Testicular connexin 43, a precocious molecular target for the effect of environmental toxicants on male fertility

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Many recent epidemiological, clinical and experimental findings support the hypothesis that environmental toxicants are responsible for the increasing male reproductive disorders (congenital malformations, declining sperm counts and testicular cancer) over the past 20 years. It has also been reported that exposure to these toxicants, during critical periods of development (fetal and neonatal), represents a more considerable risk for animals and humans than exposure during adulthood. However, the molecular targets for these chemicals have not been clearly identified. Recent studies showed that a family of transmembranous proteins, named connexins, regulates numerous physiological processes involved in testicular development and function, such as Sertoli and germ cell proliferation, differentiation, germ cell migration and apoptosis. In the testis, knockout strategy revealed that connexin 43, the predominant connexin in this organ, is essential for spermatogenesis. In addition, there is evidence that many environmental toxicants could alter testicular connexin 43 by dysregulation of numerous mechanisms controlling its function. In the present work, we propose first to give an overview of connexin expression and intercellular gap junction coupling in the developing fetal and neonatal testes. Second, we underline the impact of maternally chemical exposure on connexin 43 expression in the perinatal developing testis. Lastly, we attempt to link this precocious effect to male offspring fertility.

Introduction

A dramatically decline of human male reproductive function, associated with diseases of the male genital tract (azoospermia, cryptorchidism, cancers), has been reported for the past 20 years.¹ Many recent epidemiological, clinical and experimental findings suggest that one of the major causes of these reproductive dysfunctions could result from prenatal and

neonatal environmental chemical exposure. Even if numerous studies argue for a relationship between male infertility and environmental and/or occupational exposure, the molecular mechanisms by which these anti-reproductive compounds act are still unknown. There is now evidence that environmental pollutants may affect male reproductive function through different pathways and mechanisms. Environmental pollutants can impair male reproductive function by competing with endogenous endocrine effectors, which may conduce to altered expression of genes that play an essential role in spermatogenesis and androgen synthesis.^{2,3} Epigenetic inactivation of essential genes by these toxicants has been reported in reference 4. Environmental toxicants could also increase oxidative stress in the testis, leading to infertility⁵ through increasing DNA damage, apoptosis, impaired cell division and sperm membrane damage by peroxydation of sperm membrane lipids.⁶ Disruption of Sertoli/Sertoli and Sertoli/germ cell junctions has also been demonstrated upon oxidative stress.⁷ The latter observation supports our previous findings suggesting that intercellular junctions, specifically gap junctions, could be major targets for environmental toxicants.⁸

It is obvious that the initiation of spermatogenesis and its maintenance require a functional hypothalamo-pituitary system involving luteinizing hormone (LH) and follicle stimulating hormone (FSH) as well as local controls mediated through paracrine signals. Local intratesticular cell-cell interactions including direct contact-dependent junctional pathways are also essential not only in the regulation of mammalian spermatogenesis but also for maintaining the male phenotype.⁹ These cell-cell contacts are formed of specialized proteins involved in anchoring junctions (such as cadherin-catenin, nectin, integrin, etc.) and tight junctions (such as occludin, claudins, Zonula occludens, etc.), the main constituents of the blood-testis barrier (BTB).¹⁰ A third class of junctions present in the testis and in the male genital tract (epididymis, seminal vesicle, prostate, corpus cavernosum) concerns gap junctions and their constitutive proteins, connexins (Cx).^{11,12}

Gap junctions and their transmembranous proteins regulate numerous physiological functions, such as cell proliferation,

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Figure 1. (A) Schematic diagram of gap junction plaque, gap junction channel, gap junction hemi-channel or connexon and connexins. Gap junctions are channels formed by the docking of two hemi-channels, the functional unit of gap junctions, composed of six transmembrane proteins, connexins. Gap junction channels interconnect cytoplasms of two adjacent cells through pores, which allow the diffusion of ions and water-soluble molecules up to about 1,000 Da. (B) Schematic drawing illustrating possible arrangements of connexons to form heterotypic and heteromeric gap junction channels. Homotypic gap junction channels are structured by a single connexin and heterotypic channels by more than one connexin. Homomeric connexons are composed of a single connexin, whereas heteromeric connexons are composed of more than two different isoforms. (C) Each connexin monomer is composed of four transmembrane domains (TM1–TM4) with two extra-cellular domain (E1 and E2), essential to maintain integrity of the plasma membrane achieved by 3 sulfhydryl linkages between each connexin molecule, an intracellular loop (IL), and cytoplasmic N-terminal (NH₂) and C-terminal (COOH) domains.

