SAPIENZA
Università di Roma
Facoltà di Farmacia e Medicina

Ph.D. in
MORPHOGENESIS AND TISSUE ENGINEERING

XXXV Ciclo
(A.A. 2021/2022)

Toxicogenomic effects of direct Benzo[a]pyrene-exposure on adult testicular organotypic culture

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1. SUMMARY

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon generated by the incomplete combustion of organic compounds. BaP is an inert molecule, however, once inside cells it is bioactivated into a series of toxic metabolites that can damage cellular DNA, causing cell apoptosis and cancer (Boström et al., 2002). Liver and lung are the main responsible for the biotransformation of BaP and the toxic metabolites produced can reach peripheral organs through the circulation (Ramesh et al., 2002).

Although it is known that BaP can impair male fertility, so far it has never been demonstrated whether the testis has its own ability to metabolize BaP, generating those toxic metabolites responsible for the damage, or whether it is a BaP-resistant organ, injured by BaP products generated in other organs. For this purpose, we investigated the toxicogenomic effect of direct BaP exposure on adult testicular organotypic cultures, carrying out this study using both murine and human fresh and cryopreserved samples.

Overall, our work has shown for the first time that alterations in the expression of genes responsible for BaP metabolism occur in testis directly exposed to this pollutant, demonstrating the sensitivity of the testis to BaP. By using fresh and cryopreserved human biopsies we showed inter-individual differences and a general lower responsiveness of the cryopreserved tissues compared to fresh tissues. Furthermore, we demonstrated BaP-induced modulation in the expression of steroidogenic genes, which suggests an impairment of testosterone production in line with published data in rodents. Finally, we observed a significant increase in cell apoptosis in BaP-treated samples, suggesting that the testis has its own ability to bio-activate BaP.

2. INTRODUCTION

2.1 Overview on the testis

Testis is the male reproductive organ. It yields two main functions: the production of spermatozoa in a process called spermatogenesis, and the production of sexual hormones in a process called steroidogenesis. These functions are crucial for the maintenance of male fertility (Weinbauer et al., 1997). The mammalian testis is covered by a protective fibrous capsule, the tunica albuginea, composed mainly of collagen with some fibroblastic and smooth muscle elements (Fig 1). Besides its protective function, this capsule is also involved in regulating blood flow, temperature, pressure, and sperm movement. The capsule extends from the tunica inward, forming the testicular parenchyma. Functionally, the testis parenchyma is organized into two major compartments: the tubular and the intertubular one.

The tubular compartment is composed of seminiferous tubules which can be divided from the outer wall to the center into tunica propria, seminiferous epithelium and tubular lumen. The tunica propria contains the peritubular smooth muscle-like myoid cells, that form one (in mice and rats) to several layers (in humans and marsupials) according to the species, and contain collagen and laminin fibers (Maekawa et al., 1996). The underlying seminiferous epithelium is a highly organized tubular lining consisting of germ cells and Sertoli cells, the only somatic cell type of the seminiferous epithelium. No blood vessels, nerves or other cell types are present in the seminiferous epithelium. Spermatogenesis takes place within the seminiferous tubules. During this process, the male germ cells differentiate to form spermatozoa. Spermatozoon is the male gamete with a haploid set of chromosomes. The fusion of the spermatozoon with the female gamete generates a new diploid cell, the zygote, from which a new

individual will develop. Inside the seminiferous tubule, therefore, there are male germ cells with different degrees of differentiation. Spermatogonia are the most undifferentiated germ cells in the adult and are located in the most peripheral part of the tubule, in direct contact with the basement membrane. As the germ cells differentiate into primary spermatocytes, secondary spermatocytes and spermatids, they move toward the tubular lumen. Spermatozoa are then formed in the lumen. Sertoli cells constitute the somatic component of the seminiferous epithelium and they play a fundamental role in the control and regulation of spermatogenesis. Indeed, these cells provide nutrition, protection and support to germ cells. Furthermore, they secrete the fluid responsible for tubular lumen formation and spermatozoa transport (França et al., 2016)

The intertubular compartment, also called the interstitium, consists of a greater diversity of cell types. Blood and lymphatic vessels, nerves, mast cells, macrophage and dendritic cells, as well as fibroblasts and connective tissue fibers are found in the interstitium, and their amount varies significantly according to the species. Leydig cells are usually the most frequent cell type present, and they have the function of producing steroid hormones during the process of steroidogenesis (Svechnikov et al., 2010).

Although spermatogenic and steroidogenic activities occur, as seen, in two distinct functional compartments of the testis, they are closely linked by very complex cellular and physiological interactions. This is because the correct relationship between the two processes is essential for maintaining male fertility.

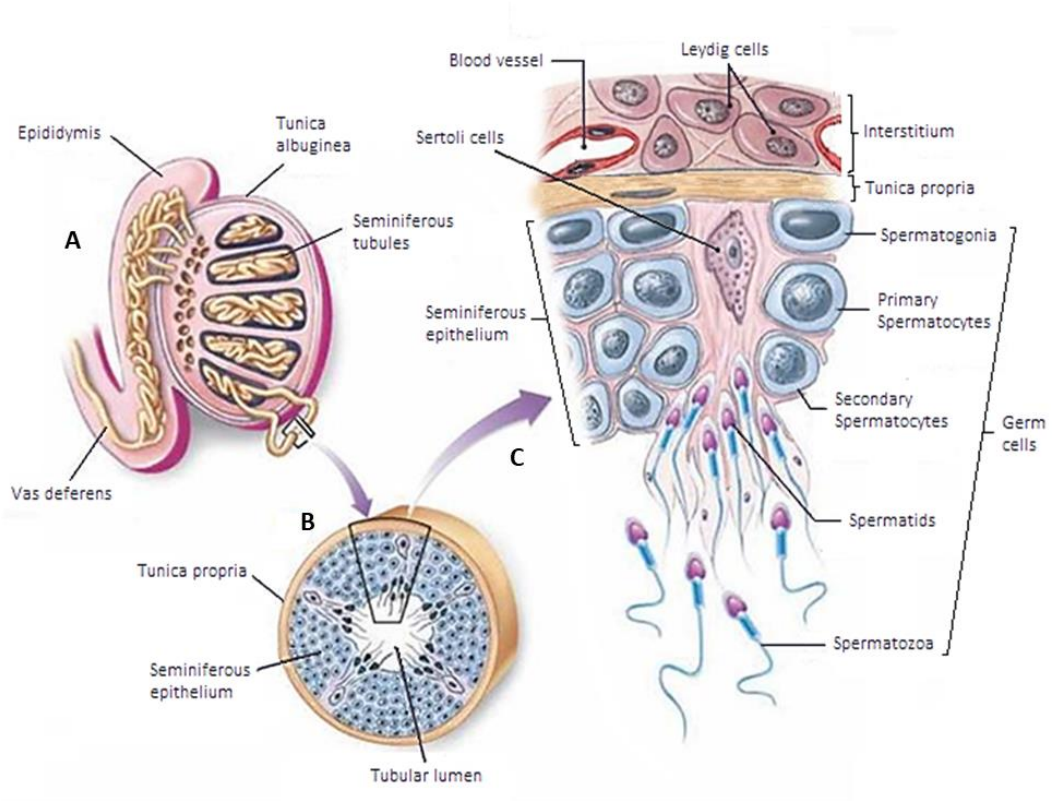

Fig. 1 *Morphology of the testis. (A) Schematic representation of the testis, surrounded by tunica albuginea and composed of seminiferous tubules. (B) Cross-section of a seminiferous tubule, consisting, starting from the outer wall towards the center, of the tunica propria, the seminiferous epithelium and the tubular lumen. (C) Detail of the cross-section of the seminiferous tubule. Inside the seminiferous tubules, where spermatogenesis occurs, there are the germ cells with different degrees of maturation (spermatogonia, spermatocytes, spermatids and spermatozoa, respectively from the periphery of the tubule to the lumen) and the somatic cells of the Sertoli. Seminiferous tubules are surrounded by the interstitium, where there are several types of somatic cells, including Leydig cells, specialized in steroidogenesis. Modified from https://schoolbag. info/biology/living/248.html*

2.1.1 Spermatogenesis

Spermatogenesis is a highly specialized testicular function. It is the differentiation process that occurs inside the seminiferous tubules and transforms the most undifferentiated germ cells into mature spermatozoa, suitable for successful reproduction. Spermatogenesis begins during puberty, and continues throughout the adult life of the male, ensuring continuous production of sperm. During their maturation, germ cells gradually move in a coordinated and organized way from the periphery to the lumen of the seminiferous tubule. At the end of this process, spermatozoa are generated and released into the tubular lumen. Spermatogenesis involves both mitotic and meiotic divisions and extensive cellular remodelling. It can be divided into three phases: (1) proliferation and differentiation of spermatogonia, (2) meiosis, and (3) spermiogenesis, that will be described below (Fig. 2).

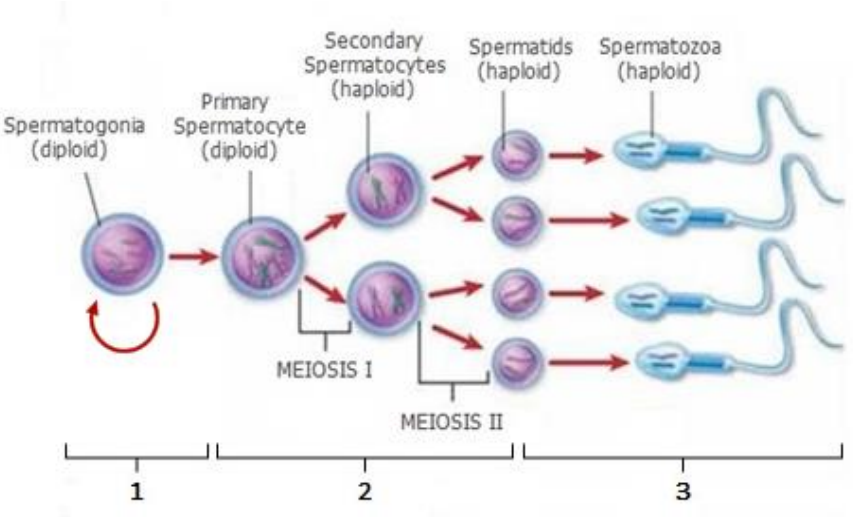

Fig. 2 *The three phases of spermatogenesis. (1) Proliferation and differentiation of spermatogonia, (2) Meiosis and (3) Spermiogenesis. Modified from https://schoolbag.info/biology/living/248.html*

Spermatogenesis starts with spermatogonia, which are located on the basement membrane of the seminiferous tubule. The spermatogonial compartment has been traditionally divided into two categories: the undifferentiated and the differentiating spermatogonia (de Rooij & Russell, 2000). Among the undifferentiated spermatogonia there are also rare, adult, unipotent stem cells, called spermatogonial stem cells (SSCs). All spermatogonia are diploid cells. In the first phase, the cells of the spermatogonial compartment undergo a series of mitotic divisions, which guarantee the maintenance of the pool of spermatogonia and SSCs that are periodically lost. At the end of the last mitotic division, the more differentiated differentiating spermatogonia generate primary spermatocytes, the diploid cells that enter meiosis.

Meiosis is the second phase of spermatogenesis. This process is composed of two successive cell divisions (meiosis I and meiosis II), not interspersed with DNA duplication. Since during meiosis I the rearrangement of chromosomal material occurs through crossing-over, at the end of meiosis II four different haploid cells, the spermatids, are formed starting from a single initial primary spermatocyte. The primary diploid spermatocyte and the four haploid spermatids are connected by an intermediate cellular phase, which forms at the end of meiosis I and enters meiosis II: the secondary spermatocyte. Secondary spermatocytes are haploid cells, two are formed from each primary spermatocyte and each in turn generates two spermatids.

The final stage of spermatogenesis is spermiogenesis, a complex process that transforms round spermatids after meiosis into spermatozoa. During this transformation, the spermatid significantly reduces its size due to the removal of cytoplasm at spermiation, and the strong compaction of the genetic material. Its shape elongates and a flagellum develops, which will make the spermatozoon a mobile cell capable of reaching the female gamete.

Another typical feature that develops in the spermatozoon is the acrosome, an apical organelle necessary to ensure fertilization of the egg cell.

2.1.2 Steroidogenesis

The second main function of the testis is steroidogenesis, a process responsible for the production of steroid hormones, cholesterol-derived hormones that act as chemical messengers in the body. Testicular steroidogenesis occurs in the interstitial compartment of the testis, within the Leydig cells. Steroid hormones produced in the gonads are called sex steroids and are divided into androgens, estrogens and progestogens. Sex steroids, especially androgens, are crucial for the completion of full spermatogenesis and for the development and maintenance of sexual male characteristics (Weinbauer et al., 1997). Androgens and probably other steroids, including estrogen, are especially necessary for spermiogenesis and sperm release. Although they regulate the maturation of germ cells, androgens control spermatogenesis through Sertoli cell intermediation, as germ cells per se do not express receptors for these hormones (Walker, 2021).

Steroidogenesis in men is controlled by the hypothalamic-pituitary-gonadal axis. Hypothalamic neurons synthesize and release gonadotropin-releasing hormone (GnRH), which in turn, binding to receptors on the anterior pituitary gland, stimulates here the synthesis and secretion of luteinizing hormone (LH). Through the circulation, LH reaches the testis and, by binding to the LH receptors on the Leydig cells, triggers the production of sex steroid hormones (Fig. 3). Briefly, when the LH receptors on the Leydig cell membrane become activated, the cAMP/protein kinase A (PKA) pathway is stimulated in the cytoplasm of these cells (Andric & Kostic, 2019). This event on the one hand promotes the

transport of cholesterol into the mitochondria and on the other induces the expression of the enzymes involved in the biosynthesis of testosterone. Cholesterol is transported to the inner mitochondrial membrane of Leydig cells thanks to the action of a multi-protein complex which includes the steroidogenic acute regulatory (called StAR or STARD1) protein (Rone et al., 2012). Here, cholesterol is converted into pregnenolone by the cholesterol side chain cleavage enzyme, a cytochrome P450 (CYP) enzyme known as CYP11A1 (or P450Scc). When pregnenolone is formed, it diffuses into the endoplasmic reticulum where the biosynthesis of sex steroid hormones proceeds via Δ4 and Δ5 pathways. These two pathways produce different sex hormones, but they converge in the synthesis of testosterone, the principal male sex hormone. At the end of testicular steroidogenesis, some testosterone is converted by 5α-reductase to the more potent androgen, dihydrotestosterone (DHT), and an even smaller part of testosterone is converted by the enzyme CYP19A1 (also known as aromatase) to 17ß-estradiol (Miller & Auchus, 2011)

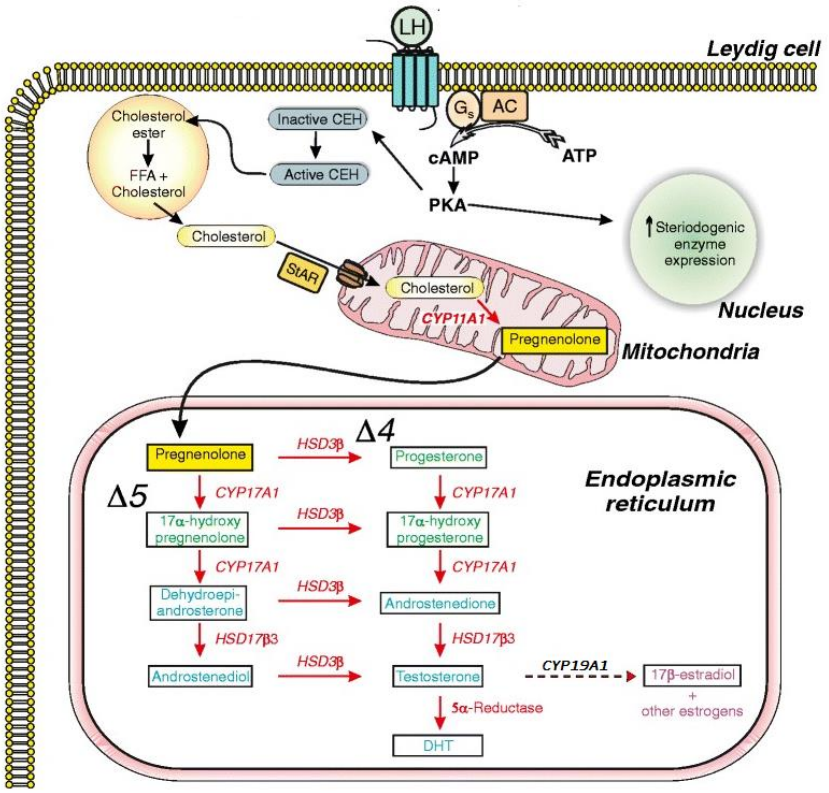

Fig. 3 *The testicular steroidogenesis. Modified from Ayaz & Howlett, 2015*

2.2 Overview on Benzo[a]pyrene

Benzo[a]pyrene (BaP) is a well-known five-ring polycyclic aromatic hydrocarbon (PAH) generated by the incomplete combustion of organic materials (Hattemer-Frey & Travis, 1991; Boström et al., 2002). It is released into the atmosphere as a component of smoke from natural activities, such as forest fires and volcanic activity, but above all from anthropogenic activities, such as industrial processes, vehicle exhaust, cigarettes, and through the combustion of fuels (such as wood, coal and petroleum products) (Boström et al., 2002; Agrawal et al., 2018). BaP persists in the atmosphere in the particulate phase for long periods of time and is therefore efficiently transported over long distances. Thanks to its lipophilic nature and low water solubility, once deposited in soil or water, it strongly adsorbs to particulates and sediments and degrades slowly over several years (Hattemer-Frey & Travis, 1991). BaP is therefore an environmental pollutant and is ubiquitously present in the surrounding environment: in the air, water, soil, some food products, some pharmaceuticals (Fig. 4).

