SHBG has been suggested to cause adverse effect reproductive such as infertility, ovarian dysfunction, endometrial cancer (Cherkasov et al., 2005), moreover SHBG has an important role during the development. Experimental exposure to phthalate in rodents has shown adverse effects due to deregulation of metabolic pathways by phthalate compounds (Desvergne et al., 2009). In fish there are scant knowledge about its structure and the site(s) of expression, although studies have highlighted that during the development of zebrafish, SHBG mRNA first appears within the liver and gut (Miguel-Queralt et al., 2004). In addition, studies have shown SHBG affinity xenobiotics further to endogenous sex steroids (testosterone and estradiol). Chen and Collogues (2018) have found an increase of SHBG in zebrafish larvae exposed to Bisphenol AF (BPAF), which is recognized as endocrine disruptor. Therefore the positivity to SHBG in our investigation suggest that similar to Bisphenol AF (BPAF) TiO_2-NPs act like endocrine disruptor. Whereas, the positivity of Prothymosin α (PTMA) was occur especially at the concentration of 4 mg/L (figure 14).

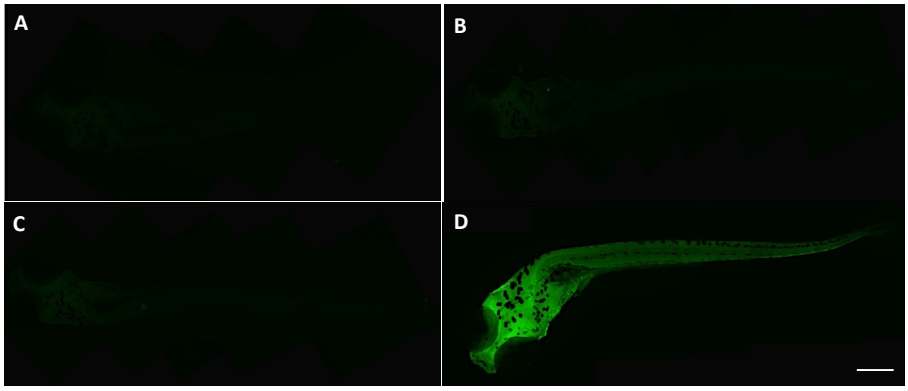

Fig.14: PTMA antibody-staining of the 144-hpf zebrafish larvae after exposure to TiO2-NPs including untreated zebrafish larvae. **A)** Zebrafish embryo unexposed to TiO_2-NPs. **B)** Zebrafish embryo exposed to 1mg/L TiO_2-NPs. **C)** Zebrafish embryo exposed to 2mg/L TiO_2-NPs. **D)** Zebrafish embryo exposed to 4mg/L TiO_2-NPs. Scale bar 420 µm

Prothymosin α (Ptma) is a small nuclear protein (109–113 amino acids depending on the species) containing more than 50% acidic amino acid residues (glutammic and aspartic) (Haritos et al.,1984) then it is considered the most acidic polypeptide in eukaryotes. It is considered a nuclear protein with a potent nuclear localization signal (NLS), although data show cytoplasmic and extracellular presence as well, under specific physiological or pathological conditions (Haritos et al., 1984; Dosil et al., 1990). Its intracellular roles are associated with cell proliferation and the cell survival. PTMA stimulates cell proliferation by sequestering a repressor of estrogen receptor activity in various cells (Martini et al., 2000), while in cell survival PTMA is able to binds Keap1 to dissociate Keap1-Nrf2 complex thus upregulating the expression of Nrf2 dependent anti-oxidative stress genes (Karapetian et al., 2005). PTMA was originally isolated from rat thymus, however, it has been identified and

studied in other Vertebrates, such as Amphibians (*Rana esculenta*; Aniello et al., 2002; De Rienzo et al., 2002), Chondroitties (*Torpedo marmorata;* Prisco et al., 2009) and the Teleostei (*Danio rerio*; Donizetti et al., 2008). In zebrafish, was found that gene ptma is duplicated: ptmaa and ptmab. Only the ptmab transcript is observed at 4 and 8 hpf of development in all embryonic cells, whereas both genes are expressed at later stages. Both are expressed in the same territories, but only the ptmaa transcript was found in the trigeminal ganglion and in endodermal pouches. In the eyes, at 72 hpf, the ptmaa and ptmab transcripts were found in amacrine cells, whereas only the ptmab transcript appeared in horizontal cells (Donizzetti et al., 2008). These evidence, highlight their function in cell proliferation and differentiation. Moreover, it was observed that PTMA expression levels vary following stimulation by estrogen, thus as well as SHBG expression, the positivity increment of PTMA in embryo zebrafish in our experimentation suggest that TiO_2-NPs act a like endocrine disruption.

11.2 Adult exposure

Daily monitoring until the end of experimentation, revealed no fish died, no abnormal behaviors like loss of equilibrium, refusal to feed and no apparent abnormalities in the body of fish. The target of our investigation has been the testis because it is know that reproductive organs are sensitive to stress such as heavy metals, pesticides, chemical compounds but also microwaves and recently the nanoparticles (Nishi and Hundal, 2013; Mehrpour et al., 2014; Rzymski et al., 2015). Reproductive toxicity is increasingly as an important part of overall toxicity, because is growing the concerns about the sensitivity of reproductive and developmental outcomes to toxic substances (Hougaard and Campagnolo, 2012). However, a large discrepancy there is currently between the number of chemicals in commerce and the number that have been evaluated for reproductive toxicity. This aspect for the nanoparticles or the nanomaterials in general is even graver. Downsizing and engineering of materials convey physical and chemical qualities that differ from that of the corresponding bulk material, thus the toxicity of nanoparticles might not be adequately assessed.

For example, the nanoparticles thanks to their nananoscale size are able to cross the blood-testes and also blood-brain barriers to accumulate in these organs (De Jong et al., 2008; Lankveld et al., 2010). Consequently, there is increased incidence of male reproductive diseases may be partly attributable to nanoparticles exposure. Among the metal oxide nanoparticles, TiO_2-NPs has received more attention due to its toxic effect on health.

The animal models used to evaluate the negative effects of TiO_2-NPs on testes are usually murine species, as reported by the data in the literature. TiO_2-NPs were able to accumulate in the testis to induce cytotoxicity and gene expression changes, leading to impairment of the male mouse reproductive system (Yoshida et al., 2006; Asare et al., 2012). Zebrafish testicles of our experiment

were also found to accumulate TiO_2-NPs at concentrations of $1{,}18x10^{-3}$ mg/Kg and $8{,}14x10^{-3}$ mg/Kg under 2mg/L and 4mg/L doses, respectively. Thanks to Syngistix Nano Application software, appeared that TiO_2-NPs had a size < 100 nm. An accumulation of TiO_2-NP in the testicular tissue that resulted in histological alteration of its architecuture, sperm malformations, and alterations in serum sex hormone levels was observed in male mice treated intragastrically with 2.5, 5, and 10mg/kg body weight of TiO_2NPs for 90 consecutive days (Gao et al., 2013). This data suggest that TiO_2- NPs get to the testes presumably via blood circulation. Although the exposure to the TiO_2-NPs can occur via various routes, generally the NPs are rapidly absorbed in the blood and distributed to tissues (De Jong et al., 2008; Sadauskas et al., 2009; Lankveld et al., 2010, 2011). For human, the oral and dermal routes are considered most relevant due to external exposure to consumer products containing TiO_2-NPs or due to environmental contaminant exposure. In addition the TiO_2-NPs are added to food and drugs as ingredients (Song et al., 2015; Vance et al., 2015; Wang et al., 2007). Firstly, the NPs enter and distribute in the exposed site and then they translocate to secondary organs such as the liver, spleen, kidneys, brain, ovaries, and testes (Mcauliffe and Perry, 2007). Skin local application of consumer products like sunscreens, cosmetics and drugs that contain TiO_2-NPs have shown no significant penetration of TiO_2-NPs as suggested by *in vivo* and *in vitro* studies (Dussert et al.,1997; Teichmann et al., 2006; Kiss et al., 2008; Sadrieh et al., 2010). The detection level of TiO_2-NPs was very low. However, TiO_2 nanoparticles (4 and 60 nm) applied topically for 60 days on the skin of hairless mice were able to penetrated through the horny layer and reached the epidermidis. They exhibited wider tissue distribution and even reached the brain. In particular the deposition of the TiO_2-NPs caused the oxidative stress that resulted in the pathological lesions, thus application of TiO_2-NPs for long-time can induce skin ageing and also may pose a health risk in human (Wu et al., 2009). One of the important NP absorption pathways is the gastrointestinal via, because it was demonstrate that multiple NPs can be absorbed from the gastrointestinal tract into the bloodstream distributed to other organs, resulting in their gradual accumulation in the whole body (Chen et al., 2013; Shi et al., 2013). Administration of TiO_2-NPs (rutile) of nominal size 500 nm by oral gavage have been shown to systemically translocate to other tissues from the rat gastrointestinal tract. TiO_2-NPs translocate mainly to systemic organs such as the liver and the spleen (Heringa et al., 2016). In young rats with high-dose TiO_2-NPs (200 mg kg^{-1}), the accumulation of TiO_2 in the liver induced histological alterations such as: liver edema, hepatic cord disarray, perilobular cell swelling, hydropic degeneration, or vacuolization; instead, in adult rats slight injury in the liver and kidney have showed. However, in both cases TiO_2-NP exposure can cause reductive stress in plasma, namely increased reduced glutathione (GSH)/oxidized glutathione (GSSG) ratios (Wang et al., 2013; Shrivastava et al., 2014). Another possible entry route of TiO_2 -NPs is via respiratory tract by occupational exposure or