differentiation, migration and apoptosis.¹³ This direct pathway of communication between adjacent cells, called gap junctional intercellular communication (GJIC), participates in the establishment of tissues and organisms by controlling cellular signaling, cell growth, differentiation, homeostasis and apoptosis.^{14,15} In the testis, the evidence that Cxs are essential for the establishment

and the maintenance of spermatogenesis comes from both observations in transgenic mice invalidated for Cx genes and clinical observations in men.¹²

Recent studies reported that testicular Cx could be molecular targets for the deleterious effect of environmental toxicants on spermatogenesis.^{16–18} However, to date, there is a lack of information (i) on a reliable link between altered Cx expression and toxicant exposure, (ii) on the developmental window during which Cx is the most sensitive to pollutants and could result in transgenerational reproductive dysfunction and (iii) on the molecular mechanisms by which toxicants impair male reproductive function through Cx disruption. In the present paper, after introducing the physiological role of Cx, its regulation and its testicular function, we propose to give an overview of Cx expression within the developing testis. We further analyze the effect of fetal and neonatal exposure to environmental toxicants on testicular Cx expression and its consequence on offspring male fertility.

Gap Junctions and Connexins

Structural aspects. Gap junctions are cluster of membranous channels at the interface between two adjacent cells, which allow efficient and direct exchange of small cytosolic molecules (<1 kDa) involved in cell physiology, such as ions (Ca²⁺), intracellular messengers (cAMP, cGMP, Ins(1,4,5)P₃...) and metabolites. As illustrated in **Figure 1**, gap junction channels are formed by two transmembrane hemi-channels termed connexons, each issued from adjacent cells. Each connexon is an oligomer of six Cx molecules. Cxs are non-glycosylated proteins with four transmembranous domains, one intracellular loop and two extracellular loops, implicated in connexon association and cytoplasmic NH₂- and COOH-termini.^{14,19} Cxs are encoded by a multigene family that contains 21 members in humans and 20 members in rodents.²⁰ Specific Cx functions have been extensively investigated by analyzing targeted mouse mutants and human genetic disorders.²¹ The Cx isoforms exhibit about 40% overall sequence identity with an highly conserved sequence for transmembranous and extracellular domains and an hypervariable sequence for the COOH-termini domain. Most cells express more than one isoform and some isoforms exhibit tissue-specificity whereas others are restricted to specific species (such as Cx33 found only in rodent brain and testis). Two major alternative nomenclatures have been proposed for Cx proteins. The most widely used is based on the molecular mass of the Cx isotype (Cxs vary in mass from 25 to 62 kDa) while the other is based on mRNA and protein sequence alignments forming two major groups named α and β .^{22,23}

Several Cx isotypes can form a connexon (**Fig. 1**). Homomeric connexons are composed of exclusively one unique Cx isoform, whereas heteromeric connexons are formed by more than one isotype. Similarly, gap junction channels can be homotypic when they are formed by two similar connexons or heterotypic when constituted by two different connexons.¹⁴ Differences in channel structure may support differences in ion or second messenger permeability between cell types.²⁴ Recently, a second group of gap junction related proteins has been identified in mammals,

the pannexins, which are structurally similar to Cx. Three pannexins have been today characterized in humans and rodents. If pannexins are structurally similar to Cx and can form hemichannels, there is, until now, no evidence that they can form gap junction channels.^{25,26}

Regulatory aspects. Cx can be controlled by several mechanisms that include gene transcription, mRNA processing, protein synthesis, post-translational modifications (phosphorylation, ubiquitination) that interfere with assembly in the Golgi apparatus, transport to the cell surface, anchoring to the cytoskeleton, regulation of internalization and degradation of the protein. All these processes have been largely discussed in previous reviews in reference 27–29. In addition, the gap junction turn-over is a highly complex process since the delivery, assembly and removal of junctional channels are actively controlled.³⁰ Indeed, Cx trafficking is regulated by a large number of scaffolding proteins, kinases, phosphatases and cell signaling molecules, which undoubtedly participate in the multiple stages of gap junction channel formation and functionality.³¹ Analysis of pathological situations revealed that mutation of Cx and epigenetic regulation of Cx gene could also be important features in the control of Cx and gap junction functionality.^{32,33}

Connexin Expression, Function and Regulation in the Developing Testis

In the developing testis, Cx expression evolves during the different periods of cell proliferation and differentiation. Several key developmental periods (fetal, neonatal, pubertal and adult) could be used as reference endpoints to analyze and understand Cx expression, cellular localization, function and regulation.