BaP is absorbed by organisms mainly by inhalation and orally, to a lesser extent by the dermal route. BaP exposure is harmful to health and causes adverse effects including carcinogenesis, teratogenicity, neurotoxicity, reproductive toxicity and immuno-suppression. It affects apoptosis, lipid metabolism and induces epigenetic modifications (Agrawal et al., 2018). However, BaP is actually an inert molecule (Gelboin et al., 1969; Shimizu et al., 2000). The real responsible for the toxic effects of BaP are some molecules that are formed during the metabolism of BaP, which takes place inside the cells (see the next paragraph).

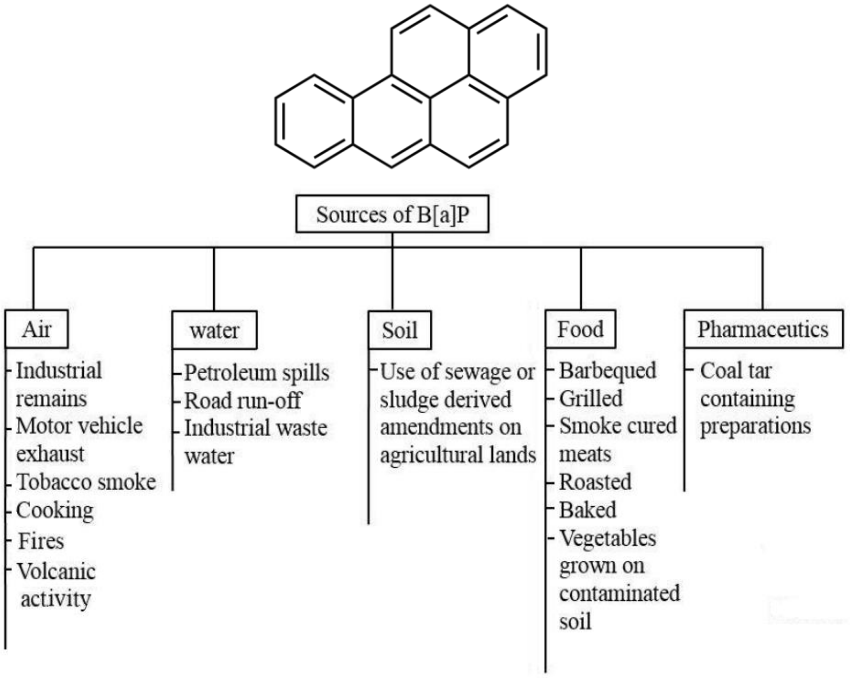

Fig. 4 *The molecular structure and sources of Benzo[a]pyrene (B[a]P). Modified from Agrawal et al., 2018*

2.2.1 Metabolism of Benzo[a]pyrene

BaP is biologically inert and requires metabolic activation to exert its toxicity. The cellular metabolic pathway triggered by BaP exposure is illustrated in Figure 5. Once absorbed by the body, BaP enters the cells, crossing the plasma membrane thanks to its lipophilic nature (Plant et al., 1985). In the cellular cytosol of almost all mammalian tissues and organs there is a ligand-activated receptor/transcription factor, called aryl hydrocarbon receptor (AhR) (Revel et al., 2003). AhR is involved in the xenobiotic metabolism and is activated by binding to numerous types of ligands, including BaP (Degroot et al., 2011). When AhR is in its inactivated state, it forms a multiprotein complex with the heat shock protein 90 (Hsp90) (Chen & Perdew,1994), hepatitis B virus X-associated protein (XAP2) (Meyer et al., 1998), and p23 protein (Kazlauskas et al., 1999). When BaP enters the cell cytoplasm, it binds to AhR which changes conformation and is activated. AhR-BaP then dissociates from the proteins of the inactive complex and binds with the aromatic hydrocarbon nuclear transporter (ARNT), which allows the receptor/transcription factor to enter the nucleus (Whitelaw et al., 1993; McGuire et al., 1994). Here, the active AhR-ARNT complex is associated with xenobiotic response elements (XREs), sequences present in the promoter of numerous genes involved in xenobiotic metabolism. It is in this way that BaP, through the activation of AhR, triggers the induction of a series of enzymes involved in its own metabolism (Matsushita et al., 1993; Whitelaw et al., 1993).

The metabolism of BaP is divided into two phases: phase I (activation), in which the transformation of BaP into toxic metabolites inevitably takes place; and phase II (conjugation/detoxification), in which the metabolites of BaP are converted into more hydrophilic molecules, suitable for excretion from the body.

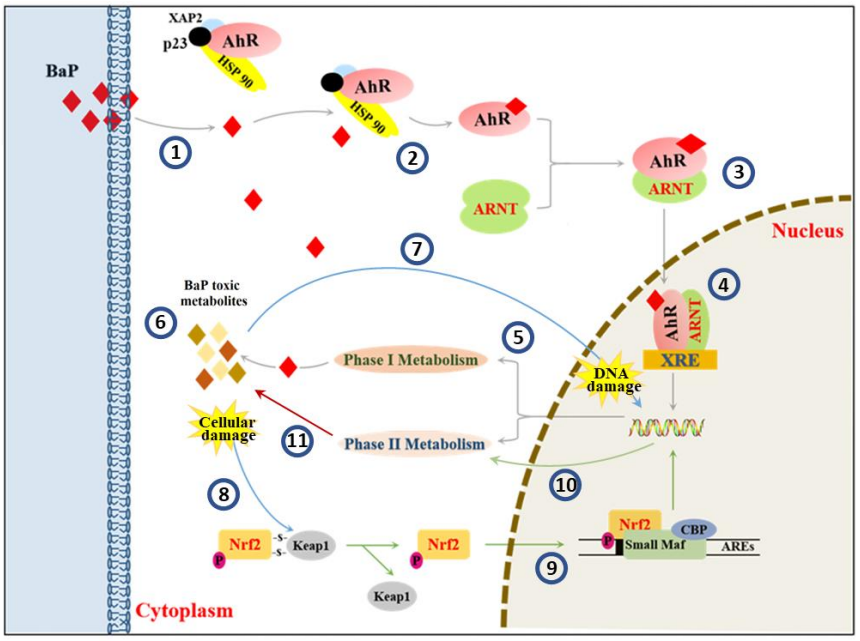

Fig. 5 *Schematic representation of metabolic pathway triggered by BaP exposure. (1) BaP crosses the cell membrane and, in the cytoplasm, (2) binds and activates the AhR receptor. (3) Upon binding to ARNT, the AhR-BaP complex enters the nucleus, (4) binds the XRE sequence on the promoter of AhR-inducible genes, and (5) induces the expression of drug-metabolizing enzymes, especially of the phase I. (6) The action of CYP1A1 and CYP1B1, together with that of EPHX1, transforms BaP into a series of toxic metabolites, some of which (7) damage DNA. (8) The presence of cellular damage (oxidative stress and DNA damage) induces the activation of NRF2. (9) Entering the nucleus, NRF2 stimulates the expression of genes having the ARE sequence on the promoter. (10) The expression of phase II metabolic enzymes is thus induced, which (11) eliminates the toxic products of BaP. Grey arrows indicate BaP/AhR-mediated effects. The blue arrows indicate the effects caused by the presence of the toxic products of BaP. Green arrows indicate NRF2-mediated action. Finally, the red arrow indicates the detoxifying effect of phase II metabolic enzymes on BaP toxic metabolites. Modified from Wang et al., 2020.*

Phase I of xenobiotic metabolism is triggered by AhR activation (Tijet et al., 2006), while phase II is triggered by the activation of another transcription factor, the nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Itoh et al. 1997; Ramos-Gomez et al., 2001). The two metabolic phases are connected to each other as NRF2 is itself an AhR-target gene (Miao et al., 2005) and as it is activated in response to ROS-induced oxidative stress and in the presence of reactive electrophiles (Ishii et al. 2000; Itoh et al., 2003), which are produced during phase I of BaP metabolism. Furthermore, several metabolic enzymes are regulated by both transcription factors.

As illustrated in figure 6, at the beginning of phase I of metabolism, BaP is converted into epoxy, passing through a radical cation form, thanks to the action of several enzymes, the most important of which are CYP1A1 and CYP1B1 (CYPs) (Shimada et al., 1999). The action of epoxide hydrolase 1 (EPHX1 or EH) transforms the epoxide form of BaP into a diol. BaP diols are converted to BaP-7,8-diol-9,10-epoxide (BPDE) by CYPs or to catechols by aldo-keto reductases (AKRs). EPHX1 can further metabolize BPDE to tetraols. On the other hand, the BaP catechol can be converted to quinone, through a cyclic redox reaction which also leads to the formation of reactive oxygen species (ROS). BaP cations, BPDE, BaP quinones and ROS are the dangerous metabolic products of BaP as they can covalently bind to cellular macromolecules such as DNA, RNA or proteins causing deleterious effects. The formation of DNA adducts, in particular, can cause strand breaks, apoptosis, mutations, malignant transformation of the cell (Muñoz & Albores, 2011; Moffat et al., 2015).

Under basal conditions, NRF2 localizes in the cytoplasm bound to the kelch-like ECH-associated protein 1 (Keap1), which targets NRF2 to proteasomal degradation (Haarmann-Stemmann & Abel, 2011). Following exposure to BaP, NRF2 activity increases not

only because NRF2 expression is induced (Miao et al., 2005), but also because expression of the Keap1 repressor is suppressed (Nguyen et al., 2010). Once activated, NRF2 dissociates from Keap1 and translocates into the nucleus. Here this transcription factor forms a heterodimer by associating with partner proteins, especially small Maf proteins, and then binds to antioxidant response elements (ARE) in the promoter region of the target genes to induce their transcription (Itoh et al. 1997). NRF2 then directly induces the expression of cytoprotective genes, including copper-zinc superoxide dismutase (SOD1) and catalase (Milani et al., 2011; Chen & Kunsch, 2004), and several uridine diphosphate–glucuronosyl transferases (UGTs) and glutathione S-transferases (GSTs) isoforms (Haarmann-Stemmann & Abel, 2011). SOD1 and catalase are important for quenching and detoxifying ROS (Chen & Kunsch, 2004), while GSTs and UGTs combine the metabolites of BaP with hydrophilic moieties to enhance the solubility in water and the consequent elimination of these toxic molecules. In particular, UGTs conjugate to glucuronosyl BaP tetraols, diols and quinines, while GSTs conjugate to glutathione BaP epoxides, diol epoxides and quinones (Moffat et al., 2015).

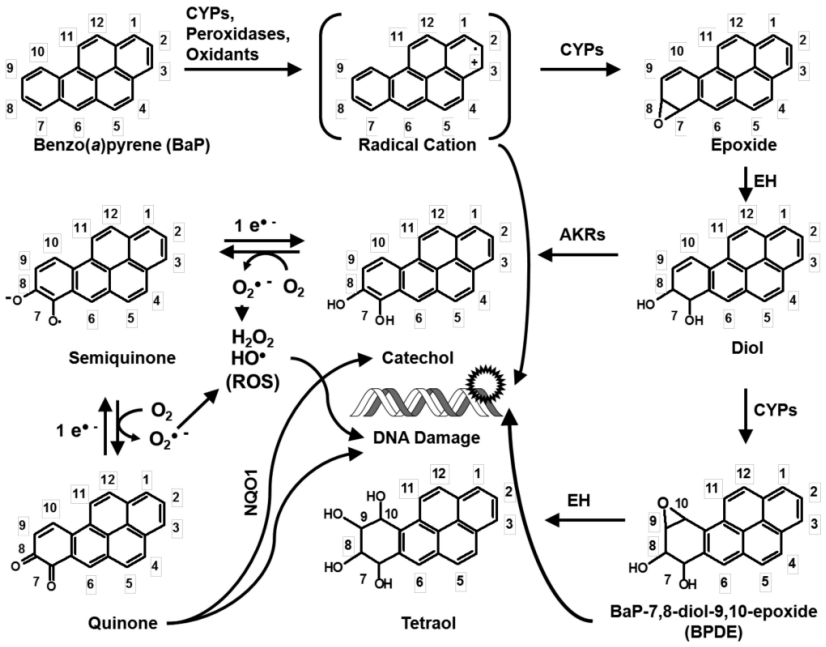

Fig. 6 *The toxic metabolites of BaP, produced during phase I of AhR-induced metabolism. Only representative BaP metabolites (modified at positions 7, 8, 9 and 10) are shown. In addition, BaP metabolism can yield many other hydroxy-, oxide-, dihydroxy- and quinone- related compounds at each of the 12 carbon atoms. From Moffat et al., 2015.*

2.2.2 Benzo[a]pyrene and male fertility

Several studies in the literature demonstrate that BaP has adverse effects on male reproductivity.

For ethical reasons, human studies on this topic concern only *in vitro* experiments that have almost exclusively evaluated sperm parameters, due to the ease of finding this type of sample. These human studies can be classified as prospective or retrospective. In prospective studies, sperm is collected from donors considered to be healthy. The spermatozoa obtained from these samples are then exposed to BaP for known times and doses (Sipinen et al., 2010; Mukhopadhyay et al., 2010; Alamo et al., 2019). In retrospective studies, instead, the analysis is carried out on sperm samples of subjects already exposed to BaP for smoking and drinking habits, for occupational history or because they live in polluted areas (Gaspari et al., 2003; Han et al., 2011; Oliveri et a., 2017**)**. Retrospective studies are by nature the most realistic, but at the same time donors are not properly exposed to BaP alone, but to a complex mixture of pollutants. Prospective studies, instead, allow to verify the exclusively BaP-mediated effects, with the disadvantage of less realism. Anyway, both types of human studies agree that BaP exposure correlates with altered sperm quality and quantity.

On the other hand, mice and rats have been extensively used to evaluate the effect of prolonged BaP exposure on male fertility in *in vivo* studies. BaP was usually administered to these animals by gavage or by inhalation and, following animal sacrifice, several tests were conducted to assess BaP-mediated damage at testicular level. In this way, it has been shown that prolonged exposure to BaP alters normal testicular morphology (Jeng et al., 2015; Sheweita et al., 2016). In fact, in rodents, following sub-chronic exposures, a considerable variation in the interstitial volume and in

the size of the seminiferous tubules is observed. The tubules become irregular, with reduced integrity. The volume of the seminiferous epithelium and the lumen is reduced and the connection with the basement membrane is lost (Fig. 7). This is also due to the BaP-mediated apoptosis that has been observed in all testicular populations: in germ cells and Sertoli of seminiferous tubules and in Leydigs of the interstitium (Jeng et al., 2015; Banerjee et al., 2016). Consequently, testicular weight is decreased in animals exposed for long periods to BaP (Ramesh et al., 2008; Archibong et al., 2008; Jeng et al., 2015; Banerjee et al., 2016). Numerous studies have also shown that BaP exposure reduces intratesticular and plasma testosterone levels (Inyang et al., 2003; Ramesh et al., 2008; Archibong et al., 2008; Chung et al., 2011; Sheweita et al., 2016; Banerjee et al., 2016). The cause is on the one hand the death of some Leydig cells, but above all the alteration in the remaining Leidygs of the activity and/or of the expression of the enzymes involved in steroidogenesis, such as STARD1, CYP11A1 and CYP19A1 (Chung et al., 2011; Sheweita et al., 2016). BaP-induced impairment of both testicular functions, spermatogenesis and steroidogenesis, results in the reduction in the quantity and quality of sperm. The spermatozoa of rodents exposed *in vivo* to BaP, in fact, show a lower density, reduced vitality and motility and have a series of morphological anomalies, such as abnormal heads and curled tails (Inyang et al., 2003; Ramesh et al., 2008; Archibong et al., 2008; Jeng et al., 2015; Banerjee et al., 2016) (Fig. 8).