also during application of antimicrobial spray containing TiO_2. A large number of studies have been performed on rodents to the absorption of TiO_2 NPs through inhalation (Kuempel et al., 2006). It is known that particles in the range of 1-5 nm deposit in nasopharyngeal, tracheobronchial and in the alveolar region, whereas 20 nm ENPs deposit to around 50% in the alveolar region (Simkó and Mattsson, 2010). The alveolar region of the lungs is the most permeable since gas exchange between blood and air is taking part here. It was observed that a small fraction of 20-nm-sized TiO_2-NPs can was transportation from the airway lumen to the interstitial connective tissue (Mühlfeld et al., 2007) and also they can distributed in the mediastinal lymph node (van Ravenzwaay et al., 2009). In addition, TiO_2-NPs produced reactive species and an increased bronchoalveolar lavage inflammatory parameters (Li et al., 2007; Hamilton et al., 2009; Liu et al., 2009). However, several studies have confirmed that inhaled NPs were transferred to extrapulmonary organs. According to Peters and Colleagues (2006), the mainly way which allows the translocation of TiO_2-NPs to secondary organs is by the blood circulation. However, clearance mechanisms in airways and alveoli are reducing the retention time of NPs in the lungs, therefore only relatively few nanosized particles can translocate to secondary organs (Simkó and Mattsson, 2010). Overall, the several data in literature revealed that bio-distribution of TiO_2-NPs in various organs depended on the exposure route as well as time and size. Moreover, the passage of NPs through biological barriers depends by their specific physical and chemical properties (i.e., size, shape, and polarity) (Mcauliffe and Perry, 2007). In our study, 30-day exposure of zebrafish to TiO_2-NPs suggest that fish uptake TiO_2-NPs by breathing and feeding. The nanoparticles were constantly resuspended in the aqueous medium, so fish are highly likely to internalize them via their gills and mouth. Gills are target organ for toxicity, because they are in continued contact with the water column, therefore they are the main entrance route for all contaminants and also for nanomaterials (Lead et al., 2018). In particular, we found accumulation of TiO_2 NPs in the gills tissue for the concentrations 2mg/L ($1{,}50X10^{-3}$ mg/Kg) and 4mg/L ($2{,}39X10^{-3}$ mg/Kg), whereas no accumulate TiO_2-NPs was found in control as expected, but also at 1mg/L concentration. Accumulated of Ag NPs in gills was also found by Griffitt and Colleagues (2013) for 32 day of exposure. Of course, the shape and physical properties of the TiO_2-NPs have rendered them more easily caught/immobilized in gills of the zebrafish; Zheng and Colleagues (2018) have demonstrated that bare NPs induced more bioaccumulation of iron than starch-coated Fe_3O_4-NPs. Our histological observations of gill tissue from controls, 2 mg/L and 4mg/L showed alterations in gill tissue morphology. There is an increasing cellularity in the interlamellar space and hyperplasia that brings a higher width of the secondary lamella respect to control (Figure 15).

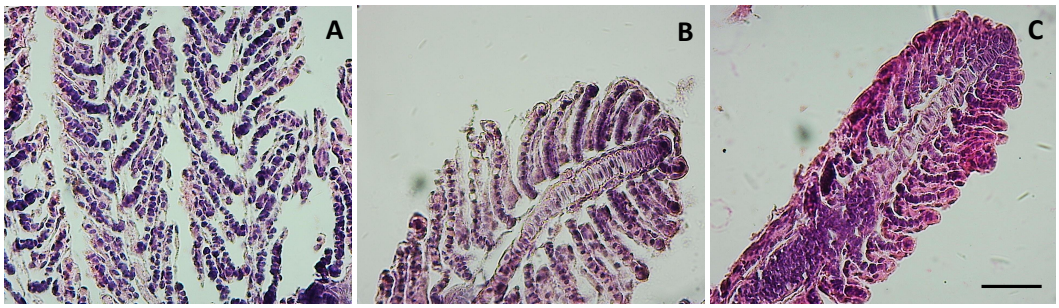

Fig.15: Histological section of gills after 30 days to exposure TiO2-NPs. **A)** CTRL gills with well-organized lamellae. **B)** 2mg/L TiO2-NPs, **C**) the cells start to proliferate becoming hypertrophic at the concentration 4mg/L. Scale bar 1500 µm

Simular results were previously observed in zebrafish exposed to other nanoparticulate metals: copper, silver. At already 24h of exposure, Griffitt and Colleagues (2009) reported 3.5-fold increase in gill filament of zebrafish exposed to copper nanoparticle, whereas silver nanoparticles caused slight changes. Also in rainbow trout (*Oncorhynchus mykiss*) exposure to TiO_2-NPs has caused some gill pathologies including oedema and thickening of the lamellae (Federici et al., 2007). Hyperplasia and fusion of secondary lamellae have been showed also in other species of fish (Ostaszewska et al., 2016; Kakakhel et al., 2021). While even if the intestine has been target organ of Ag NPs which cause injury after waterborne exposure (Osborne et al., 2015; Lacave et al., 2018), we have found that bioaccumulation of TiO_2-NPs was lower in the intestinal tract than gills. Since TiO_2-NPs after ingestion through the mouth were distributed in the digestive tract and then excreted through feces (Xiong et al., 2011), further there was not a real oral supply of nanoparticles because no real oral administration was performed. Therefore, the bioaccumulation of TiO_2-NPs detected in the intestine by ICP-MS may be a consequence of the distribution of NPs through the bloodstream. Histological examination did not reveal alterations in the intestinal tissue for all the experimental groups. Therefore, regardless of the routes of exposure, the blood flow is responsible for the absorption of NPs. In addition, macrophage clearance allows to NPs to translocate trough the circulatory and lymphatic system to many tissues and organs, as suggest several animal and human studies (Buzea et al., 2007). NPs as foreign substance are taken by mononuclear phagocytic cells which become the entry route of NPs into the tissues and cells. Also the tissue macrophages phagocytose and sequester nanoparticles, for example in the mouse model it was demonstrated a durability of Au, Ag, and SiO_2 NPs thanks their action. This probably occurred in our experiment. As mentioned, bioaccumulation of TiO_2-NPs was found in the testes of our experimental groups. Generally, this is an important process to understand when evaluating hazard and risks from NPs. The exposure and subsequent bioaccumulation of NPs is usually a precursor to toxicity, because they must be retained by the organism to cause toxicity (Luoma and Rainbow, 2008; Fabrega et al., 2011). For this reason, after the evaluation of bioaccumulation of TiO_2-NPs in testes, we exanimated the histological section of

each groups. The exposure to TiO_2-NPs caused an alteration on the spermatogenic epithelium. In zebrafish testes, seminiferous tubules are characterized by spermatogenic epithelium which is built of clusters of cells, they so called spermatocysts. In fish spermatogenesis is defined as cystic (Callard, 1996), because it is occurs in cysts that are formed when a single spermatogonium is completely surrounded by the cytoplasmic projections of one or two Sertoli cells (Pudney et al., 1993). As in all animals, the cells resulting from differentiating mitotic divisions of single spermatogonia remain interconnected by cytoplasmic bridges that synchronize developmental processes among the members of the same germ cell clone (Pudney et al., 1993; Loir et al., 1995). Thus, the testis presents cysts formed by groups of Sertoli cells that surround and assist in the development of germ cells from a single initial clone. The seminiferous tubules are separated by thin strands of interstitial connective tissue, which contains connective cells and Leydig cells. They produce androgen when stimulated by the pituitary-derived gonadotropic hormone.

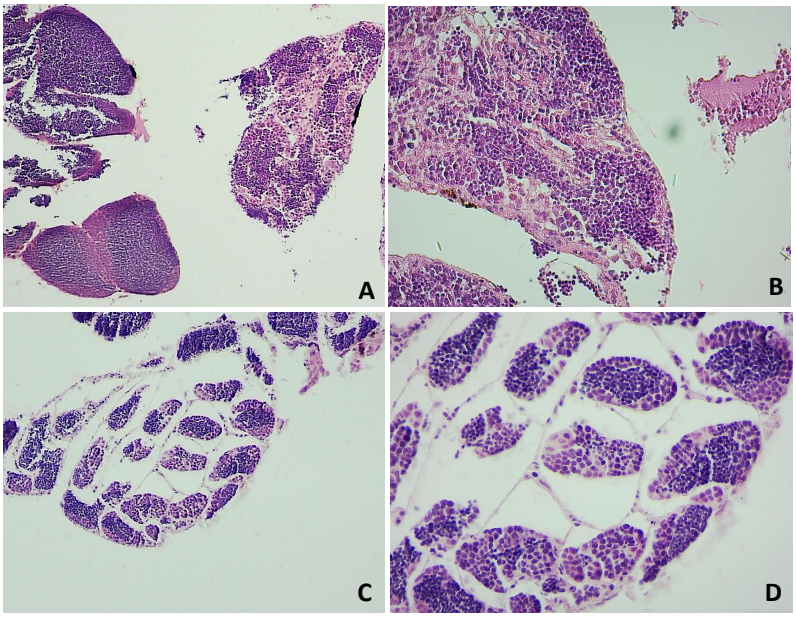

Fig.16: Histological sections of testis. Unexposed group 10X **(A)** and 40X **(B)** with normal organization of spermatogenic epithelium; **(C)** 10X and **(D)** 40X after 30 day of exposure to TiO_2-NPs (2mg/L), testis showed a detachment of the spermatogenic epithelium from the connective tissue. (E-E staining, sections 4 µm).