Connexin expression. Transcripts for Cx26, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx46 are found by RT-PCR in the fetal mouse testis.³⁴ Cx43 and Cx45 were readily amplified whereas only faint bands were obtained for Cx26, Cx31, Cx32 and Cx37. Intriguingly, Cx31.1, Cx33, Cx40, Cx46 and Cx50 were not reproducibly detected in tubules from postnatal day 6 rats.³⁵ This discrepancy could be explained by a repression of the expression of these Cxs as well as species-specificity. After birth, on day 15 postpartum, seminiferous tubules contain all of the Cx mRNAs in adult testes (Cx26, Cx31, Cx31.1, Cx32, Cx33, Cx37, Cx40, Cx43, Cx45, Cx46 and Cx50), which are present in polysomes and presumably translated.³⁵ Among these 11 Cxs, Cx43, the most widely distributed Cx in the organism, is early expressed during development^{36–38} and is the most predominant Cx in the growing testis.^{39,40}

Connexin localization in the testis. In fetal and newborn rats, and at the time of initiation of spermatogenesis, Cx43 is present between Leydig cells, between Sertoli cells and between Sertoli and germ cells.^{41–43} During this critical period of testicular development, Cx43 is first detected as separate dots or a filiform-like network (Fig. 2), which shifts from the apical to the basal regions of seminiferous tubules.^{42,44,45} From postnatal day 12 and thereafter, Cx43 appears as an intense and focal band distributed close to the base of the seminiferous tubule (Fig. 2). This pattern of expression is quite similar to that reported for occludin, an

Figure 2. Immunolocalization of connexin 43 in the wild-type mouse testis during neonatal development. From day 6 to day 27 postpartum, seminiferous tubules are populated by Sertoli cells and increasing number of maturing germ cells (A, C, E and G). Clusters of Leydig cells take place between the tubules. A spotty signal is mainly located in the adluminal compartment of seminiferous tubules at day 6 and 8 postpartum (B and D, arrows). Afterwards the immunoreactive signal appears as a focal band distributed close to the base of the seminiferous tubule (F and H, arrows) in the region of Sertoli/Sertoli cell occludin junctions, essential component of the blood-testis-barrier (x200).

integral membrane protein component of the blood-testis barrier.⁴⁶ In contrast to Cx43, only sparse results have been reported for the intratesticular localization of other Cx proteins.¹¹

At puberty, Cx43 is localized in the interstitial compartment, between Leydig cells, and also highly intermingled with proteins of tight and anchoring junctions between Sertoli cells at the level of the BTB^{12,47,48} and between Sertoli cells and germ cells: spermatogonia and spermatocytes.⁴⁹ During the first wave of spermatogenesis, the levels of Cx43 protein measured in the seminiferous tubules increase. Thereafter, its expression is related to the development and to seminiferous epithelium stages characterized by the association of specific germ cells.¹¹

Connexin function during testis development. To date, the role of Cxs in the establishment and maintenance of the spermatogenic process is still unknown. Indeed, null-mutant male mice for many Cx, such as Cx31, Cx32, Cx40, Cx46 and Cx50, exhibit normal fertility.⁵⁰ Since Cx43 is the most expressed Cx in the testis, it is likely to hypothesize that it might play a key role in the developing testis process and thereafter in spermatogenesis. Unfortunately, Cx43 knockout (KO) mice die shortly after birth due to Cx43 involvement in heart and brain development.⁵¹ The implication of Cx43 in fetal testis development is supported by the observation that male fetuses exhibit a marked depletion in primordial germ cells (PGC).⁵² This depletion in germ cells

Figure 3. Schematic drawing illustrating the potential role of Cx43-based gap junction (red structures) in the fetal/neonatal testis (left part) and in the mature testis (right part) between Leydig cells (yellow), Sertoli cells (blue) and germ cells (green).

could be explained by the essential role of Cx43 in PGCs migration in 8.5- and 11.5-d-old fetuses.⁵³ Moreover, in older fetuses, Cx43 plays an essential role in PGCs cell survival, since apoptotic loss of PGCs in the Cx43 KO mouse fetuses, due to abnormal p53 activation, was reported in reference 53. These results summarized in **Figure 3** strongly suggest that Cx43 is involved in fetal testis development by regulating PGCs survival and/or migration.^{52,53}