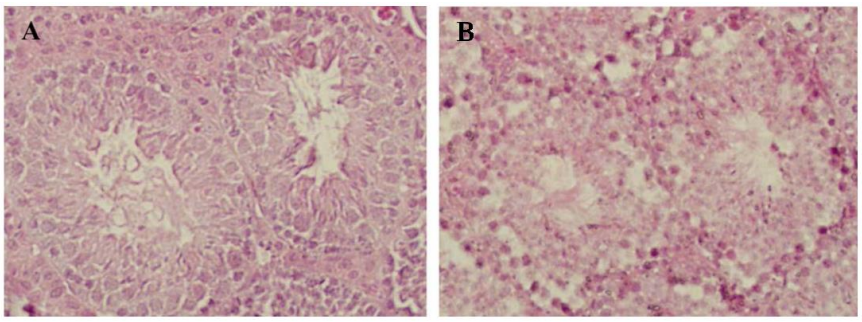

Fig. 7 *Alteration of normal mouse testicular morphology caused by exposure to BaP. (A) Control, 100x showing seminiferous tubule integrity, organized seminiferous epithelium, normal luminal space, and numbers of maturing spermatozoa; (B) 100 mg/kg/day of BaP for 30 days, 400x with noticeable variation in the diameter of the seminiferous tubules and reduced integrity, a decrease of the seminiferous epithelium, and a decrease in the luminal volume of maturing spermatozoa, increasing variance in the seminiferous tubule size, and organization with a pronounced and uneven Sertoli cell maintenance of the seminiferous epithelium. Modified from Jeng et al., 2015*

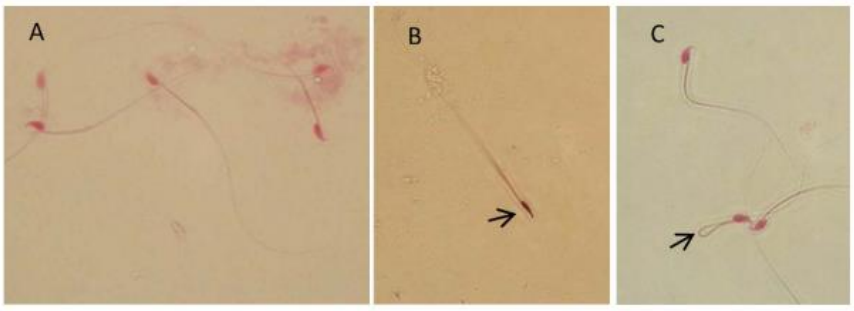

Fig. 8 *Alteration of normal mouse spermatozoa morphology caused by exposure to BaP. (A) The control group with normal morphology; (B) Spermatozoa from mice exposed to 50 mg/kg of BaP for 30 days [arrow indicates an abnormal spermatozoon head]; (C) Spermatozoa from mice exposed to 50 mg/kg of BaP for 30 days [arrow indicates an abnormal spermatozoon curled tail]. Modified from Jeng et al., 2015*

3. AIM

Benzo[a]pyrene exposure causes numerous adverse health effects (eg carcinogenesis, teratogenicity, neurotoxicity, immuno-suppression) and it has already been shown in the literature that this pollutant has also negative effects on male fertility. Rodents exposed *in vivo* for a long time to BaP showed alterations of the normal testicular morphology, with associated impairment of the two main testicular functions, spermatogenesis and steroidogenesis. Due to the apoptosis and the alteration of gene expression induced by BaP in the testicular cells, there is, in fact, a reduced production of testosterone by the Leydig cells, the death of both somatic and germinal testicular cells and there is consequently less support for spermatogenesis. This results in reduced sperm production and consequently impaired fertility in exposed adult rodents (Inyang et al., 2003; Ramesh et al., 2008; Archibong et al., 2008; Chung et al., 2011; Jeng et al., 2015; Sheweita et al., 2016; Banerjee et al., 2016). The reduction in sperm density and the appearance of morphological abnormalities in spermatozoa following exposure to BaP has also been demonstrated *in vitro* in humans, with prospective or retrospective studies that have evaluated only sperm parameters (Sipinen et al., 2010; Mukhopadhyay et al., 2010; Alamo et al., 2019; Gaspari et al., 2003; Han et al., 2011; Oliveri et a., 2017).

Although the negative effect of BaP on male fertility is already proven, the effect of direct exposure to BaP on the adult testis has never been addressed. As mentioned, BaP is an inert molecule that must be metabolized to exert its toxicity. Animal models show that the lung and liver are the major organs in which BaP metabolites are produced following oral and inhalation exposure (Ramesh et al., 2002). However, to date, it is not known whether the testis is an organ capable of bioactivating BaP or whether the observed damage to fertility is due to the action of toxic metabolites

produced in other organs and which reach the testis through the circulation.

The goal of the thesis was therefore to verify the impact on the testis of direct exposure to BaP. This study was performed on organotypic cultures made with human and mouse testicular fragments and focused on the following objectives:

a) To analyze whether the human testis can metabolize BaP, verifying the induction of the enzymes involved in xenobiotic metabolism (CYP1A1, CYP1B1, EPHX1, NRF2, SOD1, CATALASE, GST, UGT)
b) To analyze whether BaP impacts steroidogenesis, checking the expression of the genes involved in the production of steroid hormones (STARD1, CYP11A1, CYP19A1)
c) To test whether cells of the seminiferous epithelium are susceptible to apoptosis following BaP exposure, by analyzing the induction of apoptotic genes (P53 and BAX) and using the TUNEL assay.

4. RESULTS

4.1 Effects of BaP exposure on cryopreserved human testis culture

To analyze the effects of direct BaP exposure on testicular cultures we employed cryopreserved testicular biopsies (n=6). Thawed samples were used for setting organ cultures in which testis fragments were exposed to increasing doses of BaP (0 to 10 μM) for 12h. The effects of BaP on the modulation of the expression of genes of interest were evaluated using ddPCR, while the BaP-mediated apoptotic effect was studied by TUNEL assay.

4.1.1 BaP-mediated effect on the expression of phase I metabolic genes

In order to demonstrate that the testis has its own ability to transform the inert BaP into toxic metabolites, we started by analyzing the alteration of the expression of the genes responsible for the bioactivation of BaP: CYP1A1, CYP1B1 and EPHX1, by ddPCR. As can be seen from figure 9, the response obtained was heterogeneous, depending on the donor. Three out of six donors (ID # 24, 04 and 20) showed a BaP-mediated effect on the expression of genes involved in BaP bioactivation. Among these, ID # 04 is the only one that showed an increase in the expression of both CYP1A1 and CYP1B1, mediated by the highest concentrations of BaP. In ID # 20 BaP 1 and 10 μM increased the expression of CYP1A1, but did not alter that of CYP1B1, while in ID # 24 BaP 10 μM strongly increased the mRNA levels of CYP1B1, without modulating those of CYP1A1. EPHX1 transcript levels drop in culture in all cryopreserved donors. In the responsive donors ID # 24 and ID # 04 the expression of EPHX1 decreased with the highest concentrations of BaP. In ID # 20, instead, EPHX1 levels increased in the presence of all the tested BaP concentrations compared to the culture control. In the remaining

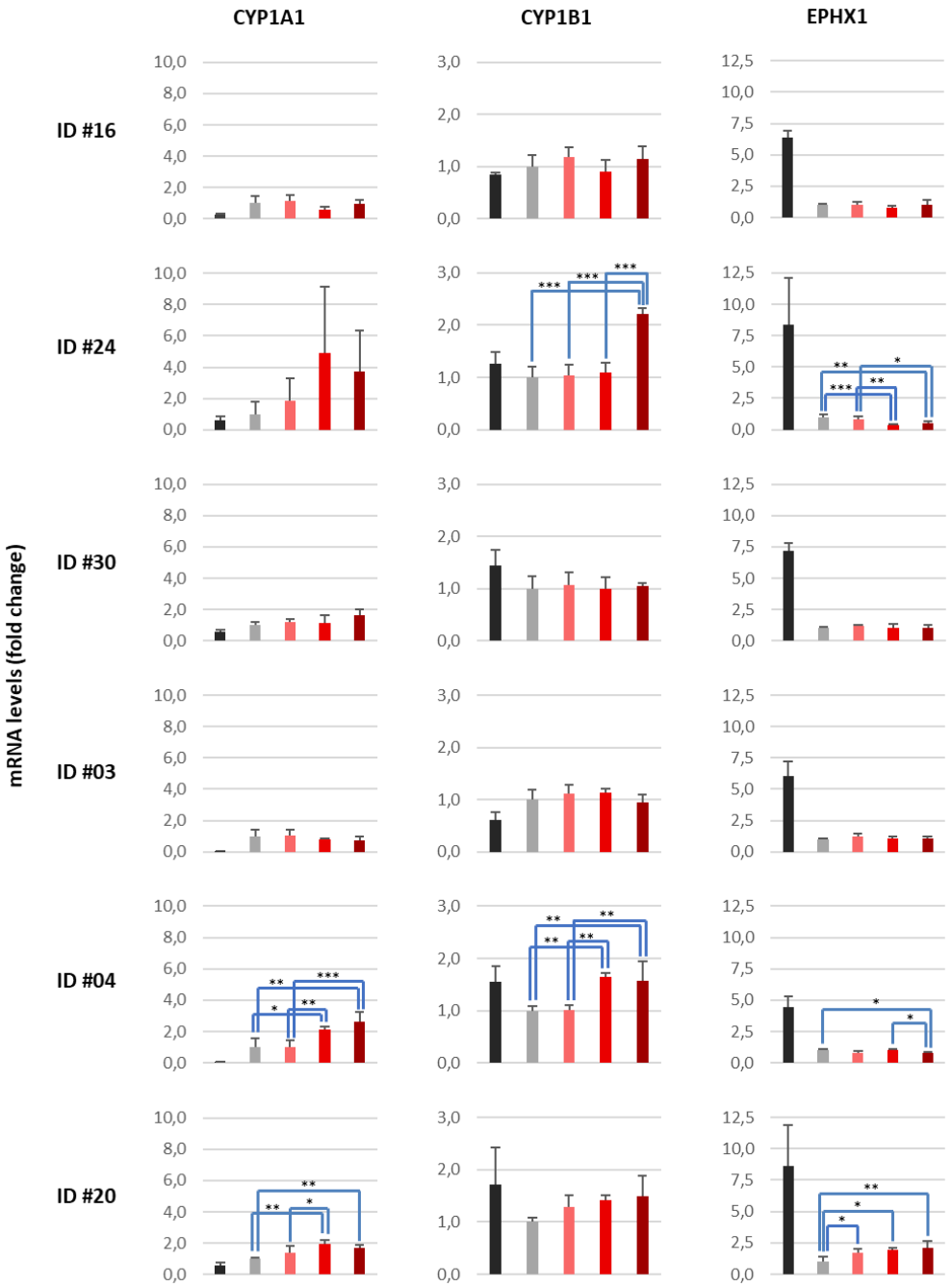
CYP1A1
CYP1B1
EPHX1
ID #16
ID #24
ID #30
ID #03
ID #04
ID #20
mRNA levels (fold change)

FIG. 9 *BaP-mediated modulation of the expression of the most important genes involved in the bioactivation of BaP in cryopreserved human testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar), cultured BaP 0.1 μM (pink bar), cultured BaP 1 μM (red bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

cryopreserved donors, ID # 16, ID # 30 and ID # 03, the expression of the genes involved in BaP bioactivation was not significantly altered.

4.1.2 BaP-mediated effect on the expression of steroidogenic genes

To verify the ability of BaP to impair steroidogenesis in our testis organ cultures, we analyzed the effect of BaP exposure on the alteration of the expression of genes encoding steroidogenic proteins/enzymes: STARD1, CYP11A1 and CYP19A1. Also in this case, the response obtained was not the same for all donors (Fig. 10). Almost no donors showed alteration of STARD1 gene. Only in ID # 24 the highest concentration of BaP caused an evident reduction in the levels of this steroidogenic gene. Since in ID # 30 only the lowest BaP concentration produced a significant effect on the expression of STARD1, we consider this effect to be negligible. ID # 04 and ID # 20 showed an increase in the expression of both CYP11A1 and CYP19A1 genes, following the exposure to BaP 1 and 10 μM. In all cryopreserved donors, CYP11A1 expression was strongly reduced under culture conditions, most noticeably in ID # 24 and less noticeably in ID # 03. In ID # 16, where we observed no alteration of any metabolic gene, BaP 10 μM caused the induction of only CYP19A1. Having considered the effect of BaP 0.1 μM on the expression of STARD1 in ID # 30 to be negligible, in donors ID # 30 and ID # 03 the effect of BaP on the expression of steroidogenic genes was still null, as seen with phase I metabolic genes.

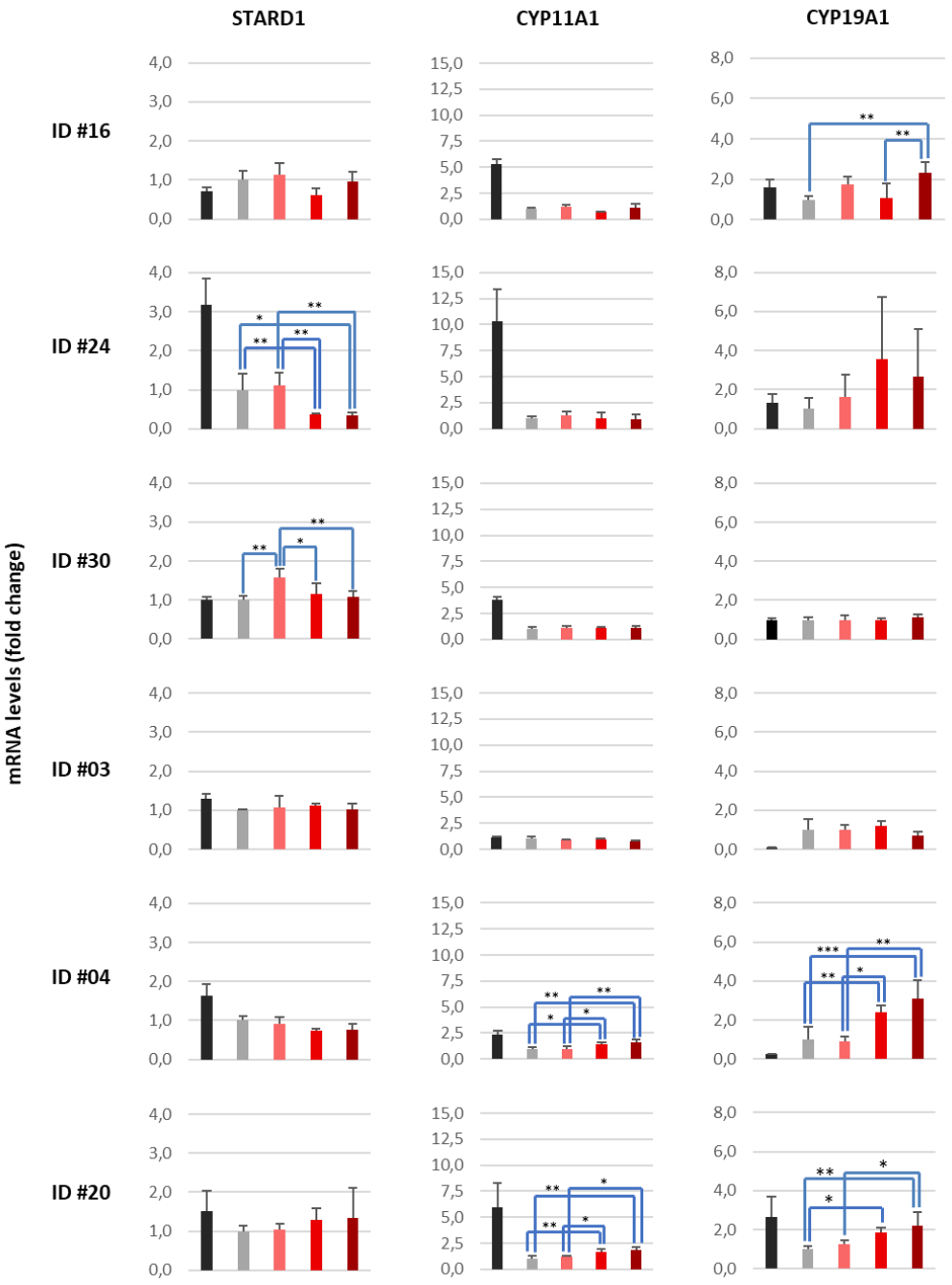
STARD1
CYP11A1
CYP19A1
ID #16
ID #24
ID #30
ID #03
ID #04
ID #20
mRNA levels (fold change)

FIG. 10 *BaP-mediated modulation of the expression of steroidogenic genes in cryopreserved human testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar), cultured BaP 0.1 μM (pink bar), cultured BaP 1 μM (red bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

4.1.3 Apoptotic effect of BaP

The apoptotic effect of BaP exposure on cryopreserved human testis cultures was evaluated by TUNEL assay (Fig. 11). For each donor, along with treated and untreated testis fragments, DNA fragmentation was also evaluated in testis fragments before cryopreservation (fresh) and immediately after thawing (T0). A greater number of apoptotic cells are observed in testis fragments under culture conditions. However, of the six donors analyzed, in only one, ID # 20, a significantly higher number of TUNEL-positive cells was observed in the presence of BaP compared to all other conditions. On the other hand, in ID # 24 a greater number of TUNEL-positive cells was observed in the culture control, rather than in the presence of BaP. These results indicate that the culture conditions themselves induce an increase in DNA fragmentation with no significant effect of BaP treatment over the control (Fig. 12).

A schematic summary of all data obtained with cryopreserved human testis is summarized in Table 1.