The exposure of TiO_2-NPs has caused a detachment of the spermatogenic epithelium from the connective tissue as shown in the figure 16, moreover in the tubules the area occupied by spermatogonia was increased, in compared to area occupied by spermatozoa that was decreased (Figure 17).

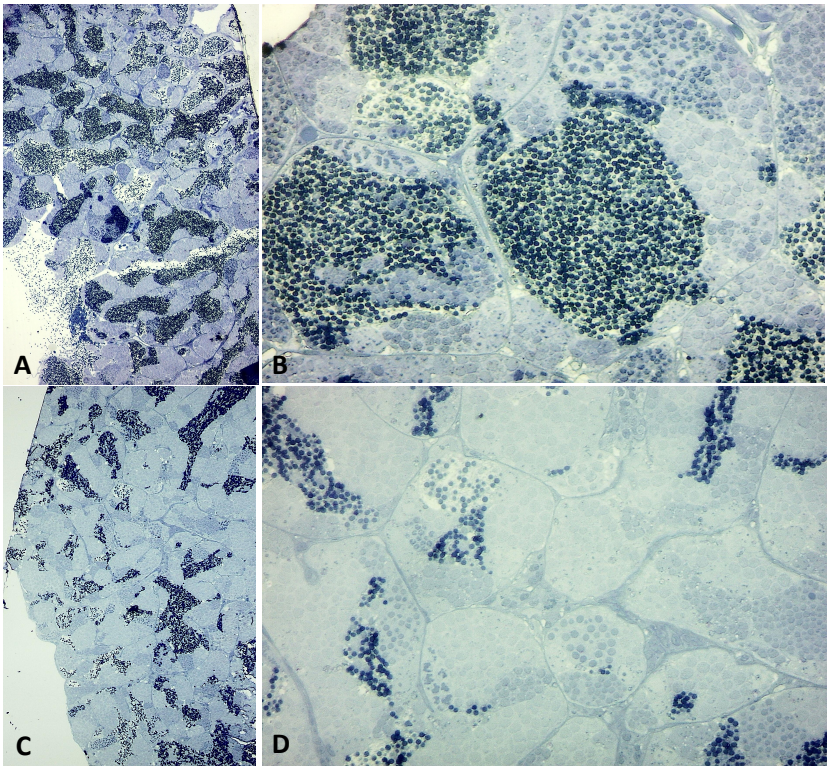

Fig.17: Histological sections of testis staining with toluidine blue (sections 0,85 µm).Unexposed group 10X **(A)** and 40X **(B)**;**(C)** 10X and **(D)** 40X after 30 day of exposure to TiO_2-NPs (2mg/L). Testis treated with TiO_2-NPs have an increase of spermatogonia compared to spermatozoa

A disordered arrangement of spermatogonia was observed at the concentration of 4 mg/L and the connective tissue presented irregularities making it difficult to distinguish from Leydig cells and connective cells (Figure 18). Whereas, testis tissues in the 1mg/L dosage group no showed changes compared with that of control.

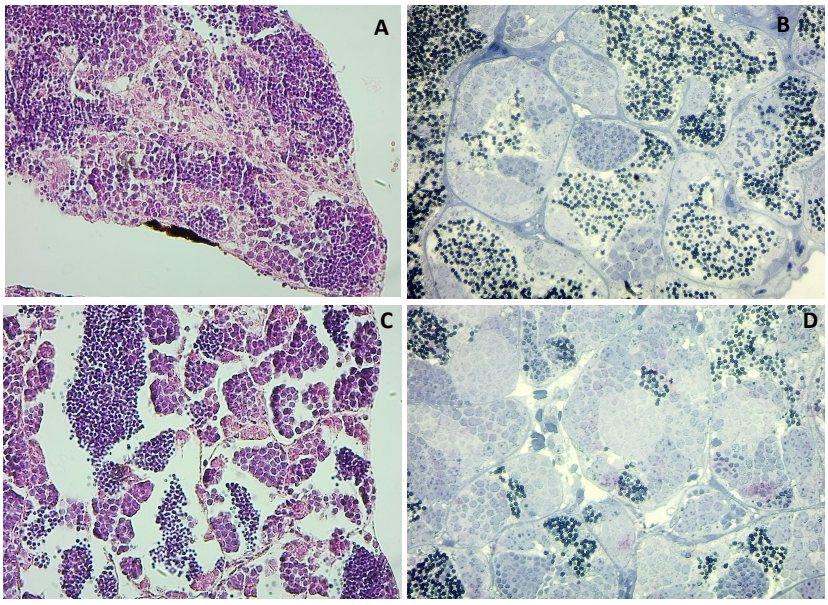

Fig.18: Histological sections of testis8.**(A)** (10X) and **(B)** (40X) unexposed group showed a good morphology and organization of tubules testis; **(C)** (10X) and **(D)** (40X) group exposed to 4mg/L TiO_2-NPs showed disorganization of tubules testis.

Previous studies on mouse, highlighted that oral administration of TiO_2-NPs caused changes in relative weights of the testis and accessory male sex organs (Morgan et al., 2017). Mice treated intragastrically with dosages of 10, 50 and 100 mg kg^{-1} of body weight (PC) anatase TiO_2-NPs for 28 days showed morphological changes in testes with a reduction in germ cell number, spherospermia, interstitial glands vacuole, malalignment, and vacuolization of spermatogenic cells (Song et al., 2017). Degenerative alterations to the cellular architecture of rat testes and epididymis with Ag-NP at 50 mg/kg were observed (Olugbodi et al., 2020), CeO_2-NPs also cause congestion and degeneration of seminiferous tubules (Adebayo et al., 2018) as well as in rats treated with ZnONPs whose toxic effect increased gradually with the increasing the dose. At the higher dose (400 mg/kg) rats have showed shrunken, disorganized seminiferous tubules with irregular basement membrane and incomplete spermatogenesis. Some tubules showed coagulative necrosis and the seminiferous tubules were almost empty from spermatids and spermatozoa (Hussein et al., 2016). In zebrafish, similar negative effects on the epithelium of testicular tubules was observed in exposure to the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) that decreased spermatozoa with concurrent increase in spermatogonia. A decreased germinal epithelium thickness in TCDD exposed males compared with controls was observed (Baker et al., 2016). Also exposure to bisphenol A (BPA) in male zebrafish (100 μg/L), caused degeneration in their testicular parenchyma, with a clear decline in their germinal epithelium cells and in their spermatozoa, moreover there was an accentuated increase of Sertoli cells (Lora et al., 2016). An impairment of spermatogenesis was associated to ethylene thiourea (ETU) with reduction of spermatogonial differentiation (Nittoli et al., 2021). Instead, there are few previous studies that have evaluated the negative effect of TiO_2-NPs on zebrafish testis. The most recent investigation was carried out by Kotil and Colleagues (2017). They have used the same our concentrations of TiO_2-NPs to evaluated ultrastructure of zebrafish testis after exposition. Their results showed that TiO_2-NPs induced autophagy and necrosis at higher doses in Sertoli cells and consequently negatively affected spermatogenic cells and testicular morphology. Also in our ultrathin sections, observed by transmission electron microscopy (TEM), the presence of vesiculation in Sertoli cells was evident at the concentration of 4mg/L with detachment of the cell membrane compared to the control (Figure 19).

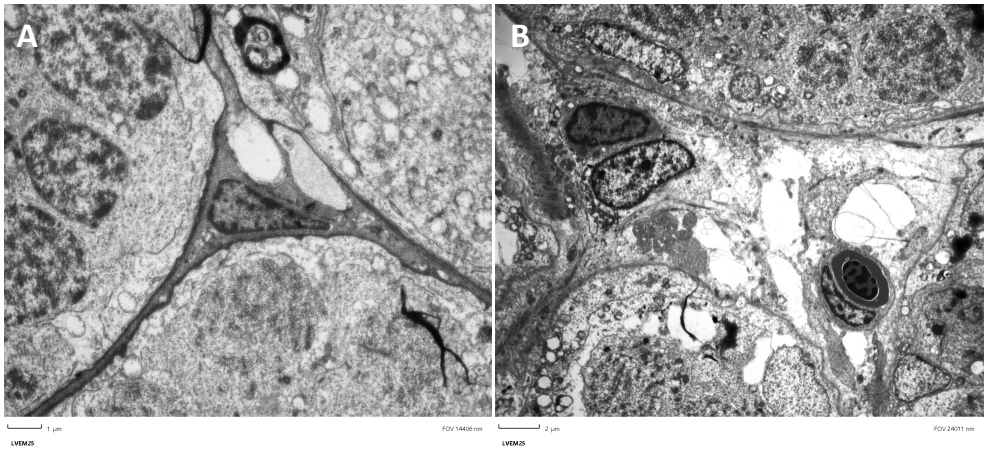

Fig.19: Ultrathin sections of zebrafish testis: **(A)** section of testis unexposed; **(B)** section of testis exposed to 4mg/L with evident vesiculation

Wang and Colleagues (2021) demonstrated that TiO_2-NPs should act as a carrier to facilitate the accumulation of 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) in zebrafish testes and result in a synergistic effect on BDE47-induced adverse reproductive outcomes via disruption of intercellular connectivity of zebrafish testes. Negative effects of TiO_2-NPs was also indicated on the female reproductive system by Wang and Colleagues (2011a). Chronic exposure of zebrafish to 0.1 mg L^{-1} TiO_2-NPs, establish a reduction in the cumulative number of zebrafish eggs after 13 weeks of exposure; thus TiO_2-NPs can significantly impair zebrafish reproduction. A sub-lethal effects of TiO_2-NPs was observed at the end of the 14-d exposure adult zebrafish to 1.0 mg l^{-1} TiO_2 (both NP and bulk), because the fish were able to reproduce; however, the cumulative number of viable embryos produced was lower (Ramsden et al., 2013). In addition to the morphological changes of testis organization, we have investigate some marker to investigate the action of TiO_2-NPs like endocrine disruptor.