In order to understand if Cx43 plays a physiological role during neonatal testis development, fetal testes from Cx43 KO mice were grafted under the kidney of wild-type adult mice. Unfortunately, the germ cell deficiency, due to altered PGCs migration and survival defects, persists after birth, giving rise to a "Sertoli cell only" (SCO) phenotype.⁵⁴ By using a Cx inhibitory mimetic peptide strategy, we reported that Cx43 between Sertoli cells controls Sertoli cell proliferation in seminiferous tubules from 7,8 d-old mice whereas Cx43 between Sertoli cells and spermatogonia regulates germ cell survival rather than germ cell proliferation.⁵⁵ Finally, the role of Cx43 in the regulation of Sertoli cell proliferation and differentiation was confirmed by original studies using testis from Sertoli cell conditional Cx43 KO mice.^{56,57} Altogether, these data suggest that, during the course of testis development, Cx43 present in the perinatal testis controls Sertoli cell differentiation and maintains the number of germ cells (gonocytes or primitive spermatogonia) through

Cx43-based gap junctions between germ cells and the adjacent supporting cells (**Fig. 3**). Although we have presently no proof, it could be hypothesized that Cx43 present in perinatal Leydig cells is essential for synchronizing androgen production, a prerequisite for germ cell development and sexual differentiation of the genital tract and central nervous system.

The role of Cx43 in adult testis was first underlined by the substitution of the coding sequence of Cx43 gene by Cx32 and Cx40 in knock-in (KI) mice (Cx43KI32 and Cx43KI40). Under these conditions, the lack of Cx43 conduced to impaired spermatogenesis with SCO seminiferous tubules.^{58,59} Reduced spermatogonia number has also been described in drosophila males carrying a null mutation in the zero population growth (zpg) locus that encodes the germ line-specific gap junction protein, innexin 4.⁶⁰ Moreover, one day after intratesticular injection of panCx inhibitory mimetic peptide, which virtually blocks all testicular Cx, seminiferous epithelium mostly contains spermatocytes and elongated spermatids whereas only primary spermatocytes and spermatogonia are present on day 15 post-treatment.⁶¹ In agreement with these data, it has been recently reported that a close Cx43-dependent coupling between Sertoli cells and pachytene spermatocytes is essential for meiotic progression of rat spermatocytes.⁶² Thus, altogether these findings suggest that Cx43-based gap junctions between Sertoli and germ cells could allow metabolic and signaling coupling, crucial

for spermatogonia proliferation and late maturation of spermatocytes. Finally, to accurately characterize the role of Cx43 in spermatogenesis, a Sertoli cell conditional Cx43 KO mouse using Cre-loxP methodology has been developed by two independent laboratories.^{56,57} These studies revealed the presence of seminiferous tubules devoid of germ cells, but with Sertoli cell proliferation, confirming that Cx43 is essential for both Sertoli cell differentiation and spermatogenesis establishment. Based on these results, one could hypothesize that adult Sertoli cells would ensure metabolic and signaling coupling to germ cells, through Cx43 gap junction channels and allow synchronization of male germ cell proliferation, differentiation and survival (Fig. 3). However, the mechanism(s) involved in this process is (are) still unknown. Nevertheless, it has been proposed in female that Ca^{2+} flux, which propagates through gap junction channels, between granulosa cells (the homologous of Sertoli cell in female) and oocytes, acts as a meiosis regulator during oogenesis.⁶³ The possibility that Ca^{2+} waves also participate in the control of male germ cell proliferation is strongly suspected.⁶⁴ If the mechanism by which Cx43 controls Sertoli cell proliferation seems to be also dependent on Ca^{2+} waves,⁵⁵ the molecular machinery and the target genes remain, to date, unknown.

It is interesting to note that Cx43 in Sertoli cell exhibits unique and specific role, since its loss in Sertoli cell Cx43 KO mice cannot be compensated by other Cxs, known to be expressed in the testis. Then, understanding the physiological regulation of this Cx during testis development can bring more evidence of the specific and crucial role played by this protein and highlights the systemic mechanism(s) controlling testis development and spermatogenic function.