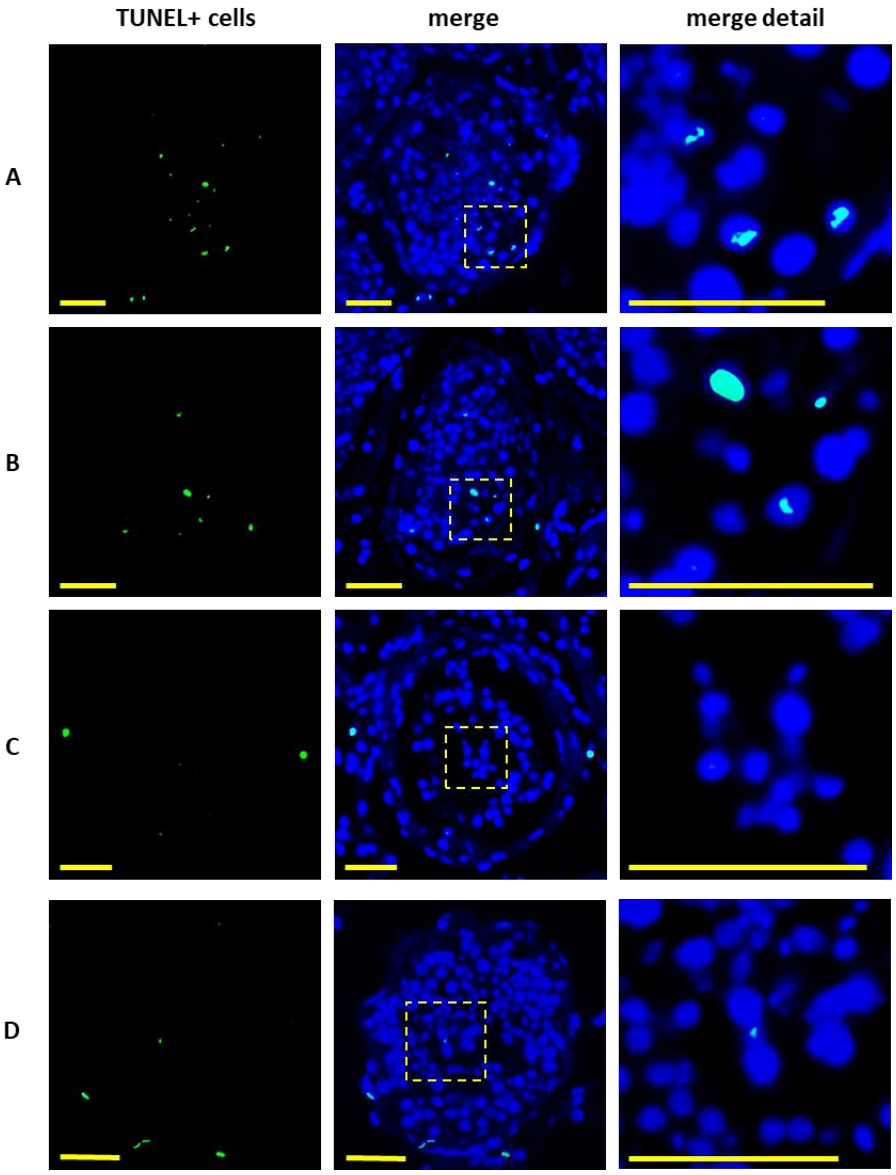

FIG. 11 *Representative images of the TUNEL assay on cryopreserved human testicle specimen (A-C) and its respective fresh condition (D). In each image of the "TUNEL+ cells" and "merge" columns a cross-section of a seminiferous tubule is represented. The "merge detail" column shows the magnification of the dashed box in the corresponding "merge". (A) Cultured BaP 10 μM, (B) cultured BaP 0 μM, (C) uncultured time zero. Yellow bar corresponds to 50 μm.*

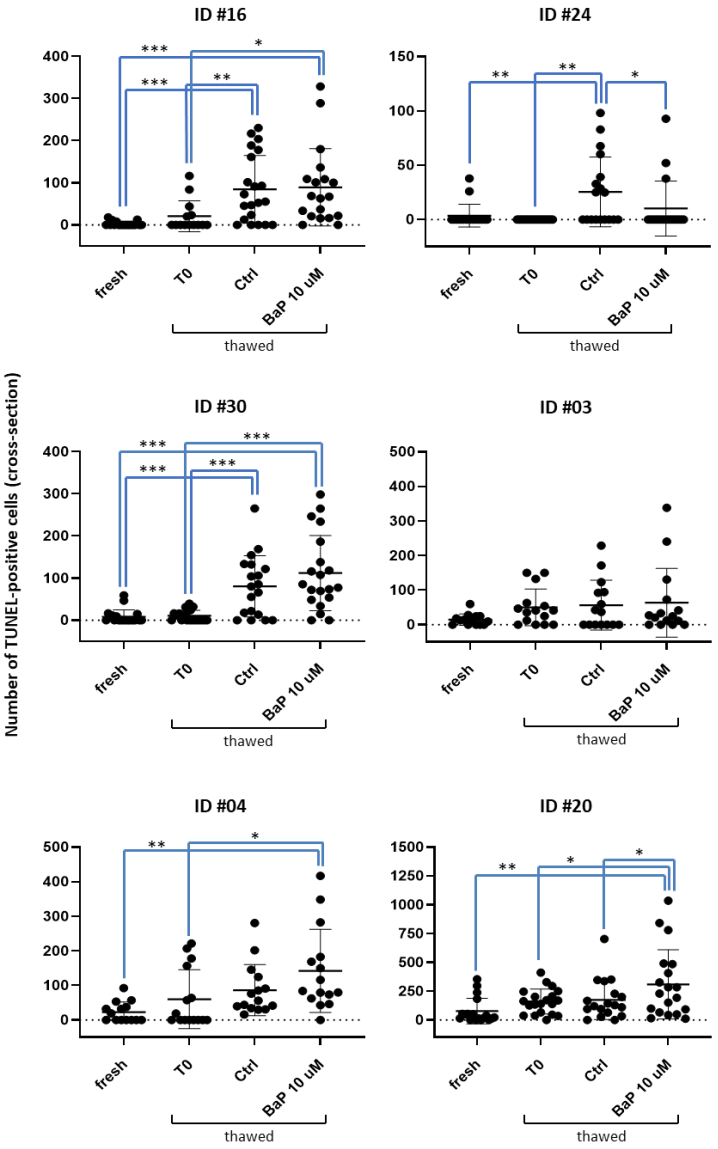

FIG. 12 *BaP-mediated apoptotic effect on human cryopreserved testicular biopsies measured with TUNEL assay. Each point corresponds to the number of TUNEL-positive cells within a cross-section of a seminiferous tubule. For each experiment, 15-20 cross-sections were counted for each experimental condition. Data are expressed as mean ± SD. * p<0.05 ; ** p<0.01 ; *** p<0.001*

4.2 Effects of BaP exposure on fresh human testis culture

We next investigated the effect of BaP exposure on fresh testis fragments that were not cryopreserved (n=6). Also in this study we exposed testicular fragments to BaP for 12h and at the end of the culture we performed the same analyses conducted for the cryopreserved testicular fragments, using the ddPCR and the TUNEL assay. However, since in cryopreserved cultures the BaP-induced effects were observed only at the highest doses of BaP, this time the fresh testicular fragments were treated with BaP 10 μM.

4.2.1 BaP-mediated effect on the expression of phase I metabolic genes

In contrast to what was observed in cryopreserved samples, in all fresh samples BaP caused the alteration of the expression of at least one of the genes involved in BaP bioactivation (Fig. 13). In ID # 34 and ID # 36 the modulation of all three phase I metabolic genes analyzed was observed. In both donors, BaP induced the expression of CYP1B1 and EPHX1, while CYP1A1 was increased in ID # 34, and decreased in ID # 36. BaP treatment modulated the expression of CYP1B1 and EPHX1, but not that of CYP1A1, in ID # 33 and ID # 38. Also in this case the expression of EPHX1 increased in both donors, however the levels of CYP1B1 increased in ID # 36, while decreased in ID # 33. Finally, in ID # 32 only the modulation of CYP1B1 was observed, which in the presence of BaP increased its levels, as observed in almost all the other donors. On the other hand, in ID # 40 only the alteration of EPHX1 was observed which, contrary to what was observed in the other fresh samples, reduced its transcript levels. It should be noted that, even in fresh donors as in cryopreserved samples, the expression of EPHX1 was strongly reduced in culture compared to the uncultured condition.

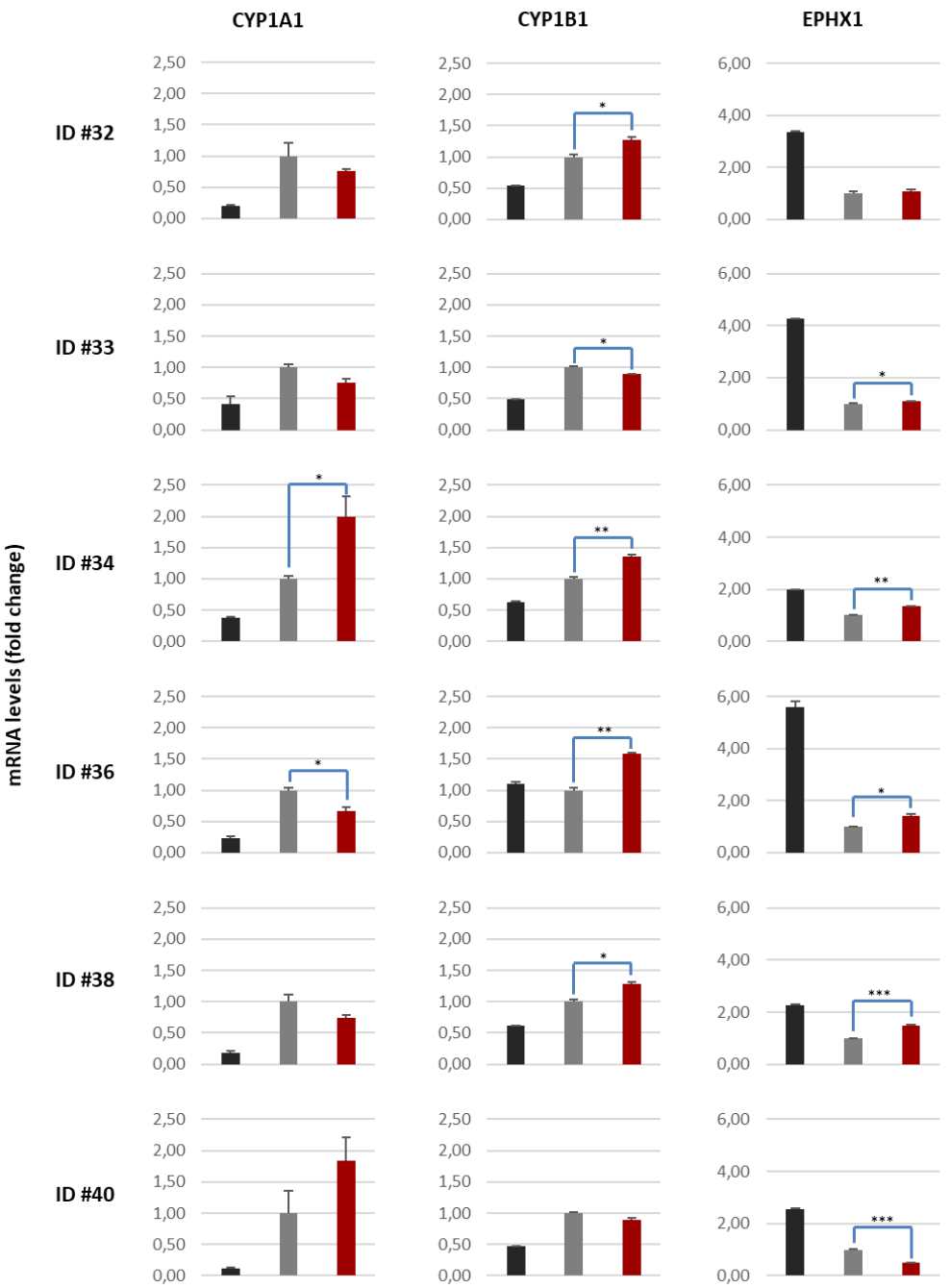
CYP1A1
CYP1B1
EPHX1
ID #32
ID #33
ID #34
ID #36
ID #38
ID #40
mRNA levels (fold change)
2,50
2,00
1,50
1,00
0,50
0,00
6,00
4,00
2,00
0,00
*
**
***

FIG. 13 *BaP-mediated modulation of the expression of the most important genes involved in the bioactivation of BaP in fresh human testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

4.2.2 BaP-mediated effect on the expression of steroidogenic genes

As for phase I metabolic genes, the expression of steroidogenic genes was altered and BaP-mediated modulation of at least one gene was observed in all samples (Fig. 14). ID # 34, ID # 36 and ID # 38 all showed an increase in STARD1 and CYP11A1 expression levels, but no effect on CYP19A1 modulation. ID # 40 was the only donor in which the alteration of all three steroidogenic genes analyzed was observed, however, together with a BaP-dependent increase in CYP19A1 levels, a reduction in the expression of STARD1 and CYP11A1 was observed. In the presence of BaP, in ID # 33 both STARD1 and CYP19A1 expression decreased, while there was no change in CYP11A1 levels. Finally, ID # 32 was the only fresh donor in which modulation of only one of the three steroidogenic genes analyzed was observed: BaP induced increased expression levels of STARD1 and no modulation for CYP11A1 and CYP19A1.

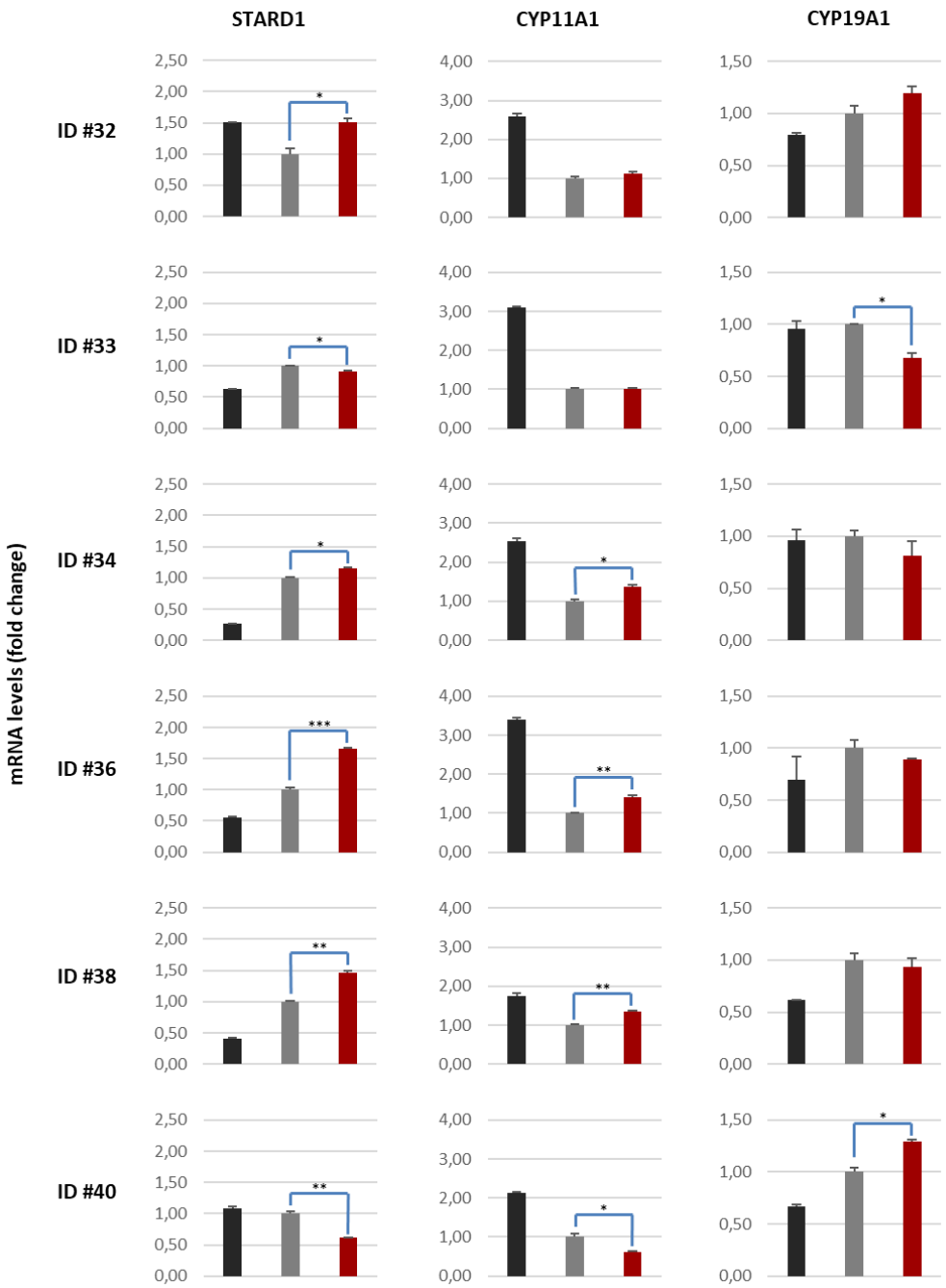
STARD1
CYP11A1
CYP19A1
ID #32
ID #33
ID #34
ID #36
ID #38
ID #40
mRNA levels (fold change)

FIG. 14 *BaP-mediated modulation of the expression of steroidogenic genes in fresh human testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

4.2.3 Apoptotic effect of BaP

The apoptotic effect of BaP 10 μM on fresh human testis cultures was evaluated by TUNEL assay (Fig. 15). As with cryopreserved tissue, even with fresh tissue, a greater number of apoptotic cells are generally observed in the culture conditions than in the uncultured condition. However, unlike cryopreserved tissue, this time a significant difference between the BaP and control conditions was shown in half of the donors (specifically ID # 34, ID # 36 and ID # 40), demonstrating the presence of a BaP-dependent apoptotic effect on fresh human testis cultures (Fig. 16). Therefore, we subsequently also analysed which types of testicular cell populations can undergo BaP-dependent apoptosis: the germ cells, the Sertoli cells or both. To this end, we performed TUNEL assay on fresh human testicular BaP-treated samples, followed by labelling with markers for germ cells (VASA) or Sertoli somatic cells (FATE1). As shown in Figure 17, our results demonstrate that both germ cells and Sertoli somatic cells can be TUNEL-positive and thus both testicular cell types are susceptible to BaP-induced apoptosis.

A schematic summary of data obtained with fresh human testis is summarized in Table 1.