Although the studies on the hazardous effects of TiO_2-NPs on the testis, the detailed molecular mechanisms associated to testicular injury and alteration of reproduction have not been fully explained. The endocrine system seems to be a target of TiO_2-NPs (Tassinari et al., 2014; Miao et al., 2015) consequently the male reproductive system is particularly highly susceptible (Meena et al., 2015). Testis has also an endocrine function, because they are responsible for the secretion of male sex hormones, in particular testosterone (T). Testosterone is the main hormone androgen, that is produced from cholesterol by Leydig cells through a process called steroidogenesis, Leydig cells are the sex steroid-producing cells in the testis. This cells are hold in the interstitial compartm ent of testis with blood vessels, nerve fibres, macrophages, mast cells, peritubular myoid cells, fibroblasts (De Waal, 2009). The Sertoli cells, in addition to interacting with the cells of spermatogenesis, perform an endocrine function through the production of ABP (androgen-binding protein), which concentrates testosterone favoring spermatogenesis. NPs have been reported to affect also Leydig and Sertoli cells

because after internalization they induce cytotoxicity and gene expression changes (Yoshida et al., 2006; Komatsu et al., 2008; Asare et al., 2012). We have investigate the expression of sex hormone-binding globulin (SHBG) protein. As mentioned sex hormone-binding globulin (SHBG) is a glycoprotein produced in the liver, but also in the testis, uterus and placenta (Lin et al., 2010; Hammond, 2011). In the blood of all vertebrate species with the exception of birds, SHBG acts as a carrier of androgens and estrogens regulating their bioavailability (Siiteri et al., 1982). An ortholog (shbg) has been so far identified in zebrafish being expressed in the digestive tract, liver, gills, pancreas, and testis (Miguel-Queralt et al., 2004, 2008; Bobe et al., 2010). SHBG in zebrafish, has the same function, then it is involved in the transport, regulation and action of steroids in zebrafish circulation (Tokarz et al., 2013). The results of immunohistochemical analysis revel that SHBG was expressed in control groups in regular way, because the cysts have maintained their morphological organization, as explained above, however a positive expression remained in the exposed group, in particular positivity was higher in the group exposed to 2mg/L than 4mg/L. It was evaluated by image J software, in addition as shown in the figure 20, the positivity was related to the cells that around the seminiferous tubule cysts.

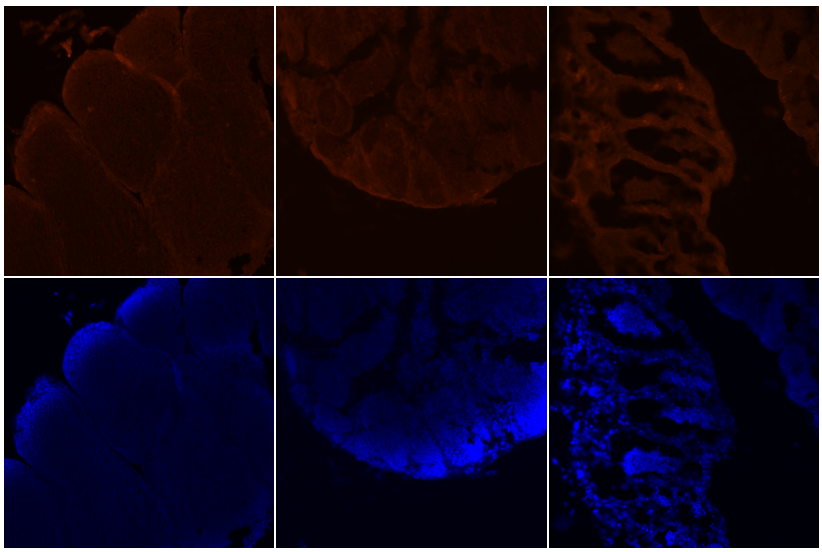

Fig.20: SHBG expression in testis tissue. Nuclei blue (DAPI) and red fluorescent of SHBG protein. A positivity staining was observed in the 4mg/ L concentration (40X).

In mammalian, the testis produces an SHBG homolog, known as the testicular androgen-binding protein (ABP) which is produced and secreted by Sertoli cells primarily under the influence of follicle-stimulating hormone and then secreted into the seminiferous tubular lumen to regulate androgen availability in the male reproductive tract (Joseph, 1994). While the single human SHBG gene is also expressed in the germ cells of the testis, and an SHBG isoform accumulates in the sperm

acrosome to binds steroids as well as the plasma SHBG (Selva et al., 2002, 2005). In fish, Shbg has been found in the plasma of an elasmobranch, the skate (*Raja radiata*) (Freeman and Idler, 1969) and of a teleost, in the rainbow trout (*Oncorhynchus mykiss*) (Fostier and Breton, 1976) a few decades ago, and subsequently identified and studied in a wide variety of fish species. In particular, the Shbg alpha form (Shbga) is clearly the ortholog of the mammalian Shbg and has been found in all investigated teleost species. In contrast, the Shbg beta form (Shbgb) initially identified in rainbow trout (Bobe et al., 2008) has subsequently been found in other salmonid species (Miguel-Queralt et al., 2009). Studies of the SHBG steroid-binding characteristics in several fish species (Mak and Callard, 1987; Ovrevik et al., 2001) have shown that its affinity for endogenous sex steroids (testosterone and estradiol), and xenobiotics (Milligan et al., 1998; Tollefsen, 2002; Pryce-Hobby et al., 2003) varies between species. There is also some evidence that plasma SHBG levels fluctuate in fish during the reproductive cycle (Foucher et al., 1992; Hobby et al., 2000). Shbg (Shbga) exists as a homodimeric glycoprotein characterized by a common structure composed by two laminin G (LG) like domains that contain two sets of conserved cysteines that form intramolecular disulphide bridges. As secreted proteins, a signal peptide sequence is cleaved from the precursor protein to give rise to the mature protein. A part from its role as a steroid carrier protein, there is some evidence that this protein can mediate steroid signaling by binding to membrane associated proteins (Hryb et al., 2002). Atlantic sea bass (*Dicentrarchus labrax*) (Miguel-Queralt et al., 2005), rainbow trout (Bobe et al., 2008) and coho salmon (Miguel-Queralt et al., 2009) and zebrafish (*Danio rerio*) (Miguel-Queralt et al., 2004) are the fish species in which Shbg (Shbga) sequences have been characterized. In zebrafish, the tissue distribution of shbga transcript was detected in digestive tract and hepatopancreas (Miguel-Queralt et al., 2004). In addition, a low expression was detected in testis using RT-PCR (Miguel-Queralt et al., 2004). However, several data indicate, in agreement with mammalian literature, that liver is the main expression site of Shbga in teleost fish (Bobe et al., 2008; Miguel-Queralt et al., 2009). In some species, it was also found a significant expression of Shbg in several other tissue, which suggest that the circulating Shbga could has extra-hepatic origin. In mammals, a local expression of Shbg in several target organs has also been evidenced and associated with a modulation of the steroidogenic signal (Hryb et al., 2002). Similarly, non-hepatic expression sites of Shbga in teleost could also be associated with local action in target organs. For this reason the transcription of shbg in fish, could depend by different variant which have to still identified. It is not known how expression of the SHBG gene is regulate (Miguel-Queralt et al., 2004). Our results evidence that an expression of SHBG could be increased in the presence of NPs. This was supported by the result of gene expression of SHB in the testis, that indicate an increase expression of SHBG gene for the 2 mg/L and 4 mg/L concentrations compared to control group. Unfortunately, the biological importance

of SHBG in fish is not studied as well as in mammals. May be reasonably hypothesized that the function of Shbg proteins expressed locally in target organs and tissues could be different from the circulating of Shbg. As in mammals, fish Shbg protein is involved in sex steroid transport, regulation, action and at light of our evidence Shbg could improve the spermatogenesis process because their localization in testis could bring a higher intake of androgen hormone or TiO_2-NPs could act like androgen hormones. This hypothesis is support by gene expression result of SRD5A2 gene. This gene in all vertebrate encode for the steroid enzyme 5-αreductase α-polypeptide 2 (SRD5α2), an enzyme of spermatogenesis (Hering et al., 2014). The action of this enzyme is convert testosterone (T) to dihydrotestosterone (DHT). Overall, androgens hormone play an important role in controlling sexual differentiation and spermatogenesis in males. In particular the 5α-dihydrotestosterone (DHT) has been considered the most potent androgen in mammals, birds, reptiles and amphibians (Prahalada et al., 1997; Avila et al., 1998; Bissegger et al., 2014), while the 11-ketotestosterone (11KT) is the major androgens in fish with an important role in the development of the gonad and spermatogenesis (Miura et al., 1991; Bhandari et al., 2004; Martyniuk et al., 2013). 5α-dihydrotestosterone (DHT), play a physiologically important role in some fish species and it is associated to spermatogenesis. However, the molecular mechanisms underlying androgen function in fish are still largely unknown, although the androgen receptors of several fish species have been cloned and characterized at the molecular level (Ikeuchi et al., 1999; Sperry and Thomas, 1999; Takeo and Yamashita, 1999; Touhata et al., 1999; Kim et al., 2002; Wilson et al., 2004) as well as the enzymes of the general pathway leading to the formation of steroid hormones. Enzyme 5-α reductases is a membrane-associated NADPH-dependent enzyme that catalyzes the irreversible steroid specific reduction of C19 3-keto-Δ4–5 steroid to 5α-reduced metabolites. It is bound to endoplasmic reticulum membrane thanks the α-helices in his structure (Liang al., 1985; Yokoi et al., 1996; Scaglione et al., 2017; Cantagrel et al., 2010). In the human SRD5α2 is expressed throughout the body, but mainly in male secondary sexual organs such as the prostate, epididymis and penis (Thigpen et al., 1993). In the fish it is expressed in liver, brain, ovary and testis (higher than in ovary) (Martyniuk et al., 2013). Overall, the SRD5α2 action is a key step in the androgen synthetic pathway because converts testosterone into dihydroxytestosterone directly in the targeted tissue. Several evidences have documented consequences of a lack SRD5α2 activity that brought a decreases of 5α-DHT levels in those tissues. Generally, a decrease in 5α-DHT synthesis lead to adverse effects such as: reduction of the prostate weight in mammalian species (human: Kang et al., 2014, Park and Choi, 2014, Mendonca et al., 2016; rats: George et al., 1989, Giatti et al., 2016, Enatsu et al., 2017; mice: Mahendroo et al., 2001), reduction of weight in the epididymis (rat: George et al., 1989, Garcia et al., 2012), seminal vesicles (rat: George et al., 1989, Cayatte et al., 2006, Enatsu et al., 2017), corpus cavernosum (rat: Zhang et