In addition to the control exerted by Cx43 gap junction channel, there is now compelling evidence that Cx43 can also drive the spermatogenic process through regulation of tight and anchoring junctions that are closely intermingled with each other at the level of the BTB, crucial for the establishment and the maintenance of Sertoli cell polarity. This hypothesis was first supported by previous studies in continual (guinea pig) and seasonal (mink) breeder testes, indicating that Cx43 between Sertoli cells participates in the formation and regulation of the BTB.⁶⁵ Other recent studies revealed that the blockage of Cx functions by using a pan-connexin peptide concomitantly leads to a diminished occludin expression and upregulated N-cadherin expression.⁶¹ Since Cx and occludin are present within a protein complex at the membrane, it has been proposed that the dysregulation of Zonula occludens 1 (ZO-1), a common adaptor shared by occludin- and connexin-associated protein complexes,⁶⁶ indirectly hampers the stability and integrity of the occludin-associated protein complex. This new property of Cx43, which completes and reinforces its role in spermatogenesis, has also been supported by a new set of data demonstrating that Cx43 controls tight and anchoring junction proteins involved in the blood-testis barrier integrity.^{64,67,68}

Altogether, these data suggest that Cx43, through at least these two ways, can play a pivotal role in the establishment, maintenance and control of the reproductive testicular function. Because of its key role in these processes, it could be hypothesized that Cx43 dislocation, during critical periods of development,

conduces to abnormal testis development, which further leads to male reproductive dysfunction in adulthood.

FSH/LH effects on testicular connexin 43. Gonadotropins are key hormones in the control of spermatogenesis. Thus, the possibility that Follicle Stimulating Hormone (FSH) controls Sertoli cell proliferation and differentiation, at least partly through Cx43, has been hypothesized since a previous study demonstrated that FSH increases the levels of Cx43 mRNA, reduces cell proliferation and enhances differentiation of granulosa cells, the homologous of Sertoli cells in the ovary.⁶⁹ In Sertoli cells, it has been demonstrated that FSH and its second messenger, cAMP, upregulate the electric and functional coupling,^{70,71} and concomitantly modify the intracellular localization of Cx43 in Sertoli cells.⁷² Recently, we reported that exposure of seminiferous tubules to FSH and cAMP leads to reorganization of the Cx43 gap junction plaques, which appear less discontinuous and thicker as compared with the control plaques.⁷³ This morphological modification was accompanied by a slight coupling increase. However, others were unable to demonstrate that the effect of FSH on maturation of Sertoli cell functionality takes place regardless of the presence of Cx43-based gap junctions.⁷⁴

Contradictory results have also been reported on the effect of Luteinizing Hormone (LH) on Leydig cell Cx43 expression. LH/human chorionic gonadotropin diminished in vitro and in vivo Cx43 mRNA and protein in rat Leydig cells and caused a redistribution of Cx43 to the plasma membrane.⁷⁵ Studies on the TM3 Leydig cell line demonstrated that increased testosterone secretion in response to LH was associated with reduced intercellular communication, suggesting that gap junction mediated coupling could be the modulator of hormone secretion in Leydig cells and consequently of spermatogenesis.⁷⁶ However, other authors reported that the in vitro LH-stimulated androgen production was not different between testis from wild-type and Cx43 KO mice suggesting that the LH-stimulated steroidogenic activity of Leydig cells was not compromised by the absence of Cx43.^{77,78} Altogether, from these contrasting studies, it is not possible, to date, to really appreciate the true role played by the gonadotropins LH and FSH in the control of testicular Cx43.

Testicular diseases associated with impaired connexin expression. Since Cxs play a pivotal role in tissue homeostasis, impaired expression of Cx has been associated with numerous human pathologies, including cancer.^{32,79} In the testis, earlier morphological studies described severe disorganization of gap junction structure in men with impaired spermatogenesis.⁸⁰⁻⁸⁴ More recently, it has been reported that Cx43 mRNA and protein are absent within seminiferous tubules but are still present in Leydig cells in azoospermic patients with Sertoli cell only (SCO) syndrome.⁸⁵⁻⁸⁷ However, no testicular alteration was reported in men with Cx gene mutations.^{88,89} As reported for other tumoral tissues, Cx43 expression is totally altered in testis cancer. Indeed, Cx43 protein is undetectable in human testis infiltrated with carcinoma-in situ (CIS) or in seminoma,⁹⁰ and aberrantly delocalized within the cytoplasm of seminoma cells.⁹¹⁻⁹³ In contrast to Cx43, Cx26, another Cx usually not expressed by normal human

testis, and Cx40 are both upregulated in infiltrated tubules with spermatogonial arrest, CIS-only and seminoma.⁹⁴