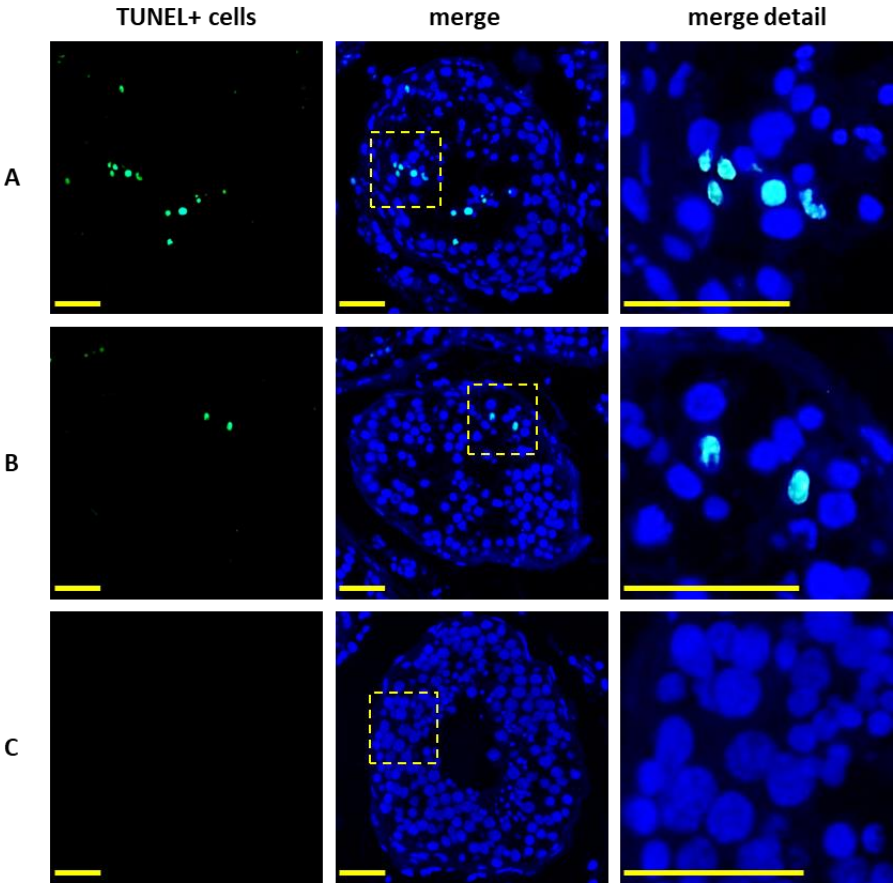

FIG. 15 *Representative images of the TUNEL assay on fresh human testicle specimen. In each image of the "TUNEL+ cells" and "merge" columns a cross-section of a seminiferous tubule is represented. The "merge detail" column shows the magnification of the dashed box in the corresponding "merge". (A) Cultured BaP 10 μM, (B) cultured BaP 0 μM, (C) uncultured time zero. Yellow bar corresponds to 50 μm.*

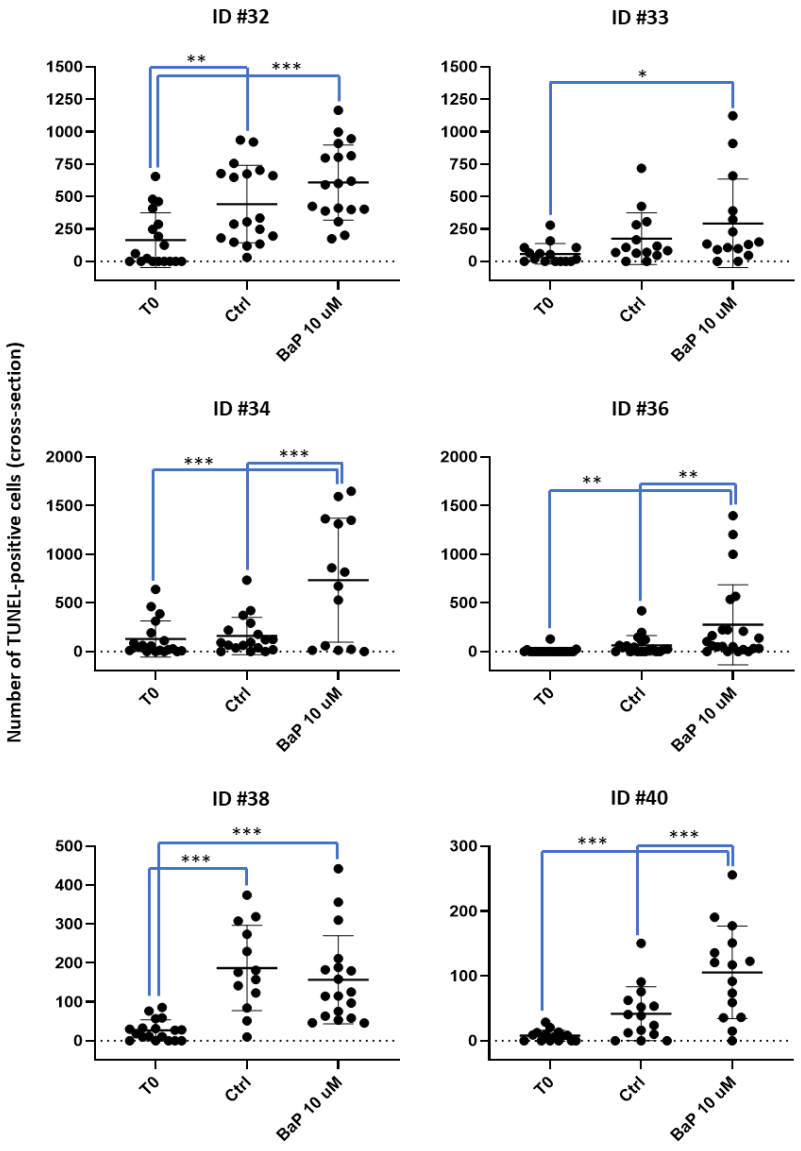

FIG. 16 *BaP-mediated apoptotic effect on human fresh testicular biopsies measured with TUNEL assay. Each point corresponds to the number of TUNEL-positive cells within a cross-section of a seminiferous tubule. For each experiment, 15-20 cross-sections were counted for each experimental condition. Data are expressed as mean ± SD. * p<0.05 ; ** p<0.01 ; *** p<0.001*

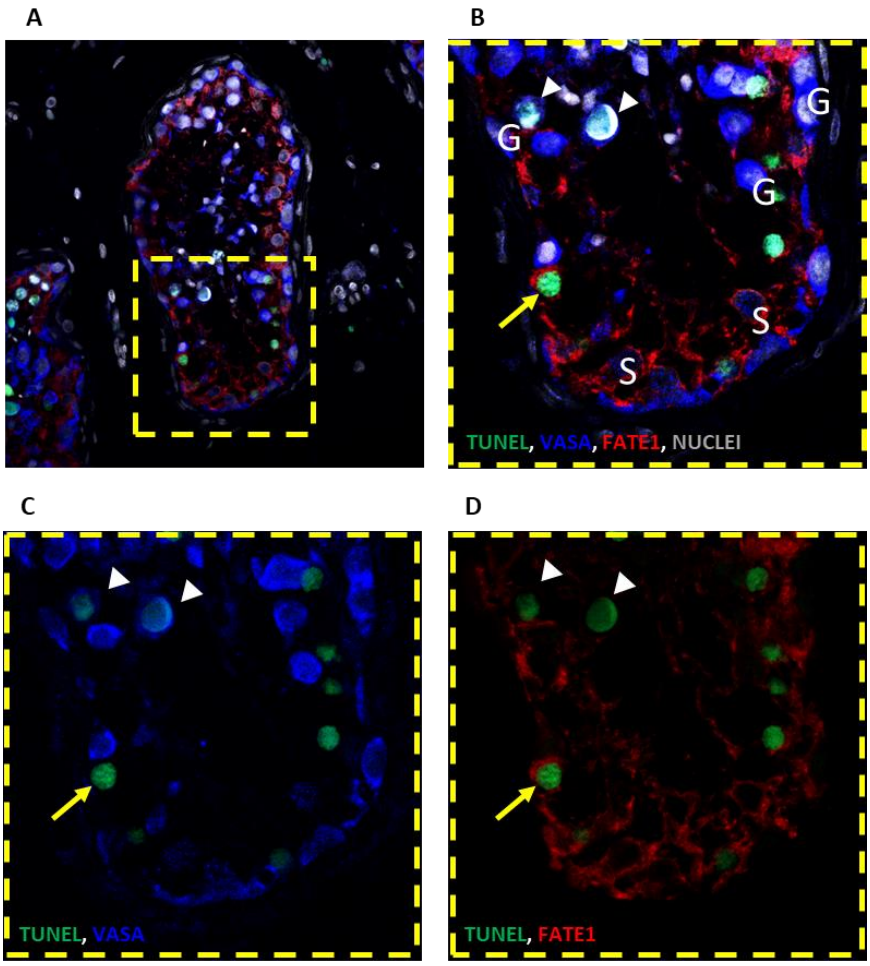

FIG. 17 *(**A**) Representative image of the TUNEL assay on BaP-treated fresh human sample, followed by labelling with markers for germ cells (VASA, in blue) or Sertoli somatic cells (FATE1, in red). Nuclei are labeled in grey, TUNEL positive cells in green. (**B-D**) Enlargement of the detail in the dotted yellow box, showing BaP-induced apoptosis (TUNEL positive cells) of both germ cells (**B**,**C**) and somatic Sertoli cells (**B**,**D**). White arrowheads indicate apoptotic germ cells (labelled blue and green), yellow arrows indicate apoptotic Sertoli cells (labelled red and green). G: germ cells; S: Sertoli cells.*

		CYP1A1	CYP1B1	EPHX1	STARD1	CYP11A1	CYP19A1	TUNEL+
cryopreserverd testicular tissue	ID #16	—	—	—	—	—	↑	—
	ID #24	—	↑	↓	↓	—	—	↓
	ID #30	—	—	—	—	—	—	—
	ID #03	—	—	—	—	—	—	—
	ID #04	↑	↑	↓	—	↑	↑	—
	ID #20	↑	—	↑	—	↑	↑	↑
fresh testicular tissue	ID #32	—	↑	—	↑	—	—	—
	ID #33	—	↓	↑	↓	—	↓	—
	ID #34	↑	↑	↑	↑	↑	—	↑
	ID #36	↓	↑	↑	↑	↑	—	↑
	ID #38	—	↑	↑	↑	↑	—	—
	ID #40	—	—	↓	↓	↓	↑	↑

TABLE 1. *Summary of the in vitro BaP-mediated effect on the human testis. Dashes (-) represent no effect on gene expression or apoptosis, while arrows indicate whether gene expression or apoptosis increases (up arrow) or decreases (down arrow) in the presence of BaP.*

4.3 Effects of BaP exposure on mouse testis culture

In parallel with the human study, we investigated the toxicogenomic effect of direct BaP exposure also on adult mouse testis cultures. Murine testis fragments were treated or left untreated with BaP 10 μM for 24 hours (n=5). For each culture, similar to what has been done in humans, we evaluated the effect of BaP on the modulation of genes of interest and on apoptosis. The modulation of the expression of genes of interest was evaluated using RT-PCR, while the BaP-mediated apoptotic effect was studied using both TUNEL assay and RT-PCR.

4.3.1 BaP-mediated effect on the expression of metabolic and steroidogenic genes

Also in mouse cultures, we evaluated the effect of BaP exposure on the expression of several genes, whose alteration is cause or effect of the BaP-mediated damage. We analyzed the BaP-induced modulation of the genes involved in the bioactivation of BaP (encoding the phase I metabolic enzymes) (Fig. 18A), in the detoxification of the toxic metabolites of BaP (encoding the transcription factor NRF2 and the phase II metabolic enzymes) (Fig. 18B) and in steroidogenesis (encoding STARD1, CYP11A1 and CYP19A1) (Fig. 19). Of all genes analyzed, none exhibited a BaP-dependent alteration, with the sole exception of CYP1B1 which increased its expression levels in the presence of BaP. Of note, EPHX1 and UGT-1A6 levels were strongly reduced in culture and, even if less, also GST-M1 and CYP19A1 levels decreased in culture condition.

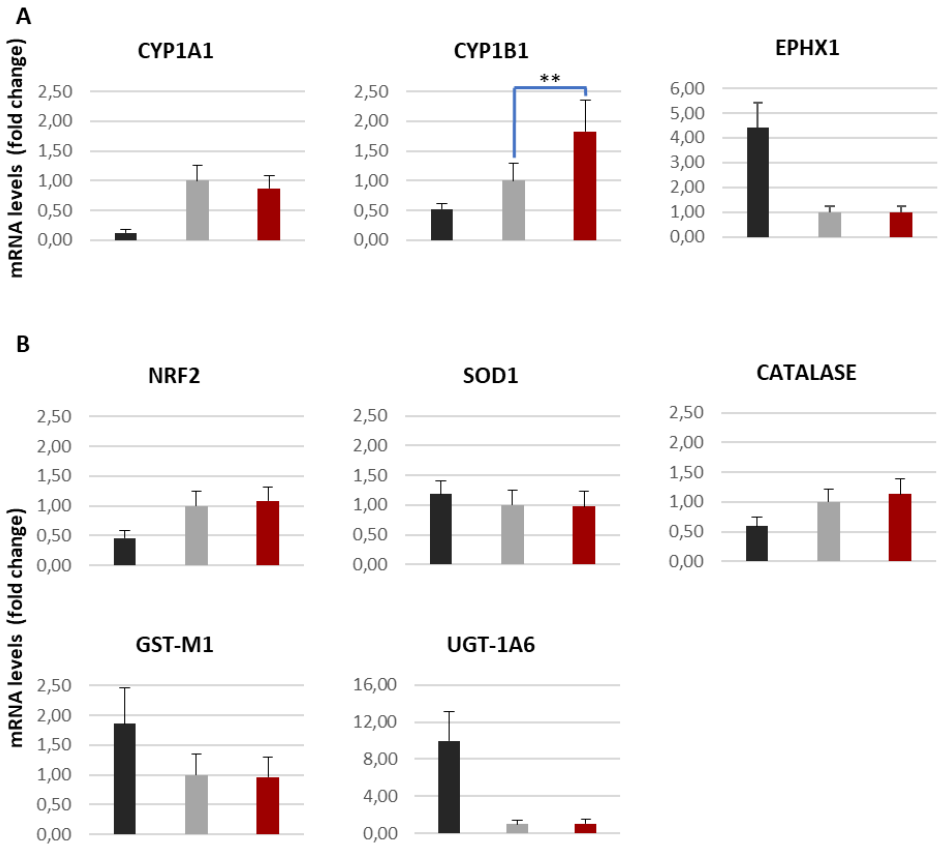

FIG. 18 *BaP-mediated modulation of the expression of the most important genes involved (**A**) in the bioactivation of BaP and (**B**) in the detoxification of its toxic metabolites in mouse testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. N = 5. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

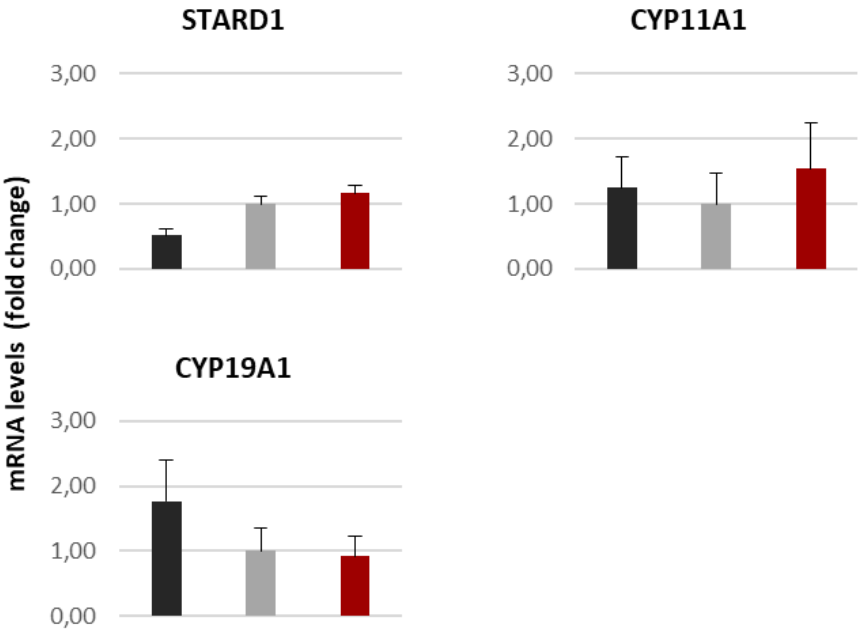

FIG. 19 *BaP-mediated modulation of the expression of steroidogenic genes in mouse testis culture. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. N = 5. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

4.3.2 Apoptotic effect of BaP

The apoptotic effect mediated by BaP in mouse cultures was studied on the one hand by evaluating the alteration of the expression of the apoptotic genes P53 and BAX, on the other hand using the TUNEL assay. As observed in almost all the other genes studied in mouse testis, the expression of P53 and BAX was not altered by the presence of BaP (Fig. 20). However, the results of the TUNEL assay demonstrated a large increase in the number of apoptotic cells in the mouse testis after exposure to BaP (Fig. 21). In line with the data obtained in human fresh samples, also in this case BaP induced an apoptotic effect on treated mouse testis cultures (Fig. 22).