al., 2012, Enatsu et al., 2017), and Cowper's gland (rat: Cayatte et al., 2006); defect in the male genital tubercle development (human: Kang et al., 2014, Mendonca et al., 2016; monkey: Prahalada et al., 1997; rats: Clark et al., 1993; rabbit: Kurzrock et al., 2000) and disruption of erectile function (rat: Pinsky et al., 2011). Others negative effects of decrease in 5α-DHT are on spermatogenesis and the structure of seminiferous tubules. Studies have shown a decrease in the production of spermatocytes, spermatids (Urbatzka et al., 2009; Kang et al., 2014) and hypotrophy of seminiferous tubules (Ryhorchuk et al., 1997; Vidigal et al., 2008). Others negative effects of decrease in 5α-DHT are on spermatogenesis and the structure of seminiferous tubules/spermatocysts. Studies have shown a decrease in the production of spermatocytes, spermatids (Urbatzka et al., 2009; Kang et al., 2014) and hypotrophy of seminiferous tubules (Ryhorchuk et al., 1997; Vidigal et al., 2008). The lack of 5α-DHT mainly affects the Sertoli cells, because they are involved in spermatogenesis by supporting of germ cells development, as well as playing an important role in the structure of the seminiferous tubules and the maintenance of the blood-testis barrier (Wang et al., 2009; Huhtaniemi, 2018). This impact could be linked to high intratesticular T concentration in the testes, which is secreted by Leydig cells and it important to promote spermatogenesis (Wang et al., 2009; Huhtaniemi, 2018). Then intratesticular T is probably converted locally into 5α-DHT by Sertoli cells which express SRD5α2, for this reason 5α-DHT is implicated in spermatogenesis and the maintenance of the seminiferous tubules in Sertoli cells. Nevertheless, our results differ from the negative effects due to the lack of enzyme activity. The expression of the SRD5α2 was increased in the gonads exposed to nanoparticles, this consequently leads to its greater activity and therefore production of DH. Then exposure to TiO_2-NPs not alter the spermatogenesis because they encourage the activity of enzyme SRD5α2. Similar date were observed in fish treated with 5α-reductase inhibitors (5ARIs) which spermatogenesis is unaltered or even increased because levels of T and 11KT are increased (Margiotta-Casaluci et al., 2013; García-García et al., 2017). Since the synthesis of SRD5αs is regulated by the androgens they produce, in the light of our results it can be hypothesized that nanoparticles may behave like androgens. It is known that SRD5αs possess androgen response elements (AREs) in their promoter sequences (Flood et al., 2013). In the promoter of SRD5α2, there are two classes of AREs. One is a classic ARE with a palindromic repeat of AGAACA, which is functional and can also act as a progesterone response element (PRE) (Matsui et al., 2002). The second is a selective ARE constituted of a partial direct repeat of AGAACA (Kerkhofs et al., 2012). Despise the presence of AREs in promoter, their regulation by ARE is more complicated than the simple activation of AR by androgens. For example, in rat's prefrontal cortex, SRD5α2 is upregulated by the administration of T and 5α-DHT in both sexes, SRD5α1 is down-regulated in male when treated with T and 5α-DHT, while it is upregulated in females only after 5α-DHT treatment (Torres

and Ortega, 2003). In T-treated rats, SRD5α1 is negatively regulated in the testes, whereas SRD5α2 is unaffected, but is overexpressed in the prostate (Pratis, 2003). All those results show that differential regulation is probably linked to co-regulators of AR, such as the pioneer factors (Pihlajamaa et al., 2015). Pioneer factors are able to bind to condensed chromatin and in turn help transcription factor like AR to bind it. The interaction between pioneer factors and AR is known to be tissue specific. Then a similar act could be carry out by nanoparticles, to explain the increasing expression in the exposed groups.

Unfortunately, no unified theory about how hormone levels change after exposure to NPs exists. Some study showed that low-dose (1 mg/kg/dose) AgNPs intravenously injected into male CD1 mouse serum, cause significative increased of the intratesticular testosterone (T) (Garcia et al., 2014), whereas in another study with CeO_2-NPs treat decreases in T, FSH, LH, and prolactin (PRL) were discovered (Adebayo et al., 2018). Indeed, these changes might be influenced by different factors, such as particle type, size, and time of exposure. Therefore research of how NPs affect hormones should be conducted and the lack of *in vitro* studies is still a problem.

Instead, the immunohistochemical investigation about cytochrome P540 (Cyp19b) confirmed the ability of nanoparticles to induce oxidative stress. As shown in the figure 21, positivity was found in the expression of P540 in the groups exposed to TiO_2-NPs (2mg/L and 4mg/L concentration) compared to control that not shown positivity for the biomarker. Software Image J pointed out greater response for Cyp19b for 2mg/L concentration compared to 4mg/L.

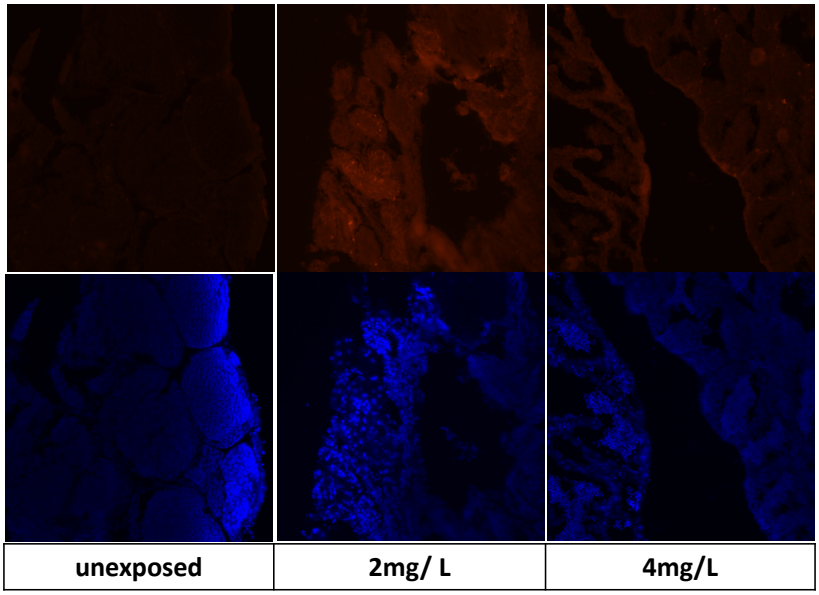

Fig.21: P540 expression in testis tissue. Nuclei blue (DAPI) and red fluorescent of P540 protein. A positivity was found at 2mg/L and 4mg/L.

CYPs are a large superfamily of enzymes capable to metabolizing several substances including steroids, pharmaceuticals and xenobiotic compounds. They catalyze mixed-function oxidation reactions, then induction of their catalytic activity, measured as ethoxyresorufin-O-deethylase (EROD) activity, and expression (protein and transcript) is a useful biomarker of exposure to xenobiotic. In addition, their catalyze activity leading to activation or inactivation of many endogenous and exogenous chemicals, with consequences for normal physiology and disease processes (Goldstone et al., 2010). An important consequence is oxidative stress. Several studies have reported that oxidative stress is induced by nanomaterials of varying chemical composition such as fullerenes, CNT, and metal oxides (Vallyathan and Shi, 1997; Bonner et al., 2007). In fish, TiO_2-NPs causes oxidative stress with the production of reactive oxygen species (ROS) (Zhu et al., 2011; Xiong et al., 2011) and also titanium dioxide nanoparticle aggregates (NM-TiO_2) cause oxidative stress on zebrafish embryo (Faria et al., 2014). Instead, the investigations about oxidative stress by TiO_2-NPs on testis are insufficient. It is known that oxidative stress is a common pathway of toxicity and disease that may be cause by many pollutants. It may be induced through oxidizing (e.g. hydrogen peroxide, H_2O_2) or photo-oxidizing (e.g. fluoranthene) agents that react with oxygen producing reactive oxygen species (ROS) (Bar-Ilan et al., 2012, Fu et al., 2012, Miller et al., 2012). Since the reactive oxygen species (ROS) are able to affect the physiology, growth, and survival in aquatic organisms (Filho, 1996; Pandey et al., 2003), they, like mammals, developed an antioxidant defense systems for neutralizing the toxic effects of ROS (Pandey et al., 2003). Antioxidant enzymes like SOD, CAT, GPX, POD and low-molecularweight, nonenzymatic antioxidants (e.g., GSH) are important components of the antioxidant defense system in animals (Van der Oost et al., 2003, Zhang et al., 2004). In our study, an increase transcription of antioxidant enzymes, such as glutathione peroxidase (GPX) and especially superoxide dismutase (SOD) was observed. Figure 22 shown the results of qRT-PCR of all genes investigated.