Effects of Environmental Toxicants on Connexin 43 in the Developing Testis

As discussed in the previous chapter, there is strong evidence that Cx43 expression in the fetal and neonatal testis is a prerequisite for normal establishment of spermatogenesis and future fertility in adulthood. Thus, it is obvious that impairment of testicular Cx43, during these critical periods of development, could result in inappropriate germ cell survival, development and differentiation, and consequently could lead to infertility of male pups. Indeed, it is now well established that environmental toxicant exposure during these periods represents a more considerable risk to animals and humans than exposure during adulthood. Fetal genital tract development is particularly sensitive to environmental pollutants for a specific gestational time-window from 15.5–17.5 d in rat⁹⁵ and 8–14 weeks in humans.⁹⁶ A second key period for the reproductive development of male pups occurs after birth, during which the perturbing agents can be transferred to the pups via lactation. Throughout these two perinatal periods, somatic cells of the genital tract differentiate into cells that acquire mature functions and become representative of the adult testis. It has been suggested that pollutant exposure, during these periods, could be the cause of a matrix of reproductive problems, such as reduced sperm counts, cryptorchidism, hypospadias and testis cancer. All these physiological disorders, resulting from prenatal Leydig and Sertoli cell dysfunction with secondary androgen insufficiency and impaired germ cell development, have been regrouped in the formulation of “testicular dysgenesis syndrome.”⁹⁷ This concept of environmental influence has been supported by experiments in laboratory animals exposed to high doses of a major representative testicular toxicant, dibutyl phthalate.^{98,99} In addition, it is noteworthy that such perinatal exposure to pollutants alters not only the fertility of the first generation F(1) of male offspring but also their subsequent second F(2) and third F(3) generations.^{100,101}

As summarized in Table 1, environmental toxicants can damage reproductive function through different characterized mechanisms and pathways: (1) by acting as endocrine disruptors through receptors; (2) by altering many genes that are essential for spermatogenesis and testosterone synthesis; (3) by exhibiting epigenetic effects and by inducing oxidative stress within the testis. For example, the consequences of phthalate esters, carbofuran and bisphenol A exposure on male reproductive function have been well documented. These toxicants can alter testosterone synthesis and insulin-like factor required for normal development of male genitalia,¹¹¹ decrease sperm motility and sperm count, increase abnormal sperm and induce defects during spermiogenesis, such as acrosomal granule and abnormal nucleus.^{134–137} In addition to these defects, disruption of ectoplasmic specialization between Sertoli cells and between Sertoli and germ cells has also been reported for BPA and cadmium, suggesting a direct effect of these toxicants at the cell/cell contact.^{7,119,126,137}

Environmental toxicants and connexin expression. It is well established that gap junctions and Cxs are potential targets for many environmental compounds that could exhibit cell type/tissue/organ specificities and induce distinct pathological consequences (embryo lethality and neurotoxicity of the central nervous system, skin hyperplasia, teratogenesis and reproductive dysfunction).¹⁶ Generally, the adverse effects of these chemical compounds on Cx can occur at the transcriptional, translational and posttranslational levels¹³⁸ and may affect many organs such as liver¹³⁹ and testis.¹⁷

The result of environmental toxicants on male reproductive function, specifically mediated through Cx alteration, could be a consequence of effects that occur at several levels: hypothalamus/pituitary, thyroid, testis (Fig. 4). At the gonadal level, by using a Sertoli cell line, we reported that gap junctions and Cx43 expression are particularly altered in response to a large number of testicular toxicants, such as toxins, xenoestrogens, pesticides, herbicides and heavy metals.^{130–132} During perinatal development, Sertoli cell junctional proteins (namely adhesion, gap and tight junctions) appear as highly sensitive targets for a perturbing chemical such as BPA.¹⁴⁰ In agreement with such hypothesis, it has been reported that neonatal exposure to an anti-androgen flutamide induces a long-term adverse effect on spermatogenesis through alteration of Cx43 gap junction and permanent impairment of both Sertoli and Leydig cell functions.¹¹⁷ In the testis of 19-d-old rats fetuses, in utero exposure to a part of phthalate esters affects many genes involved in cholesterol transport and steroidogenesis, intracellular lipid and cholesterol homeostasis, insulin signaling, transcriptional regulation and oxidative stress but also Cx43 gene.¹⁴¹ These observations suggest that the gap junction protein can also be a target for these toxicants in the fetal testis. Moreover, maternal exposure during gestation to tributyltin chloride (TBT), a chemical agent commonly used in many biocides, markedly reduced the levels of Cx43 in the fetal gonad.¹²³ This study also demonstrated that maternal TBT exposure conducted to a permanent damage of spermatogenesis in the testis of male pups. Similarly, postnatal toxicant exposure, through lactation, and prepubertal treatment of animals resulted in a reduction of testicular Cx43 expression, associated with germ cell sloughing and infertility.^{117,140,142} Other authors reported that exposure of immature rats via lactation to a mixture of xenoestrogens (17- α -ethinyl estradiol, bisphenol A and alkyl phenols) exerted long-lasting deleterious effects on epididymal sperm concentration and motility and at the same time reduced Cx43 expression in the seminiferous epithelium, suggesting that the intercellular coordination of testicular function may be affected.¹²⁴ In addition to disrupt Cx43 gap junction functionality, recent studies demonstrated that a blockade of the channel coupling by BPA gives rise to signs of germ cell loss, resulting from Sertoli cell blood-testis barrier impairment (BTB).^{68,120,126} These results argue for our recent findings that the levels of occludin, N-cadherin and β -catenin, components of the BTB, are altered in the testes of Sertoli cell Cx43KO^{-/-} mice.⁶⁴ If the data described above support the possibility that such a mechanism is operating in the mature testis, it is likely that the effect of testicular toxicants could be somewhat different during the rat fetal/neonatal sensitive period