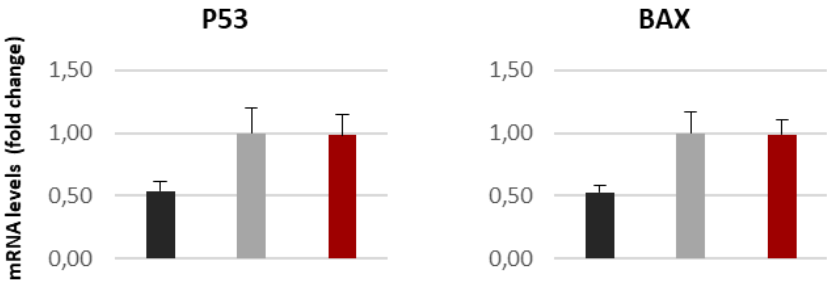

FIG. 20 *BaP-mediated apoptotic effect in mouse testis culture measured as alteration of the expression of apoptotic genes. The color of the bars on X-axis represents respectively: uncultured time zero (black bar), cultured BaP 0 μM (grey bar) and cultured BaP 10 μM (dark red bar). Y-axis represents mRNA fold change, with cultured BaP 0 μM (culture control) taken as 1. N = 5. Data are expressed as mean ± SEM. * p<0.05 ; ** p<0.01 ; *** p<0.001*

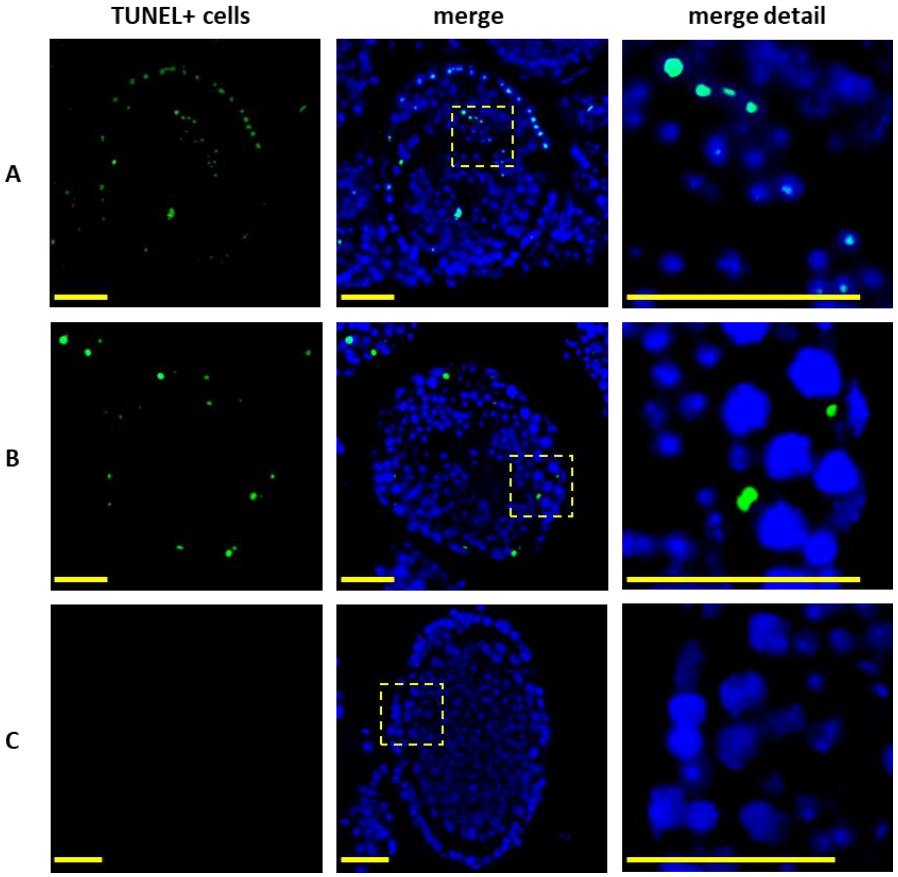

FIG. 21 *Representative images of the TUNEL assay on mouse testicle specimen. In each image of the "TUNEL+ cells" and "merge" columns a cross-section of a seminiferous tubule is represented. The "merge detail" column shows the magnification of the dashed box in the corresponding "merge". (A) Cultured BaP 10 μM, (B) cultured BaP 0 μM, (C) uncultured time zero. Yellow bar corresponds to 50 μm.*

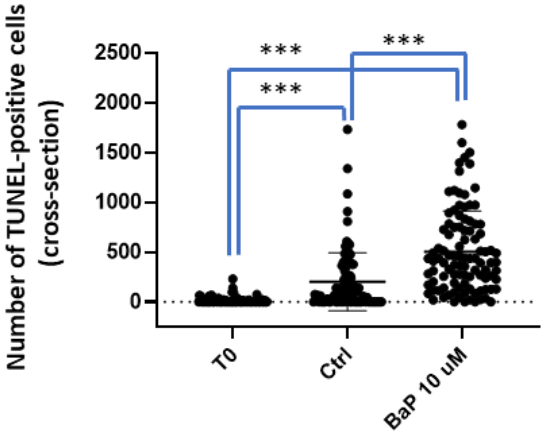

FIG. 22 *BaP-mediated apoptotic effect in mouse testis culture measured by TUNEL assay. The graph is the result of the sum of the TUNEL assays carried out on 5 different mouse cultures (N=5). Each point corresponds to the number of TUNEL-positive cells within a cross-section of a seminiferous tubule. For each TUNEL, 15-20 cross-sections were counted for each experimental condition. Data are expressed as mean ± SD. * p<0.05 ; ** p<0.01 ; *** p<0.001.*

5. DISCUSSION

With this study we wanted to broaden the knowledge of the harmful effects of BaP on male reproductive tissue. Although all published data show that exposure to BaP can compromise male fertility, is not clear yet whether the testis can be directly damaged by BaP or if its functions are impaired by toxic metabolites of BaP mainly produced in other organs such as the liver and lung (Ramesh et al., 2002). In the present work, we showed that in human testis organ culture model, BaP induced the alteration of both metabolic and steroidogenic genes, and it could induce DNA fragmentation of testicular cells. Moreover, we detected a difference in BaP responsiveness between fresh and cryopreserved testicular tissue and a general inter-individual difference in response to the BaP-induced effect. Interestingly, mouse testis fragments directly exposed to BaP did not report any modulation of the genes studied, except for the CYP1B1 gene. The induction of CYP1B1 alone, however, appeared to be sufficient to ensure the bio-activation of BaP, as demonstrated by the significant increase in apoptotic events in mouse testicular cells. Therefore, our work demonstrated that both human and mouse testes were susceptible to direct exposure to BaP.

Here we showed that in both human and mouse testis BaP mediated the induction of the main enzymes involved in phase I (bioactivation) of xenobiotic metabolism: CYP1A1, CYP1B1 and EPHX1 (Shimada et al., 1999). BaP is an inert molecule and its toxicogenomic action is triggered by the production of toxic metabolites (Gelboin et al., 1969; Shimizu et al., 2000). Our results obtained in both humans and mice indicated a general lack of BaP-mediated induction of CYP1A1 paralleled by an overall significant increase in CYP1B1 expression. From the literature, it is known that the expression of CYP1A1 and CYP1B1 is regulated by the transcription factor AhR, which is activated after binding ligands

such as BaP (Shimada et al. 2002; Shimada et al. 2003). Furthermore, the BaP-mediated induction of these two CYPs after *in vitro* exposure has already been demonstrated in rodents, for example in liver and lung organ cultures (Harrigan et al., 2006; Pushparajah et al., 2008a) as well as in humans, for example in oral and lung epithelial cells cultures (Wen & Walle, 2005; Chang et al., 2006) and in liver organ culture (Pushparajah et al., 2008a). Increased CYP1A1 and CYP1B1 mRNA levels have also been demonstrated in the testis of rodents exposed to BaP *in vivo* (Shimada et al., 2003; Banerjee et al., 2016). Therefore, the lack of BaP-dependent CYP1A1 induction both in human and mouse samples was unanticipated. On the other hand, the observation of a general induction of the CYP1B1 gene in both humans and mice was a very interesting finding, as the CYP1B1 enzyme appears to be more active in the bioactivation of BaP than CYP1A1 (Shimada et al., 1999). Furthermore, it must be considered that among the CYPs enzymes CYP1A1 is one of the least expressed constitutively in the testis, unlike CYP1B1 which is among the most expressed (Shimada et al. 2003; Bièche et al., 2007) Hence, it is conceivable that CYP1B1 could be the CYP isoform mainly implicated in the BaP bioactivation in the testis. EPHX1 also plays a very important role in the phase I metabolism of BaP (Shimada et al., 1999), but it is not an AhR target gene, it is induced by the activation of another transcription factor: GATA-4 (Zhu et al., 2004). However, *in vivo* studies on rodents have shown that, after exposure to BaP, EPHX1 increases its expression in numerous organs such as lung, spleen, liver, colon and glandular stomach (Zuo et al., 2014). Furthermore, *in vitro* studies on both rodent and human cultures have also shown that BaP can increase the protein and mRNA levels of EPHX1, as well as the catalytic activity of this enzyme (Pushparajah et al., 2008b). Our result on fresh human tissue agreed with the induction of EPHX1 after exposure to BaP, which was observed in almost all donors. On the other hand, in

cryopreserved human tissue we had a variable donor-dependent effect. In fact, in one-half of the donors we observed an increase or decrease in expression based on the donor, while in the other half modulation of EPHX1 did not occur. Despite the data in the literature and the result obtained in fresh human tissue, BaP did not induce the expression of EPHX1 in the mouse testis.

Using mouse testis samples, we evaluated the BaP-mediated alteration of the expression of NRF2 and of some phase II metabolic enzymes. Among the latter, we have chosen to study the alteration of the SOD1 and catalase genes, involved in the elimination of ROS potentially produced in the testis by BaP metabolism (Lorentzen & Ts'o, 1977; Briedé et al., 2004; Banerjee et al., 2016), and the alteration of the GST and UGT genes, which are considered the most important enzymes in containing BaP toxicity in reproductive tissues (Srivastava et al., 1999; Ramesh et al., 2001). Indeed, genes encoding for phase II metabolic enzymes are mainly induced by the transcription factor NRF2 (Itoh et al. 1997; Ramos-Gomez et al., 2001) and it has been shown that in the presence of BaP there is an increase in expression and activity of this transcription factor. NRF2, in fact, is a target gene of the AhR (Miao et al., 2005) and in response to oxidative stress and the presence of reactive electrophiles, which are generated during BaP metabolism, the accumulation of the NRF2 protein in the nucleus occurs, due to the weakening of the interaction between NRF2 and its cytoplasmic repressor Keap1 (Ishii et al. 2000; Itoh et al., 2003). Our results showed that the expression levels of NRF2 and of NRF2-induced phase II metabolic enzyme genes were unaffected by BaP treatment, even though CYP1B1 was induced in the same timeframe. The lack of phase II metabolic enzyme induction could be attributed to a low level of BaP’s toxic metabolites in target cells, the presence of which should induce the expression of detoxifying and antioxidant phase II metabolic enzymes. This

hypothesis requires further analysis. Moreover, also the future study of the nuclear translocation of Nrf2 protein in our experimental model in the presence of BaP would be an excellent method to verify the activation of the BaP detoxification pathway in our study (Buckley et al., 2003).

The partial modulation of phase I metabolic genes and the absence of modulation of phase II metabolic genes indirectly suggested the bioactivation of BaP, followed by an accumulation of toxic metabolites in testicular tissue. Several studies conducted *in vivo* on rodents have shown that BaP can alter the testicular steroidogenesis, in fact, following BaP exposure, a reduction in intratesticular and serum levels of testosterone was observed (Inyang et al., 2003; Ramesh et al., 2008; Archibong et al., 2008; Chung et al., 2011; Banerjee et al., 2016). In particular, the lowering of testosterone levels was associated with the alteration of the protein and mRNA levels of steroidogenic enzymes (Chung et al., 2011; Sheweita et al., 2016). Therefore, in our testicular fragments of humans and mice exposed *in vitro*, we evaluated the effect of BaP exposure on the alteration of the expression of some genes encoding enzymes/proteins involved in steroidogenesis such as STARD1, CYP11A1 and CYP19A1. Fresh human testis fragments were once again more responsive than cryopreserved ones to BaP exposure. In fact, our results reported that in fresh human tissue there was a general modulation of the steroidogenic genes, which was lacking in most cryopreserved human tissue cultures. In fresh human testis, the expression of STARD1 was altered in all the studied donors, even if, contrary to what was expected, in most cases an increase in its expression was observed. On the other hand, the increase in CYP11A1 mRNA levels observed in most fresh tissue donors was in line with the literature (Chung et al., 2011). Thus, at least in fresh human tissue, the alteration of the mRNA levels of steroidogenic genes could suggest

impaired steroidogenesis. In the future, quantification of testosterone released into the culture medium in the presence or absence of BaP will provide direct evidence of the impairment of this testicular function.

Using the TUNEL assay, we demonstrated a general significant increase in apoptotic events in both human and mouse testis after exposure to BaP. Interestingly, in mice this increase in BaP-mediated apoptosis was not associated with an induction of the expression of apoptotic genes such as p53 and BAX. The TUNEL assay has been used in the past to show an increase in the number of apoptotic cells in the testis of rodents exposed to BaP *in vivo* (Jeng et al., 2015; Banerjee et al., 2016). Therefore, our results obtained *in vitro* were in line with the literature, even though fresh tissue was more sensitive compared to cryopreserved tissue. Moreover, thanks to the use of specific markers, we have demonstrated that both germ cells and somatic Sertoli cells are susceptible to BaP-induced apoptosis, in agreement with what was observed after *in vivo* exposure (Jeng et al., 2015; Banerjee et al., 2016). Curiously, in mouse testis culture, BaP-induced apoptosis as measured by TUNEL assay was not associated with an increased expression level of apoptotic genes, including p53 and BAX, although this has been demonstrated in the testis of rodents exposed to BaP *in vivo* (Banerjee et al., 2016). This suggests that *in vitro* exposure to BaP could induce p53-independent cell apoptosis (Wang et al., 2022). However, before drawing this conclusion, it will be necessary to verify whether the observed lack of increase in the level of p53 RNA corresponds or not to an increase in the protein level, since the post-translational stabilization of p53 protein is known to play an important role in the induction of cell cycle arrest and apoptosis.

All data obtained in this study showed differences in the extent of the BaP-induced effects in fresh versus cryopreserved human

testicular tissue. Indeed, fresh human tissue was more susceptible to BaP exposure than frozen tissue, as demonstrated by the greater effect on gene modulation and apoptosis (Table 1). The cryopreservation of testicular tissue has been extensively studied in recent times as an experimental strategy for the preservation of fertility in young individuals at risk of infertility (Picton et al., 2015; Onofre et al., 2016). Some studies have shown that, even after long incubations in culture, cryopreserved human testes biopsies maintain structural and functional characteristics equal to fresh testicular tissues, showing no significant differences in tissue integrity, tubular cell proliferation/apoptosis, and testosterone production, confirming the usefulness of cryopreservation of testicular tissue (Kvist et al., 2006; Portela et al., 2019). Nevertheless, it has been demonstrated that long-time-cryopreserved testicular tissue may be less responsive than fresh tissue to gonadotropin exposure *in vitro*, and that testicular freezing can alter the pattern of steroid hormone production, possibly due to impaired activity of some steroidogenic enzymes (Mularoni et al, 2020). This lower responsiveness of frozen tissue to treatments in culture, also observed in our study, could be due to the long nitrogen storage period of cryopreserved testicular biopsies and to an incomplete reactivation of the enzymatic activity after thawing. It is in fact known that at low temperatures the biological metabolism of living cells drastically diminishes thereby stopping enzymatic and chemical reactions (Onofre et al., 2016).

Another feature that emerged during our study on the human samples was a general inter-individual difference in the BaP-induced effect, well seen in Table 1. These differences between human donors in BaP-mediated responses (from gene alteration to apoptosis) may be primarily due to a different starting level of PAH exposure in donors during their lifetime due to smoking habits, diet, occupational exposure and environmental pollution

(residential heating, automobile exhaust, industrial power generation, incinerators) (Boström et al., 2002). Another explanation for the donor-dependent BaP-mediated response could be due to inter-individual variations in xenobiotic metabolism. In humans, the genes coding for the enzymes that metabolize xenobiotics and the genes coding for the receptors/transcription factors regulating the expression of enzymes are characterized by polymorphisms (Thier et al., 2003). It was demonstrated that variants of CYP1A1 and CYP1B1 can differ in terms of inducibility and enzymatic activity and this can result in a variable effect induced by exposure to BaP (Schwarz et al., 2001; Shimada et al., 2001; Aklillu et al., 2005). Instead, the variants of EPHX1 have been shown not to differ much in their level of activity towards BaP metabolism (Hosagrahara et al., 2004), although combinations of EPHX1/CYPs variants may affect susceptibility to xenobiotics (Lin et al., 2000). Furthermore, the receptor/ transcription factor AhR is also polymorphic with most of the polymorphisms identified in the transactivation domain, consequently, AhR variants could regulate the expression of AhR-dependent genes (such as xenobiotic metabolism genes) with a different efficiency (Rowlands et al., 2010). To confirm the presence of inter-individual differences in BaP metabolism among our donors, the measurement and phenotyping of the BaP metabolites produced and released into the culture medium would be a reliable method of analysis, as suggested by Hecht (Hecht et al., 2006).

In conclusion, we have shown that BaP can cause alteration of gene expression and apoptosis in the testes of humans and mice directly exposed in organ culture. Therefore, to the best of our knowledge, this study demonstrates for the first time that the testis of mammals, such as mice and humans, has its own ability to metabolize BaP and that the BaP-induced impairment of male

fertility may be due to the action of toxic metabolites produced by testis itself, and not only by organs mainly responsible for xenobiotic metabolisms, such as the lung and liver. Although further studies will be needed in the future, this discovery contributes to increasing our knowledge on the action of environmental pollutants on fertility, a topic of great interest in recent times.

6. MATERIALS and METHODS

6.1 Human testicular tissue collection

Testicular biopsies were obtained from 6 heart-beating organ donors (age range: 30–53 years, median 41 years) at the Policlinico Umberto I Hospital at the Sapienza University of Rome. The cause of death of the donors was mainly haemorrhage, stroke or accidents. Tissue samples were collected from February 2011 to November 2017 during the multiorgan harvesting surgical procedures, that include perfusion through the aorta of the abdominal organs as well as simultaneous drainage of the perfusion fluid (Celsior solution) (Muciaccia et al., 2013). After collection, biopsies were transported to the laboratory in ice-cold phosphate-buffered saline (PBS) and processed within one hour. One part of each biopsy was immediately fixed in PFA, while some were placed in cryovials with a cryoprotective solution containing sterile Minimum Essential Medium (MEM), DMSO (1.1 M), fetal bovine serum (20%) and sucrose (0.15 M). Cryovials were frozen in dry ice for 15 min, then transferred to -80°C for 24–48 h and finally into liquid nitrogen.