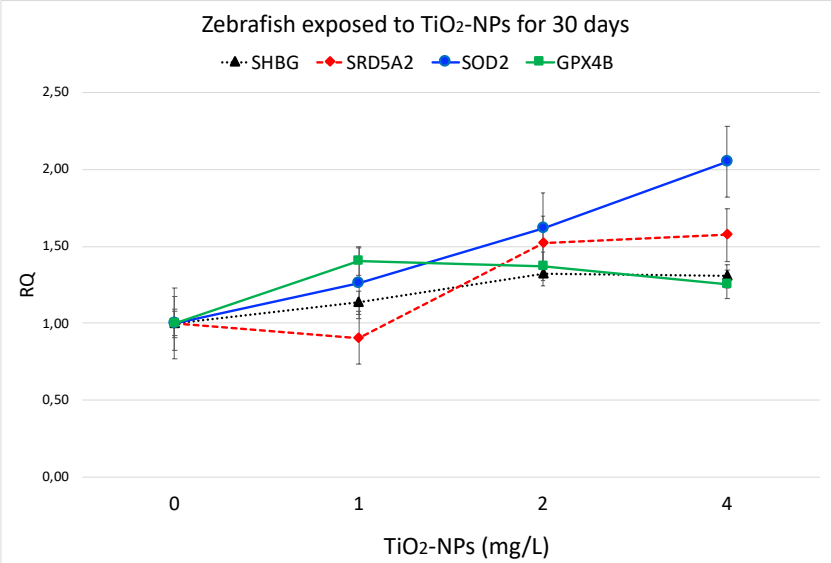

Fig.22: Results of qRT-PCR of all genes investigated.

It is clear that TiO_2-NPs are able to induce oxidative stress also in the testis. Superoxide dismutase (SOD) is the first enzyme of defense against oxidative stress by elimination ROS. It is responsible for catalyzing the dismutation of the superoxide radical into hydrogen peroxide which in turn is degraded by CAT and GPX (Lin et al., 2016; Asakura and Kitahora, 2018). Thus, SOD enzymes are an important antioxidant defense for nearly all cells exposed to oxygen (Celardo et al., 2011; Nash and Ahmed, 2015), moreover it is very sensitive to the stress of pollutants and can be used as oxidative stressed signal for the early warning of environmental pollution. Instead, the depletion of SOD activity occurs when the antioxidant defense system is overwhelmed by ROS (Van der Oost et al., 2003). Fortunately, our results showed that exposure to the lower concentration (1mg/L) motivated the SOD to eliminate generated ROS as a protection mechanism against oxidative stress and a major increase it was observed at the higher concentration (4mg/L). Then antioxidant defense of fish are induced by a oxidative stress due to TiO_2-NPs, and it not overwhelm the detoxifying or antioxidant mechanisms.

Glutathione peroxidase (GPX) as CAT, metabolize hydrogen peroxide to oxygen molecule (Pi et al., 2010), moreover it plays an important role in maintaining intracellular redox balance (Knapen et al., 1999). Induction in mRNA level of GPX gene was higher at 1mg/L concentration than 4mg/L.Fortunately, our results showed that antioxidant defense of fish are induced by a mild oxidative stress due to TiO_2-NPs, and it not overwhelm the detoxifying or antioxidant mechanisms.

11.3 *In vitro* assay

In vitro assays are efficiently useful to identify and understand the toxicology effects of different molecules (Tice et al., 2000; Culot et al., 2008; van Woudenberg et al., 2012), for example the evaluation of nanoparticles (NPs) is usually based on the use of *in vitro* cell assays to predict their toxicity before subjecting to animals (Casals et al., 2012). The toxic effects of nanoparticles in cell cultures are linked by an increasing oxidative stress and inflammatory cytokine production (Choi et al., 2015; Oberdörster et al., 2005a; Senapati et al., 2015; Tarantini et al., 2015); moreover, their cytotoxic effects are closely related to intracellular uptake process (Yu et al., 2009; Choi et al., 2010). Evidences showed that nanoparticles can be taken up by the mitochondria and nucleus, causing mitochondrial damage, DNA damage and also mutation that can bring to cell death (Li et al., 2003; Savic et al., 2003; Geiser et al., 2005; Porter et al., 2007; Foldbjerg et al., 2011; Xu et al., 2014b; Yoisungnern et al., 2015). Although, more studies have shown that the testicles are susceptible to titanium toxicity, and the male reproductive system is a TiO_2-NP health risk target, are poor the investigation *in vitr*o about the effect of TiO_2-NPs on human spermatozoa (Orazizadeh et al., 2014; Miura et al., 2017). Sperm parameters, such as sperm number, viability, abnormalities, and motility, have been extensively studied *in vivo* studies that reported a decline with increasing concentrations of NPs (Morgan et al., 2017; Song et al., 2017). In mammals, the quality of gametes plays an important role in gametogenesis, then the influence of nanoparticles on male fertility can cause decrease in the quality and quantity sperm or have negative consequences for the development of the offspring (Boisen et al., 2001; Anway and Skinner, 2008; Braydich-Stolle et al., 2010). In particular sperm motility is a reliable predictor for sperm quality and fertilization success (Au et al., 2002). Good sperm motility is very important for active swimming along the female tract and for penetration of oocyte. Although the literature data have reported a decrease of sperm motility after *in vitro* exposure to TiO_2-NPs (Pawar and Kaul, 2014; Santonastaso et al., 2019), our results not report significant reduction in motility of spermatozoa at the end of exposure. On the contrary the progressive motility was increased at the highest concentration (500 ppm) and was statistically significant compared to control ($p < 0.05$). Also viability was not changed by exposure to TiO_2-NPs ($p < 0.05$), in fact the percentage of dead spermatozoa was not increased compared to control. An improvement in sperm parameters was observed by Afifi and Colleagues (2015) after treatment with ZnONPs. Sperm count and motility increased in ZnONPs treated diabetic rats, because the ZnONPs have the ability to protect the sperm from the deleterious effect induced by diabetes. Our positive results can be explained by the swim-up technique used. The spermatozoa are selected by their ability to swim in the culture medium, so in our experiment the TiO_2-NPs don't interfere with this ability, neither it is statistically reduced compared to the control. Nevertheless more reports suggest that NPs

compromise male fertility, due to their cytotoxic effects (Song et al., 2016). They can affect the different functions of cell thanks their ability to interact with cellular macromolecules. NPs are able to interact to DNA, mitochondria and various proteins (Chenthamara et al., 2019). Our scanning analysis have shown that the TiO_2-NPs are able to interact with the sperm plasma membrane and in particular the microanalysis have shown their localization on the sperm head (Figure 23).

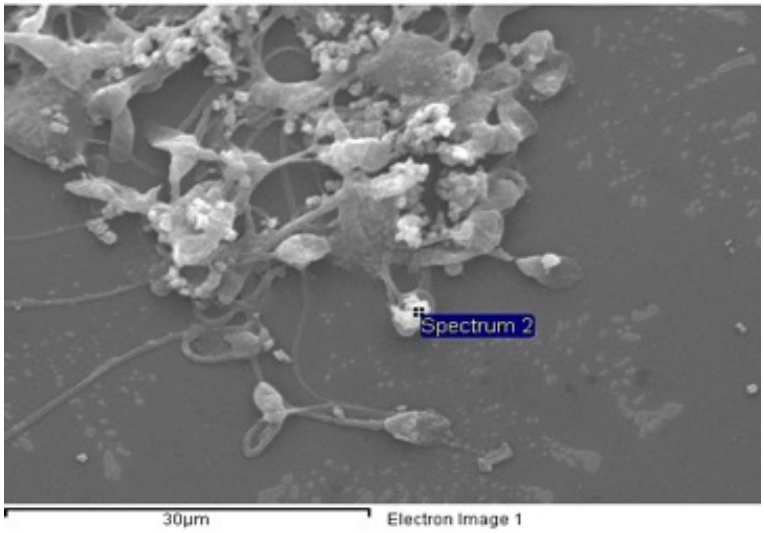

Fig.23: SEM–EDX analysis shows TiO2-NPs on the sperm head.

This can lead to their internalization. Several studies have shown that nanoparticles can penetrate into the spermatozoa (Wiwanitkit et al., 2009; Yoisungnern et al., 2015; Santonastaso et al., 2019) and can result in DNA damage. Sperm DNA damage can be defined as any chemical change in its normal structure. The most common disturbance, is the sperm DNA fragmentation (sDF) that affecting the genetic material in the form of single or double strand breaks and may be triggered by the defective packaging of the DNA during spermatogenesis, but also by processes of cell death and oxidative stress associated with environmental conditions and chemicals substances (Sakkas and Alvarez, 2010, Muratori et al., 2015, 2019). Our results obtained by Sperm Chromatin Dispersion Test (SCD) have shown significantly increased DNA damage at all concentrations (Figure 24), in addition the TUNEL assay highlighted the presence of single strand breaks in the DNA (Figure 25).