Table 1. Exemplified mechanism of actions and pathways of several environmental toxicants known to exert reproductive toxicity in animal and in vitro studies

Alterations	Mechanisms	Pathways	Toxicants	Réf.
Alteration of the endocrine system	Disrupted hormone synthesis, storage, release transport and clearance	xenoestrogens and anti-androgens that exert mimetic effects genomic, non-genomic, epigenetic effects	BPA, alkylphenol, octylphenol, dimethoate, PCB153	102-108
	Disrupted hormone/receptor recognition and binding		Triphenyltin	
	Disrupted post receptor activation		Cyproterone acetate, vinclozolin	
Alteration of steroidogenesis	Androgen deficiency	reduced expression of steroidogenic factors: StAR, CYP17A1, CYP11A1, 3 β -HSD	DEHP, DBP, BBP, HCH	109-112
Induction of oxidative stress	Increased expression of apoptosis genes	DNA damaging, death receptors DR4,5,6 (Fas-signaling pathway suggested)	HCH, DDT, DEM, TBHP, MEHP, BCA, flutamide	111-118
	Disruption of the blood-testis barrier associated with junctional proteins internalization	PI3K/c-Src/FAK and cytokines/ MAPKs signaling pathways	TCCD, BPA	7, 67, 68, 119, 120
Impairment of Gap Junction Intercellular Communication	Downregulation of Cx	Cx promoter hypermethylation	BHP	33, 121, 122
		not determined	Flutamide, PCB153, alkyl phenol, EE2, BPA, TBT, TCCD, xenoestrogens, pesticides, herbicides, heavy metals	17, 106, 117, 123-125, 130-132
	Docking of toxicant in gap junction channel pockets	Erk1/2, endocytosis of TJs and AJs	BPA	126
	Internalization of gap junctions associated with altered Cxs phosphorylation	MAPKs (Erk1/2)/c-Src	HCH	127-129
		c-Src	TCCD	125, 128
		not determined	DDT, EE2, DES, LNG	130, 131

since the BTB is not structurally functional at this time. Indeed, the mouse testis enters pubertal maturation on days 8–10 post-partum,¹⁴³ and in the immature testis tight junctions, the main constituents of the BTB, are absent whereas gap junctions are common.¹⁴⁴ Previous studies demonstrated that the formation of the BTB in the mouse is delayed by neonatally administration of diethylstilbestrol (DES).¹³³ Interestingly, this potent estrogenic

pollutant is known to abolish GJIC in Sertoli cells by inducing Cx endocytosis and degradation.¹³¹ Thus, it is possible that, before controlling the BTB dynamics in the adult testis, Cx43 and gap junctions actively participate in the establishment of BTB in the neonatal testis and that exposure to testicular toxicants during this critical period impairs the kinetic of this process. Altogether, from these data, one can hypothesize that the control exerted by

Figure 4. Schematic drawing showing the different levels at which Cx43 expression could be affected by exemplary environmental toxicants during fetal and neonatal development. The consequences on Sertoli, Leydig and germ cell proliferation and differentiation are indicated in red. GnRH: Gonadotropin Releasing Hormone; LH: Luteinizing Hormone; FSH: Follicle Stimulating Hormone; TSH: Thyroid Stimulating Hormone; T4: thyroxine; BTB: blood-testis barrier; PCB 153: Di-ortho 2,20,4,40,5,50-hexachlorobiphenyl. HCH: γ -hexachlorocyclohexane; TBT: Tributyltin chloride.