Additional testicular material from other 6 adult patients was included in the study as fresh testicular biopsies. The material had been obtained from patients referred to the andrology clinic at the Department of Urology U. Bracci at Policlinico Umberto I Hospital, Rome, mainly due to fertility problems (obstructive azoospermia, testis cancer or hydrocele). The testicular material has been taken from regions of healthy tissue and once it arrived in the laboratory it was immediately used. Part of the biopsies was directly fixed in PFA 4% in PBS O/N at 4°C, the remainder was used for organ culture.

6.1.1 Ethical approval

The use of human material for our study was approved by the Ethical Committee of the Hospital Policlinico Umberto I, according to national guidelines for organ donation as issued by the Italian Ministry of Public Health. For each cryopreserved testicular sample, the free and informed consent of the family concerned was obtained at the time. For each fresh testicular sample, patients visiting the Department of Urology U. Bracci at Policlinico Umberto I Hospital, gave their written and oral consent before any leftover biopsy tissue was used for research studies. Patient tissue was used in accordance with the Helsinki Declaration following approval from the local medical research ethics committee (permit no. H-3-2013-175).

6.2 Mouse testis collection

C57/BL6 mice were housed in a climate-controlled (21 ± 2°C) animal room at a constant 12/12-hr light/dark cycle, with free access to mouse chow. Adult male mice (2-5 months of age) were sacrificed with CO_2, the testes were removed, deprived of tunica albuginea and transferred to a plate with PBS. All procedures were performed in accordance with protocols approved by the Sapienza University Animal Care and Use Committee.

6.3 *In vitro* culture of human and mouse testis fragments

Before culturing, cryopreserved human testicular samples were thawed at 37°C in a water bath for 2 min, washed three times in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) and then incubated for 1 h at 34°C to dilute the cryoprotectant and

equilibrate in the culture medium. Fresh human biopsies and mouse testes were washed in PBS.

Both human (frozen/thawed and fresh) and mouse testicular tissues were then cut into 2-4 mm fragments and placed in organ culture dishes on steel grids coated with agar 2% in dH_2O. At this point, the testicular fragments were wetted at the air-medium interface with culture medium containing DMEM supplemented with 2% glutamine 0.2M, 1% non-essential amino acids, 2% penicillin (100 U/ml)/streptomycin (100 mg/ml), 0.08% gentamicin, 1.5% HEPES 1M pH 7.7 (complete medium). 0.025 or 0.15% DMSO (Sigma-Aldrich) or BaP (Sigma-Aldrich) 0.1, 1 or 10 µM were added to the complete medium at the beginning of each new culture. We chose 10 µM as the highest dose of BaP as this is the most used concentration in *in vitro* toxicity studies (Chang et al., 2006; Harrigan et al., 2006; Pushparajah et al., 2008a/b; Sipinen et al., 2010; Guarnieri et al., 2021). Since in our preliminary study we observed the formation of precipitates in the culture medium using concentrations higher than 10 µM, to test more doses we decided to use the lower concentrations of 0.1 µM and 1 µM in our first experiments on cryopreserved human tissue. Also these lower doses are in the range of BaP concentrations used in the literature (Wen&Walle, 2005; Chang et al., 2006; Harrigan et al., 2006; Pushparajah et al., 2008a/b; Sipinen et al., 2010). Given the absence of significant effects with the lower doses of BaP on the cryopreserved human coltures, only the highest concentration of BaP 10 µM was used for fresh human and mouse tissue cultures. For each culture, an uncultured condition called "time zero" (T0) was made. Human and mouse testicular fragments were cultured for 12h and 24h, respectively, at 34°C in a humidified atmosphere of 5% CO_2 in the air. At the end of incubation, after a brief washing in PBS, the fragments intended for the molecular study were frozen at -80°C in 200 µL of Trizol reagent (Sigma-Aldrich)

while waiting to extract the RNA. Instead, the fragments intended for the histological study were fixed in PFA 4% at 4 °C O/N. While on the one hand for all cultures, both human and murine, the cultured/uncultured conditions intended for the morphological study were carried out in monuplicate, on the other hand the conditions intended for the molecular study were carried out in duplicate for the frozen human tissue and for mouse tissue, while in monuplicate for fresh human tissue due to a smaller amount available.

6.4 RNA extraction and reverse transcription

Total RNA from the fragments intended for the molecular study was extracted using the Trizol-chloroform-isopropanol method. Briefly, the testicular fragments frozen at -80° C in 200uL of Trizol per condition, once thawed on ice, were homogenized using a pestle and a 1 mL syringe. Following the addition of another 300 µL of Trizol and 100 µL of chloroform, each sample was vigorously shaken and left at room temperature (RT) for 2-3 minutes. The centrifugation of the samples at 12000 RCF for 15 min at 4° C led to the formation of 3 separate phases: at the top was the aqueous phase containing the RNA, in the middle was the phase with proteins and DNA, at the bottom was the organic phase containing lipids and residues of membranes. For each sample, the aqueous phase was collected and 250 µL of isopropanol was added to it, to favor the precipitation of RNA. The samples were then manually shaken and left at RT for 10 min. Thereafter, they were centrifuged at 12000 RCF for 10 min at 4° C and the formed RNA pellet was recovered by eliminating the supernatant. The RNA pellet of each sample was washed with 500 µL of 70% ethanol, centrifuged at 7500 RCF for 5 min at 4° C, then allowed to dry at RT after removing the ethanol supernatant. In the final step, 20 µL

of Molecular Biology Water (Sigma-Aldrich) was added to the dry pellet of each sample. Subsequently, the samples were either frozen again at -80° C or stirred for 5 min at 55-56° C at 750 RPM to be immediately quantified.

The extracted RNA was quantified by spectrophotometer (Nanodrop Microvolume Quantitation of Nucleic Acids). Subsequently, cDNA was synthesized from RNA, using a High-Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies, Italy) following the manufacturer's instructions. The reaction mix of each sample consisted of a total of 20 µL, containing 10 µL of Buffer 2x, 0.7-1 µL of retro-transcriptase enzyme, RNA and finally water for molecular biology to reach the volume. Based on availability, 600-1000 ng of RNA were reverse transcribed for each human donor, and 2000 ng of RNA for each sacrificed mouse. The thermal cycler used for reverse transcription was set as follows: 1st step at 37° C for 60 min (extension phase), 2nd step at 95° C for 5 min (inactivation of the retro-transcriptase enzyme), 3rd final step at 4° C (for storage of samples until recovery). When not in use, the cDNA was stored at -20° C.

6.5 Droplet digital PCR (ddPCR)

Gene expression analysis of BaP effects on human testicular samples was evaluated using droplet digital PCR (ddPCR), thanks to the collaboration with Professor's L. Casarini research group (Unit of Endocrinology, Dept. Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy). ddPCR reactions were performed in technical duplicate, using the QX200 Droplet Digital PCR (ddPCR™) System (Bio-Rad Laboratories Inc.), as previously described (Sperduti et al., 2021). For each primer pair, 20 µl of reaction mixture containing ddPCR™ EvaGreen Supermix (#186-4034; Bio-Rad Laboratories,

Inc., Hercules CA, USA), nuclease-free water, forward and reverse primer probes (at the final concentration of 125 nM each) and 10 ng of cDNA was prepared. PCR droplets were generated on the QX200™ Droplet Generator (Bio-Rad Laboratories, Inc.) and amplified as follows: 5 min enzyme activation at 95°C; 40 cycles of 30 s each for DNA denaturation at 95° C, 60 s for primer annealing and extension at 58° or 60° C; 5 min at 4° C followed by 5 min at 90° C for signal stabilization. All temperature variations were performed with a ramp rate of 2° C/s. For each primer pair, the most appropriate annealing and extension temperature was defined following a temperature gradient ranging from 60˚ C to 56˚ C: CYP1A1, CYP1B1, EPHX1 and RPS29 genes were analyzed at 58° C, while CYP11A1, CYP19A1 and STARD1 were analyzed at 60° C. The ddPCR reactions were analyzed using the QX200™ Droplet Reader and the number of positive droplets was established by the QuantaSoftTM software, version 1.7.4.0917 (Bio-Rad Laboratories, Inc.). The primers used are listed in Table 2.

6.5.1 ddPCR evaluation

For the analysis of the human ddPCR data, for each gene of interest, the absolute amount of target gene revealed for each experimental condition was normalized to the mean of the respective absolute detected amount of the RPS29 gene, used to normalize gene expression levels as previously validated (Svingen et al., 2014). For each donor, the raw values of each condition were then folded to the mean of the respective culture control values.

Human gene		Sequence
RPS29 NM_001032.5	Fw	CGCTCTTGTCGTGTCTGTTCA
	Rw	CCTTCGCGTACTGACGGAAA
CYP1A1 NM_000499.5	Fw	TGTCCTTCAGCCCAGACTCT
	Rw	GCTCACATGCTCTTCCAGGT
CYP1B1 NM_000104.4	Fw	CACTGCCAACACCTCTGTCT
	Rw	AAAGTTCTCCGGGTTAGGCC
EPHX1 NM_000120.4	Fw	ATCTCCTCCCAGCGCTTCTA
	Rw	ACCTCACCCACTTTTCAGGC
STARD1 NM_000349.3	Fw	AAGAGGGCTGGAAGAAGGAG
	Rw	TCTCCTTGACATTGGGGTTC
CYP11A1 NM_000781.3	Fw	ACCAAGAACTTTTTGCCCCT
	Rw	ATGTCCCCCGAGTAATTTCC
CYP19A1 NM_000103.4	Fw	TACATTATAACATCACCAGCATCG
	Rw	TCATAATTCCACACCAAGAGAA

TABLE 2. *List of the primers used for ddPCR*

6.6 Real-Time PCR (RT-PCR)

The BaP-mediated alteration of gene expression in mouse testis cultures was evaluated using Real-Time PCR (RT-PCR).

The RT-PCR analysis was performed in duplicate for each sample with PowerUp SYBR Green Master Mix (Applied Biosystems, Life Technologies, Italy) in Quant Studio 7 Flex System (Applied Biosystems, Life Technologies, Italy). Each reaction mix consisted of a total of 10 µl, containing 7 µl of "mix sample" (containing 5 µl 2X SYBR Green Master Mix, 100 ng of specific cDNA and water for molecular biology to get to volume) and 3 µl of "mix primers" (containing a final concentration of 300 nM of each specific forward and reverse primers and water for molecular biology to get to volume). Transcript levels were measured with two-step thermal cycling conditions: initiation at 95° C for 20 s, then 40 cycles at

95° C for 1 s and 60° C for 20 s (fluorescence measurement). The temperature of 95° C was reached with a ramp rate of 2.63° C/s, while the temperature of 60° C was reached with 2.42° C/s. Each sample was run in duplicate. Primer sequences, designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast), are listed in Table 3.

6.6.1 RT-PCR evaluation

Mouse RT-PCR data obtained were analyzed with the comparative $2^{-\Delta\Delta Ct}$ method using RPS29 to normalize gene expression levels. The final result was obtained by adding for each experimental condition the raw values obtained from 5 different murine organ cultures (n=5). The mouse data were then presented as the fold change in gene expression relative to the mean of the sum of the control samples from all five organ cultures.

6.7 Dehydration and inclusion of testicular fragments

Human and mouse testicular fragments fixed in PFA 4% O/N were dehydrated and then paraffin-embedded. In detail, the fragments of each condition were incubated with increasing concentrations of ethanol (70%, 80%, 90%, 95% and 100% ethanol), each for 30 min at 37° C. The fragments were then incubated for 30 min in toluene, the first time at 37° C, the second time at 60° C. Finally, always at 60° C, the incubation with toluene/paraffin (1:1) for at least 1.5 h and two incubations of 30 min with paraffin were performed. At the end of the process, the fragments of each condition were embedded in paraffin blocks, from which histological slides were obtained by cutting 5 µm thick sections at the microtome.

The histological slides were used to perform the TUNEL assay.

Mouse gene		Sequence
RPS29	Fw	TTCCTTTCTCCTCGTTGGGC
NM_009093.3	Rw	TTCAGCCCGTATTTGCGGAT
CYP1A1	Fw	GTTTTCTTTTGGGAGGAAGTGGA
NM_009992.4	Rw	TCCAAGGCAGAATACGGTGA
CYP1B1	Fw	TGCCTGCCACTATTACGGAC
NM_009994.2	Rw	CCACAACCTGGTCCAACTCA
EPHX1	Fw	CAGAGGCATCCAGCAAGAAAG
NM_001312918.1	Rw	GGGAATAAATGTTTTTGGGCACC
NRF2	Fw	GGACATGGAGCAAGTTTGGC
NM_010902.5	Rw	CCAGCGAGGAGATCGATGAG
SOD1	Fw	GAAAGCGGTGTGCGTGC
NM_011434.2	Rw	TTGTCCCCATACTGATGGACG
CATALASE	Fw	AGAGGAAACGCCTGTGTGAG
NM_009804.2	Rw	GCGTGTAGGTGTGAATTGCG
GST-M1	Fw	AGTCTAAAGGTGGTGACAGCC
NM_001374678.1	Rw	CACTTGGGCTCAAACATACGG
UGT-1A6	Fw	GCACAGGTCTCTAACCTTACTATCT
NM_145079.3	Rw	TCCTCGTTCACTGAGATGTTCT
STARD1	Fw	CACACATTTTGGGGAGATGCC
NM_011485.5	Rw	GTTGGCGAACTCTATCTGGGT
CYP11A1	Fw	GGTTCCACTCCTCAAAGCCA
NM_019779.4	Rw	GGATCTCGACCCATGGCAAA
CYP19A1	Fw	TGAGGCCAAATAGCGCAAGA
NM_007810.4	Rw	TTCCCAGACAGTAGCCAGGA
P53	Fw	GTATTTCACCCTCAAGATCCGC
NM_001127233.1	Rw	GCAGTTTGGGCTTTCCTCCT
BAX	Fw	ATGGAGCTGCAGAGGATGAT
NM_007527.3	Rw	TGAAGTTGCCATCAGCAAAC

TABLE 3. *List of the primers used for Real-Time PCR*

6.8 TUNEL (TDT-mediated dUTP-biotin nick end Labelling) assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is a method for detecting DNA fragmentation and was used in this study to evaluate the apoptotic effect mediated by BaP exposure in testis cultures.

Briefly, deparaffinization and rehydration in six descending stages were performed on the histological slides (10 min of toluene twice and 10 min for each descending alcohol: 100%, 90%, 80% and 70% ethanol). After washing with dH_2O and then with phosphate buffer saline (PBS), antigen retrieval was performed using Target Retrieval Solution (pH 6.0; Dako) heated up in a microwave oven at 700 W for three times (2 min the first time with a lid, 1 min the last two times without the lid). The slides were then cooled with dH_2O washes. After a new wash in PBS, the slides were treated with Triton 0.5% PBS for 20 min and then washed twice with BSA 2% in PBS. DNase I (1 U/µL in PBS; Sigma-Aldrich) was transferred for 20 minutes to the sections designated as the positive control of the TUNEL assay, followed by two washes in 2 % BSA. Subsequently, the sections were incubated for 1h at 37° C with Terminal Deoxynucleotidyl Transferase (TDT) end labelling solution (1:10 TDT enzyme in label solution) from In Situ Cell Death Detection Kit Fluorescein (Roche). In this step, the sections designated as the negative control of the TUNEL assay were incubated with only the label solution without the TDT enzyme. At the end of the assay, after vigorous washing with 2% BSA, the histological slides were stained with Hoechst (1:3000-5000 in PBS) for 10 min for cell nuclei staining, washed again in PBS and finally closed using buffered glycerol (glycerol 60% in TRIS 0.1M pH 9.5) as a mounting medium.

To establish which testicular cell types were susceptible to BaP-mediated apoptosis, before nuclei staining, fresh human testicular tissue slides were incubated with rabbit anti-VASA (ABCAM AB13840) and mouse anti-FATE1 (ABCAM AB139275), specific markers for germ cells and Sertoli cells respectively. During this analysis, after washing the enzyme TDT, the samples were blocked in Glycine 1 M (Sigma-Aldrich, Milan, Italy) for 20 min to reduce the aldehyde-caused fluorescent background, and then they are incubated with 5% Donkey Serum (Jackson Immuno Research Europe Ltd, Newmarket, UK) in 2% BSA for 1h to avoid the unspecific antibody binding. Subsequently, the samples were incubated with the primary antibodies identifying the specific cell type (1:100 in 2% BSA) at 4° C overnight. After the washes, the sections were incubated with donkey secondary antibodies (1:200 in PBS) conjugated to Cy5 and Cy3 fluorochromes (Jackson Immuno Research Europe Ltd, Newmarket, UK) for 2 hours at RT. At the end, the nuclei were stained with DAPI (1:400 in PBS) for 10 min and the slides were closed using buffered glycerol.

6.8.1 TUNEL evaluation

To verify the apoptotic effect of BaP, at the end of each TUNEL assay, the slides were immediately observed under the fluorescence microscope (Zeiss). For each experimental condition of each sample, 15-20 cross-sections were photographed using the ZEN 3.3 Blue Edition program. TUNEL positive cells of each cross-section were manually counted using the ImageJ-win64 program and this number was normalized for the respective cross-section area measured in mm^2. Human data are represented by one assay per donor. The data from mice are the results obtained from n=5 animals. To establish whether germ cells, Sertoli somatic cells or both undergo BaP-mediated apoptosis, the analysis was performed

on images acquired with the Zeiss Airyscan 2 confocal microscope with 40x oil immersion objective.