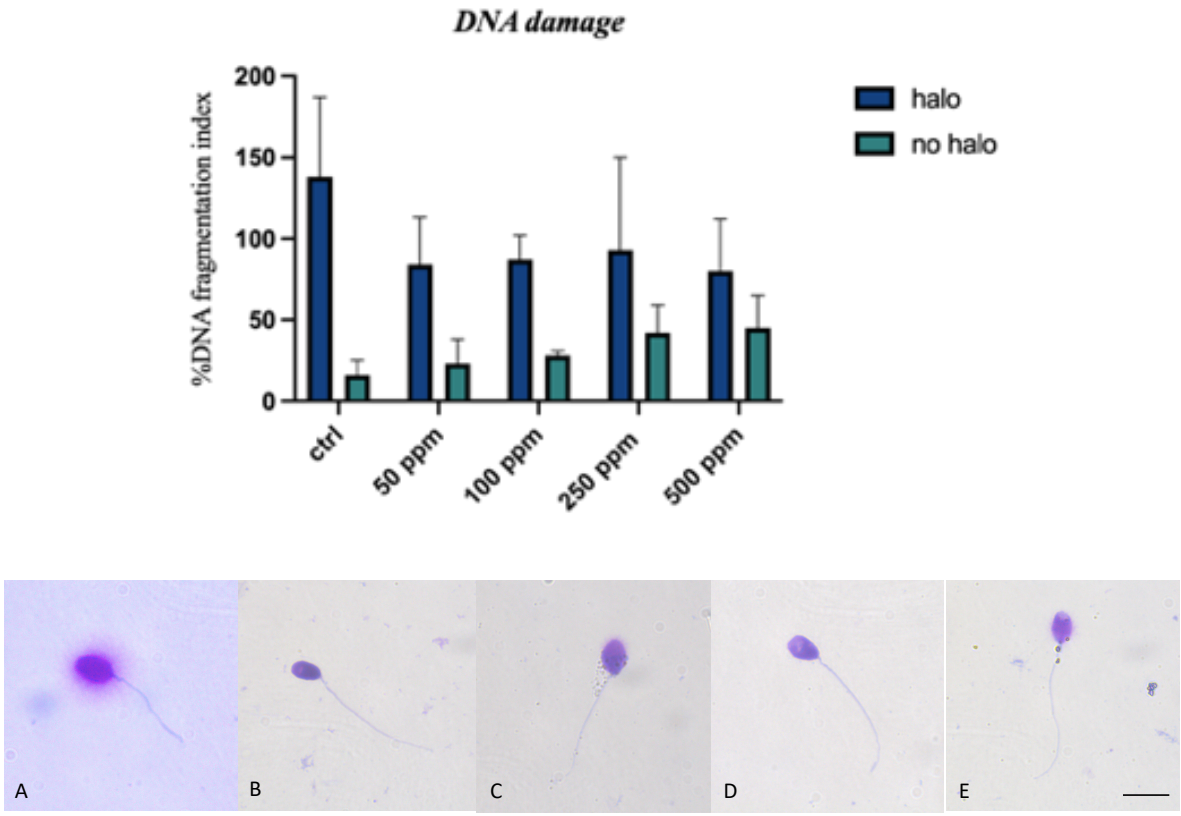

Fig.24: DNA fragmentation rate after exposure to TiO_2-NP. An increase in DNA fragmentation is evident for the 500 ppm concentration. Evaluation of the DNA fragmentation by Test (SCD) (100X). Scale bar 53 µm. **(A)** Unexposed sperm; Sperm exposed to **(B)** 50 ppm TiO_2-NPs, **(C)**100 ppm TiO_2-NPs, **(D)**250 ppm TiO_2-NPs, **(E)** 500 ppm TiO_2-NPs.

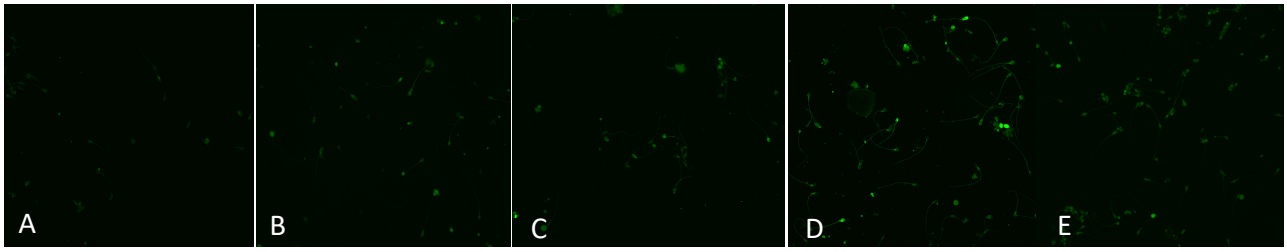

Fig.25: Evaluation of the DNA fragmentation by Tunnel Assay (40X). **(A)** Unexposed spermatozoa; Spermateozoa exposed to **(B)** 50 ppm TiO_2-NPs, **(C)**100 ppm TiO_2-NPs, **(D)**250 ppm TiO_2-NPs, **(E)** 500 ppm TiO_2-NPs.

These data are in accord to Santonostato and Colleagues (2019) which highlighted DNA fragmentation after 30 min of exposure to TiO_2-NPs (1 µg/L and 10 µg/L). Others *in vitro* study on human spermatozoa have investigated the DNA damage cause by nanoparticles. Préaubert et al. (2018) have described the capacity of CeO_2-NPs to induce significant DNA damage at very low concentrations (0.01, 0.1, 1 or 10 $mg \cdot L^{-1}$), whereas the AgNPs at concentrations of 200 µg ml^{-1} and 400 µg ml^{-1} have increased the DNA damage (Wang et al., 2017b). Many studies have confirmed that sperm DNA integrity is a prerequisite for normal fertilization and transmission of paternal genetic information to the offspring (Benchaib et al., 2007; Collins et al., 2008), because from it depends fertilization, subsequent embryo development, implantation and pregnancy (Agarwal et al., 2016).

Impaired semen parameters have been associated with infertility and reduced reproductive capacity, and in recent years DNA fragmentation has emerged as a valuable tool for evaluating male fertility (Tan et al., 2019). Evenson and Wixon (2008) in a meta-analysis study have found that the lower the sperm DNA damage, the greater the successful natural pregnancy rate, while major sperm chromatin damage increases the risk of congenital abnormalities (Kumar et al., 2012) and predisposition to childhood cancers in the offspring (Aitken and Krausz, 2001). DNA damage is occurs through the induction of oxidative stress by nanoparticles. Overall, ROS-mediated oxidative stress is a key effect for the toxicity of nanomaterials (Nel et al., 2006; Meng et al., 2009), however, physiologically the ROS are natural byproducts of the normal oxygen metabolism and they affect the cell signaling and homeostasis (Devasagayam et al., 2004). Moreover they are generated in the cellular response to exogenous substances and bacteria, then they have favorable functions at low or moderate concentrations to fight with pathogens (Dröge, 2002). In male germ cells they may have important implications in maintaining the normal functions of mature spermatozoa at physiological levels (Agarwal et al., 2006) and in spermatozoa ROS are important signaling molecules for their hyperactivation and acrosome reaction (de Lamirande and O'Flaherty, 2008). Nevertheless, an excess of ROS by external inputs such as NPs can increased the oxidative stress (OS) which results in damage DNA and apoptosis (Wan et al., 2012). Oxidative stress occurs because the ROS generation exceeds the capacity of the anti-oxidant defense mechanism. In our study, the ROS generation was monitored through DCF assay, that asses directly the production of ROS thanks the monitored of fluorescence intensity of DCF. Our results shown that exposure to TiO_2-NPs induce ROS production for all concentrations, moreover the measured ORP value was higher for the concentration of 500 ppm with a ORP mean value of 206,7 mV, compared to control (177,1 mV). This suggest that the antioxidant enzyme system of sperm cells was not able to counteract excessive presence of ROS; the excessive presence of ROS can also causes an increase concentration of the proteins involved in cell stress (Kang et al., 2008). In particular, our immunohistochemical investigation have shown that TiO_2-NPs induced heat-shock protein 70 (HSP70) expression (Figure 26).

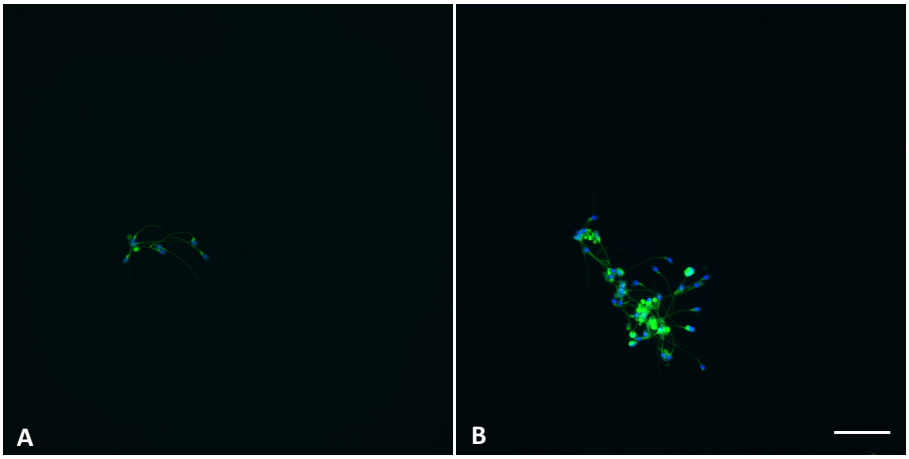

Fig.26: Localization of HSP70 on spermatozoa treated with TiO_2-NPs after 1 hour of exposure. **(A)** Unexposed spermatozoa; **(B)** Spermatozoa exposed to 500 ppm TiO_2-NPs. Nuclei blue (DAPI) and green fluorescent of HSP70 protein. Scale bar 43 µm