6.9 Statistical analysis

Statistical analysis was performed using the Sigma Plot 14.0 software (Systat Software, Inc, CA, USA). Quantitative data from the molecular study (ddPCR and RT-PCR) are shown as the mean ± standard error of the mean (SEM), while data from TUNEL analysis are shown as the mean ± standard deviation (SD).

To compare many groups, data were analyzed using a one-way analysis of variance (ANOVA). When data were assessed to differ markedly from a bivariate normal distribution, the Student-Newman-Keuls test was used. To define the significance of the differences between two groups, data were analyzed using Student's t-test. P-value <0.05 was considered significant.

7. REFERENCES

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8. LIST OF PUBLICATION

Tartarelli Irene, Tinari Antonella, Possenti Alessia, Cherchi Simona, Falchi Mario, Dubey Jitender P., Spano Furio. *During host cell traversal and cell-to-cell passage, Toxoplasma gondii sporozoites inhabit the parasitophorous vacuole and posteriorly release dense granule protein-associated membranous trails.* International Journal for Parasitology, 2020, Aug 31; S0020-7519(20)30241-1. doi.org/10.1016/j.ijpara.2020.06.012

Communications at the congress

Tartarelli Irene, Sperduti Samantha, Capponi Chiara, Stefania Fera, Pozza Carlotta, Isidori Andrea, Simoni Manuela, Casarini Livio and Vicini Elena. *Effects of Benzo[a]pyrene in human testicular organotypic culture*. 14°Congresso Nazionale SIAMS - Bologna, 21/23 ottobre 2021.

9. ATTACHMENT

Report on training, research and other activities carried out in the last year of Ph.D.

My Ph.D. project was focused at increasing the knowledge of the effects of environmental pollution on male fertility. The aim of my project was to study the effect of Benzo[a]pyrene (BaP) on testicular biopsies directly exposed in organ culture, to analyze how the male reproductive system reacts to the presence of this important ubiquitous environmental pollutant. During the first two years of my Ph.D., I began by evaluating the effect of BaP on human testicular biopsies from three cryopreserved donors at 3 hours of exposure. From these initial analyses, it appeared that BaP had no evident effect on normal testicular morphology and little/no effect on the modulation of genes involved in phase I metabolism and steroidogenesis (data not shown). Subsequently, I increased the exposure times, evaluating the effect of 12 hours of exposure to BaP both on the modulation of the genes of interest, using the ddPCR, and on apoptosis, using the TUNEL assay. Furthermore, I started to carry out similar analyses also on testicular cultures of adult mice exposed to BaP for 24 hours. The third year of my Ph.D. was the most challenging, but also the most satisfying. Indeed, I continued my study with cryopreserved human testicular biopsies, doubling the number of donors analyzed to reach an adequate number of observations. For the same purpose, I increased the number of mouse cultures, and I also evaluated the effect of BaP on the modulation of additional groups of genes, such as phase II metabolic genes and apoptotic genes. Crucial was the addition of the fresh human testicular biopsy study to the project. In fact, fresh testicular tissue was more susceptible to BaP than cryopreserved tissue and allowed us to obtain results in line with *in vivo* data described in the literature. Finally, during the last year, I started new phases of the project, were I obtained

preliminary data, which can be continued in the future. On the one hand, I started the study of a possible age-dependent effect of BaP exposure on the testis, using testicular cultures from fetal and newborn mice (data not shown). On the other hand, in adult testicular cultures of both humans and mice, I began to analyze the effect of exposure to BPDE (data not shown). BPDE is a toxic metabolite of BaP and is considered one of the most dangerous. With this new study, we could compare the testicular damage obtained with BaP (which must be metabolized) with those caused by a BaP metabolite that is ready to cause damage. In the last three months, I have been working on my Ph.D. thesis.

In the last year of my Ph.D., I participated in various training initiatives, such as congresses, courses and seminars, which are listed below.

Conferences

14° Congresso Nazionale SIAMS “Fitness & General Health”. 21-23 October 2021

5° Minisimposio su Sperimentazione Animale In Biomedicina “Dal topo all'uomo: aspetti evolutivi, fisiopatologici e traslazionali”. 29 October 2021

Webinar "Vaccini Anti Covid-19 E Di Routine: Pandemia Ed Esitanza Vaccinale - 3rd Vaccine Hesitancy Forum Covid-19”. 13 December 2021

Conferimento Dottorato di ricerca honoris causa in Advances in Infectious Diseases, Microbiology, Legal Medicine and Public Health Sciences a Anthony S. Fauci. 13 January 2022

STITCH Webinar “Data preparation e data analytics: il caso del diabete (progetto di collaborazione AMD-STITCH)”. 26 January 2022

STITCH Webinar “Big data e medicina. Prove di dialogo tra medicina e big data: vecchie incomprensioni e nuove alleanze”. 1-9-14 February 2022

STITCH Webinar “Progetto Eco4co. Sistemi di monitoraggio e gestione di interesse sanitario basati su servizi satellitari di comunicazione e osservazione della terra. Applicazioni alla gestione e contenimento dello stato pandemico corrente”. 21 March 2022

Conferenza Accademia Medica di Roma “New frontiers in regenerative medicine”. 14 April 2022

Simposio “Mente fatta a mano - come la mano influenza chi siamo”. 7 September 2022

Courses

Corso Teorico-Pratico di Statistica online a cura del Prof. Luca Digiacomo. 7-14-21 June 2022 and 5 July 2022

Seminars

10/11/2021 Next Generation Multiomic approaches to study cellular dynamics in skeletal muscle. **Dr. L Giordani**

29/11/2021 Il declino degli OGM in Italia: un caso esemplare dei rapporti tra scienza, politica e percezione pubblica. **Prof. A Grignolio**

12/01/2022 Gene Therapy by Tuning Transcription with DNA-Binders. **Prof. E M Surace**

13/01/2022 Human Organ Chips Clinical Mimicry in Preclinical Models. **Prof. D E Ingber**

27/01/2022 Microbioma Intestinale e rischio Cardiometabolico. **Prof. M Federici**

10/02/2022 The Last 20 Years of Interventional Cardiology, Met and Unmet Needs. **Prof. G D Dangas**

11/02/2022 Spatial transcriptomics: a meeting point between molecular biology and histology. **Dr. L Madaro**

16/02/2022 3D culture systems for evaluating microgravity effects on the male gonad. **Dr. M Berardini**

16/02/2022 Understanding the contribution of IL-6 transignalling to DMD progression and the implications for therapy. **Dr. C Boccia**

23/02/2022 CD44 isoforms and their interactomes: effects on melanoma. **Dr. C Castelli**

23/02/2022 Unveiling the HDAC4 functions in mediating the cross-talk between skeletal muscle and fibro-adipogenic progenitors in Duchenne Muscular Dystrophy. **Dr. G Cavioli**

02/03/2022 Advanced methods for 23Na-MRI of the brain: phenomenological application to multiple sclerosis. **Dr. I Egidi**

02/03/2022 Modelli neurofisiologici per l'addestramento di intelligenze artificiali nei sistemi di sicurezza degli autoveicoli. **Dr. A Giorgi**

09/03/2022 Separating neural and vascular contributions to BOLD-fMRI signal to characterize the effects of healthy and pathological ageing. **Dr. G Giulietti**

09/03/2022 β-Sarcoglycanopathy (LGMDR4): a study of muscle diversity using a Sgcβ KO mouse model. **Dr. M Gloriani**

16/03/2022 The identification of cell-cell interactions within the spermatogonial stem cell niche in normal and infertile men. **Dr. M Palazzoli**

16/03/2022 Testing behavior and prior sensorimotor brain representation in acquired amputees. **Dr. F Giove**

17/03/2022 RNA circolari nel differenziamento e nelle malattie. **Prof. I Bozzoni**

23/03/2022 Identification and differentiation of microplastics in liquids by phasor fluorescence lifetime imaging microscopy. **Dr. X Siyao**

23/03/2022 X-MET: a bioengineered muscle construct as a model to study muscle pathophysiology. **Dr M Zouhair**

30/03/2022 Unravelling the role of TPC2/calcium signalling-dependent autophagy in melanoma microenvironment remodelling. **Dr. S Barbonari**

30/03/2022 Dissecting the role of novel oncomiRs and their molecular targets in the evolution of resistance to target therapies in melanoma. **Dr. V Castaldo**

12/04/2022 Dalla scuola anatomica torinese di Giuseppe Levi alla esitanza vaccinale: scienza, politica, società. **Prof. A Grignolio**

12/04/2022 Il metodo della scienza dentro e fuori il laboratorio. Huntington, storia di un gene antico. **Prof. E Cattaneo**

13/04/2022 Retinoic Acid and proteotoxic stress to induce myeloid leukemic progenitors cell death in a bone marrow niche-like system. **Dr. F Liccardo**

13/04/2022 Role of m6A-dependent circRNAs during stress response in myeloid leukemic cells. **Dr. A Iaiza**

20/04/2022 The interplay between Endothelin receptor and integrin beta1 regulates the metastatic behavior of Ovarian Cancer Cells. **Dr. I Masi**

20/04/2022 Aptamer-conjugated gold nanoparticles for selective microRNA delivery in dystrophic muscles. **Dr. F Millozzi**

21/04/2022 Neurodegenerative Diseases: The Role of TDP-43 in mRNA transport and Localization. **Dr. N H Alami**

27/04/2022 The modulation of inflammatory stimulus induced by TGF-β upon myo-inositol treatment. **Dr. N Monti**

27/04/2022 Lab-on-Chip systems for the detection of pathogens and essential trace elements in biological samples. **Dr. M Nandimandalam**

04/05/2022 Multimodal neurophysiological characterization of driving drowsiness for professional drivers. **Dr. V Ronca**

04/05/2022 Gene Therapy for Ataxia-Telangiectasia Syndrome. **Dr. B Sabino**

11/05/2022 Human neurophysiological reaction to olfactory stimulation: a translational study. **Dr. A Vozzi**

18/05/2022 Role of N6-methyladenosine in ER stress response in triple-negative breast cancer. **Dr. B Cesaro**

18/05/2022 Cancer-nerve crosstalk in human Cholangiocarcinoma. **Dr. V De Franchis**

20/05/2022 Rbm24: an RNA binding protein required for myogenic differentiation. **Prof. R Grifone**

25/05/2022 Study of extracellular vesiscles (EVs) protein content released by dystrophic fibro-adipogenic progenitors (FAPs) treated with HDAC inhibitors (HDACi). **Dr. F Esposito**

25/05/2022 Role and function of glial cells in muscle homeostasis. **Dr. C D'Ercole**

30/05/2022 Nucleolar Protein Localization and Re-Organization in Myogenic Cells. **Prof J C McDermott**

31/05/2022 Assembly of axonal domains that promote nerve conduction: the axon initial segment and the node of Ranvier. **Prof. P J Brophy**

01/06/2022 Characterization of PCOS-induced mouse model and modulation of steroidogenesis in Granulosa cells upon treatment with myo-Ins and D-chiro-Ins. **Dr. V Fedeli**

01/06/2022 The role of Protein-Kinase C theta (PKCθ) in muscle-nerve communication and in neuromuscular disease. **Dr. G Laurenzi**

08/06/2022 Analysis of genetic characteristics of a cohort of Italian patients of a referral center for Amyotrophic Lateral Sclerosis. **Dr. L Libonati**

08/06/2022 Characterization of Atm-KD mouse models and cancer-associated Atm kinase mutations. **Dr. M Merolle**

15/06/2022 The effects of Two-pore channel 2 (TPC2) inhibitors on autophagy pathway during osteoblastogenesis and osteo-clastogenesis. **Dr. A Montaseri**

15/06/2022 Development of SARSCov-2 DNA vaccine encapsulated in functionalized lipid nanoparticles. **Dr. S Renzi**

22/06/2022 Small molecules loaded on nanocarriers protecting from membrane damage based amyloid neurotoxicity. **Dr. M Severino**

22/06/2022 Cross-task variability of EEG parameters. **Dr. I Simonetti**

30/06/2022 Learning and Neuroscience: In-presence vs Remote teaching at driving schools. **Dr. L Tamborra**

30/06/2022 Study of the role of telomeric proteins TRF1 and TRF2 in heterochromatin metabolism. **Dr. E Vertecchi**

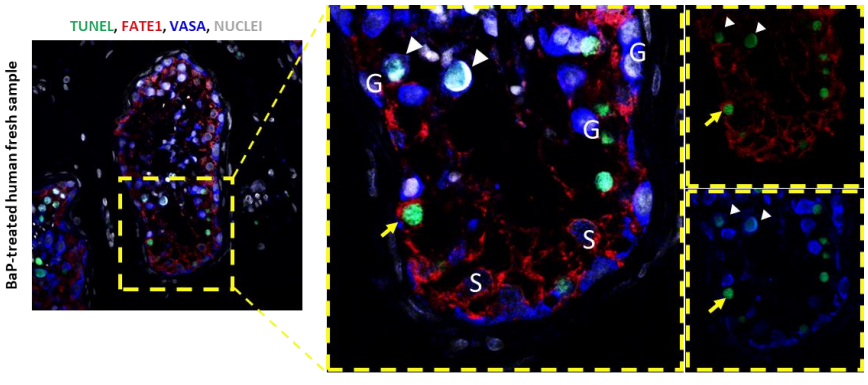
10. POINT BY POINT RESPONSE TO THE REVIEWERS

Reviewer #1

The results are well discussed, highlighting differences existing between cryopreserved or fresh human and rodent samples. Illustrations and schematic representations are fine and acceptable. The thesis is well-written and the English writing is accurate. I did not find relevant grammatical or lexical errors. I have a couple of suggestions:

1) *It would be interesting to investigate whether the pro-apoptotic effect of BaP on fresh samples involves the somatic component of the testis or the germ cell component, or both.*

We thank the reviewer for the positive comments. To answer this point, we performed TUNEL assay on fresh human testicular samples, followed by labelling with markers for germ cells (VASA) or Sertoli somatic cells (FATE1), to verify which testicular cell types are susceptible to BaP-induced apoptosis in our study. Our results showed that both cell types can undergo BaP-dependent apoptosis, as shown below, in a representative image of the results. In the enlargement, it is possible to observe that both germ cells (VASA-positive in blue, white arrow heads) and Sertoli cells (FATE1-positive in red, yellow arrow) are TUNEL-positive



(green). Our results are in line with *in vivo* studies on rodents, showing that BaP induces apoptosis in all cell populations of the testis, both in germ and somatic cells (Jeng et al., 2015; Banerjee et al., 2016).

2) *Furthermore, I would suggest investigating whether the observed lack of increase of P53, verified at the RNA level, corresponds or not to an increase at the protein level, since it is known that the post-translational stabilization of this protein plays an important role in the induction of cell-cycle arrest and apoptosis.*

The reviewer's suggestion is correct. We are currently analyzing p53 protein levels in our mouse model in the presence/absence of BaP exposure by western blot technique.

Reviewer #2

The entire work of thesis has been designed based on a good rationale and also the human material used was appropriate. An updated introduction gives the state of the art on the issue of the potential impact of BaP on male reproductive health. The methods are clearly reported and the results are sound and relevant to the field. However, I have some comments and suggestions that should be addressed, as detailed below:

1) *Add an explanation and/or References on the reason why BaP doses of 0.1, 1 and 10 µM were chosen.*

We thank the reviewer for the positive comments. We chose 10 µM BaP as the highest concentration as this is the most used concentration in *in vitro* toxicity studies (Chang et al., 2006; Harrigan et al., 2006; Pushparajah et al., 2008a/b; Sipinen et al.,

2010; Guarnieri et al., 2021). Since in our preliminary study we observed the formation of precipitates in the culture medium using concentrations higher than 10 µM, to test more doses we decided to use the lower concentrations of 0.1 µM and 1 µM in our first experiments on cryopreserved human tissue. Also, these lower doses are in the range of BaP concentrations used in the literature (Wen&Walle, 2005; Chang et al., 2006; Harrigan et al., 2006; Pushparajah et al., 2008a/b; Sipinen et al., 2010). Having obtained significant effects only with the highest dose, we used only the 10 µM dose for the subsequent experiments. These considerations and relative references were included in the Materials and Methods section of the thesis, as suggested.

2) *Fig. 9, 10, 13, 14, 18, 19 and 20 should be better described: ordinate-axis legend is lacking. Are the results reported as fold changes over time 0 taken as 1? This is not specified in the legend nor in the methods.*

Data were folded considering the culture control as 1, as described in the Materials and Methods sections. This information has been added to the description figures.

3) *Gene expression analysis of NRF2 is an interesting aspect to evaluate BaP response in the testis, however the nuclear translocation should be more informative. If this analysis is not available this limitation should be at least discussed*

This is a very good suggestion. Unfortunately, we cannot perform this analysis because of samples limitation and availability of the antibody. We have added a comment about this point in the Discussion, as suggested by the reviewer.

4) *The induction of CYP1B1 and not CYP1A1 should be better discussed. Indeed, CYP1B1 is the extrahepatic isoform of BaP metabolizing enzymes (CYP1A1 is more active in the liver, as reported in literature). Hence, it is conceivable that CYP1B1 could be the isoform mainly implicated in the BaP bioactivation in the testis? Please discuss better this point.*

We thank the reviewer to bring this up. The induction of CYP1B1 and not of CYP1A1 has been better discussed. In the Discussion was included the consideration that CYP1A1 is among the least expressed CYPs in the testis, unlike CYP1B1 which is among the most expressed (Shimada et al. 2003; Bièche et al., 2007). Also considering that CYP1B1 enzyme appears to be more active in the bioactivation of BaP than CYP1A1 (Shimada et al., 1999), it is entirely plausible that CYP1B1 is the CYP isoform most implicated in BaP metabolism in the testis.

5) *Given the English language, P-values throughout the text must be modified by replacing the comma with a dot in the decimal number.*

As requested, the comma has been replaced with a dot in the decimals.