Heat shock proteins (HSPs) are the major molecular chaperone proteins in eukaryotic cells, that originally evolved to solve problems in protein folding (Mayer et al., 2010). In mammals, the HSPs are commonly divided according to their molecular weight into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40 and HSP27 (HSPB) families. In physiological conditions they are constitutively expressed to assist in the folding and trafficking of proteins, in the assembly of multi-protein structures, and in the translocation of proteins across membranes (Bukau and Horwich 1998). HSPs are present in the cytosol and different cellular components including mitochondria, endoplasmic reticulum, and nucleus, although their expression levels exhibit cell-type-specific patterns. The well-investigated HSPs in mammals are those with molecular masses of 60, 70, 90, and 110 kDa (20) and it is known that the stress triggers the release of HSPs into extracellular spaces. Indeed stress proteins such as HSPs 27, 60, 70, 90 and 110, are released from cells in a variety of circumstances, and subsequently interact with adjacent cells or may enter the bloodstream (Calderwood et al., 2007). The expression level of HSPs in cells is changed when the cell exposure to different stresses such as hyperthermia, hypothermia, hypoxia, hyperoxia, oxidative stress, viral infection and energy depletion (Kregel, 2002; Wang et al., 2014b; Li et al., 2015). HSPs have a protective function, because they allow maintenance of cellular homeostasis under stressful/lethal (Shiber and Ravid, 2014). Membrane bound or extracellularly located HSP60 and HSP70 are thought to act as physiological alarm signals for cell trauma (Calderwood et al., 2007, Multhoff, 2007). Under oxidative stress conditions, the heat shock proteins 70 (Hsp70) family are expressed highly in various cell types of the germinal epithelium (Eddy, 1995; Vaziri et al., 1998; Khosravanian et al., 2014), because they allow to recovering the DNA and RNA damage in germinal epithelium (Eddy, 1995; Lamb et al., 2010). HSPs have been identified in the surface of sperm cell membrane of bull, boar, mouse, rat, and human. Role of HSPs is to ensure the progression and maintenance of sperm physical traits and sperm function during normal fertilization (Hiyama et al., 2014; Nixon et al., 2015) or after

exposure of sperm cell to different environmental challenges (thermal stress or freezing) (Mestril and Dillmann, 1995; Daugaard et al., 2007; Zhang et al., 2015c). For this reason, under stress conditions sperm cells resistance to environmental challenges is strengthened by the increased expression levels of HSP70 (Mestril and Dillmann, 1995; Daugaard et al., 2007) as well as we have observed in our experimental conditions.

The exposure to TiO_2-NPs induced stress to sperm cell that have increase the expression of Hsp70. In male rats, the administration of TiO_2-NPs has exert a cytotoxic and genetoxic damage in the testicular that caused an increased heat-shock protein 70 expression (Rezazadeh-Reyhani et al., 2015). This high levels of cellular stress, may significantly affect subsequent embryo development if these spermatozoa are used for fertilization. Moreover, the spermatozoa respond to the presence of metal NPs with the expression of metallothioneins (MTs), as shown in figure 27

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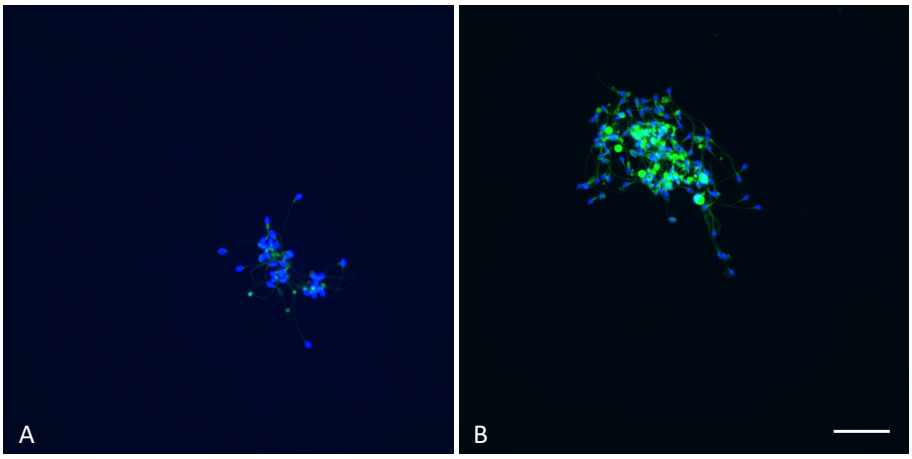

Fig.27: Localization of MT on spermatozoa treated with TiO_2-NPs after 1 hour of exposure. **(A)** Unexposed spermatozoa; **(B)** Spermatozoa exposed to 500 ppm TiO_2-NPs. Nuclei blue (DAPI) and green fluorescent of MT protein. Scale bar 43 µm

Metallothionein MT has been identified in human male genital organs (Suzuki et al., 1991, 1993). In the rat testis, metallothionein has been observed in the seminiferous tubules, especially those containing differentiating spermatogenic cells (Nishimura et al., 1990). Although the biological and physiological function of the metallothionein (MTs) in the male genital organs is unclear, our results highlighted that the MTs expressed by spermatozoa maintain their biological role of detoxification from metals. A higher positivity was observed for 500 ppm concentration.

Finally, the presence of SHBG was observed on the connecting piece of spermatozoa (Figure 28). Previous studied have demonstrate that human SHBG accumulates in the acrosome of sperm in transgenic mice, and it differ from plasma SHBG (Selva et al., 2002). Its function in the acrosome is unknown, but it binds steroid ligands with high affinity. Then the positivity increasing with the concentration of TiO_2-NPs can be connected to the activity of TiO_2- NPs like endocrine disruptor as well as explain in the results of adults zebrafish exposed to TiO_2-NPs.

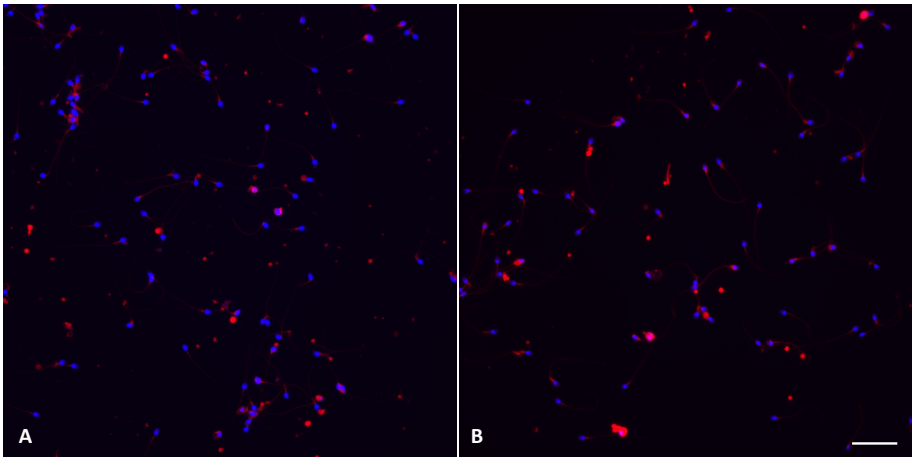

Fig.28: Localization of SHBG on spermatozoa treated with TiO_2-NPs after 1 hour of exposure. **(A)** Unexposed spermatozoa; **(B)** Spermatozoa exposed to 500 ppm TiO_2-NPs. Nuclei blue (DAPI) and red fluorescent of SHBG protein. Scale bar 43 µm

12. Conclusions

Fertility and successful reproduction are of vital importance to sustain a species. In recent years, industrialized countries have recorded a declining trend in male reproductive health which has been compromise by the endocrine disruptors. Among the classic endocrine disruptors also nanoparticles have demonstrated the ability to induce toxicity on the reproductive system, for this reason there is a greater need for knowledge of NP-induced reproductive toxicity, since the production of engineered nanoparticles is continuously increasing by the Nano Revolution, therefore the risk of exposure to nanoparticles is very common. Through acute and long-term TiO_2-NP exposure experiments in zebrafish respectively on embryos and adults, this research has improved knowledge on the action of TiO_2-NPs as endocrine disruptors. Our results have showed that TiO_2-NPs not interfer with the development of embryos zebrafish, neither cause premature death in embryos, however the embryos have showed an alterations on beat heart, body length and above all an increase of the oxidative stress with the production of ROS and the expression of biomarker associated to endocrine disruptors. The expression of the SHBG protein corresponding to the androgen binding protein (ABP) is increased in the presence of TiO_2-NPS both in zebrafish larvae and in male gonads. This result is important because ABP is known to increase with xenobiotics. An increase in its gene expression has been recorded in the testes and consequently it can be assumed that the TiO_2-NPs have an androgenic-like effect, as suggested by the increase of gene expression of SRD5A2, an enzyme which convert testosterone (T) to dihydrotestosterone (DHT). Bara and Kaul (2018), have showed that ZnO-NPs *in vitro* improved the steroidogenic activity of Leydig cells. This upregulation of spermatogenesis has consequently caused an alteration in testicular morphology which can be reflected in altered spermatogenesis. Oxidative stress also plays an important role. Finally, *in vitr*o experiments on human spermatozoa have shown that the toxic effect of TiO_2 nanoparticles is linked to an increase in sperm DNA fragmentation, while the parameters of viability and motility were not so much altered.
This can certainly depend on the exposure time considered as well as on the shape and size of the nanoparticles. Our results give additional information to the data in the literature on the toxicity of TiO_2-NP whose knowledge is therefore never enough because for example the toxic effects of engineered nanoparticles focus mainly on embryonic development and little on the reproductive system.

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