Cx43 on spermatogenesis is not only mediated via transfer of signaling molecules through gap junction channels, but also directs transcriptional regulation of protein components of the BTB.

In addition to direct effects at the gonadal level, environmental toxicants can disrupt reproductive function through indirect effects on the central nervous system (Fig. 4). Indeed, it is well established that environmental toxicants disrupt coordination and synchronization of GnRH release.¹⁰⁸

During gestational and lactational periods, maternal exposure of dimethoate (*O,O*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate), a broad-used systemic organophosphate insecticide/acaricide, adversely affected the pituitary-testicular axis of neonatal mice by reducing LH and testosterone levels and further caused reproductive dysfunction of male adult mice.¹⁰⁵ Since Cxs can play a part in the coordination and synchronization of GnRH release by GnRH neurons¹⁴⁵ and in the initiation of puberty,¹⁴⁶ it is possible to speculate that toxicants affect male reproductive

function by means of a deleterious effect on Cx expression at the central nervous system.

Adverse effects of reprotoxic agents could also be exerted by an alteration of the thyroid-gonadal axis (Fig. 4). Indeed, it is recognized that thyroid hormones, with FSH and androgens, participate in the control of neonatal Sertoli cell proliferation and differentiation, and that this control is partly exerted through testicular Cx43.^{147,148} Persistent reduced thyroxine levels, after PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) exposure, disturbs perinatal Cx43 Sertoli cell expression and subsequently impairs spermatogenesis.¹⁰⁶ Altogether, these data argue for Cx43 as one of the major molecular target for toxicants in the perinatal testis. However, they do not give more information either on the mechanism(s) by which Cx43 could be disrupted or on the nature of the testicular cell types, in which the gap junction protein is affected by the toxicants.

Effects of toxicants on connexin 43 in the developing testis: cell specificity. In the testis, the somatic cells, Leydig and Sertoli cells, are the first targets for environmental toxicants. However, it is unknown whether Cx43 is similarly affected in both cell types. Leydig cell number increases during the initial 2–3 mo following birth and is associated with the increase in plasma testosterone. Thus, it is questionable if Cx43, the only Cx expressed in these cells, is essential for Leydig cell development. In fetal and neonatal Leydig cells Cx43 allows to maintain synchronous androgen secretion essential for masculinization of the genital tract and of the nervous central system as well as for germ cell development (Fig. 3). During gonadal development, testicular toxicants could induce Leydig cells androgen deficiency,¹⁰⁹ which further impairs proliferation of Sertoli cells,¹⁴⁹ and consequently their nursery functions for developing germ cells.¹⁵⁰ The vital role of Sertoli cell proliferation throughout the neonatal period is supported by the observations that the number of sperm in adulthood is determined by the number of Sertoli cells.^{151,152} Thus, any effector that impairs the viability and functionality of Sertoli cells as well as their proliferation rate, during critical periods of development, could induce serious secondary damages of spermatogenesis. However, the importance of Cx43 in Leydig cell function was not validated by Cx43 KO mice strategy, concluding that the presence of Cx43 is not essential for androgen production.¹⁵³ Consequently, it has been proposed that the main deleterious effect of reproductive toxicants on fertility was, for the most part, not associated with disrupted Cx43 gap junction in Leydig cells. Interestingly, fetal phthalate exposure disrupts gametogenesis without alteration of steroidogenesis in mouse and human fetal gonad, suggesting that the main effect of these toxicants probably occurs through Sertoli cell dysfunction.^{154,155}

Previous findings reported that prenatal and neonatal exposure of mice and rats to bisphenol A (BPA) leads to severe spermatogenic impairment of male pups.^{137,156,157} These anti-reproductive effects have been mainly attributed to perturbations caused by the chemical on Sertoli cell junctions. In agreement with these observations, reduced communication between Sertoli cells and gonocytes has been reported in the developing testis after in utero exposure to di(n-butyl)phthalate (DBP).^{158,159} Other studies reported that the deleterious spermatogenic effect

of this chemical could result from failure of gonocytes to migrate toward the basal membrane postnatally¹⁶⁰ and consequently to gonocyte dysgenesis.¹⁵⁸ Another toxicant, BPA, given to prenatal and neonatal animals, conduces to spermatogenesis impairment of male pups associated with Sertoli cell junction disruption.^{137,156} Other studies demonstrated that the impaired GJIC consecutive to environmental toxicants was associated with downregulation of Cx43 expression in the fetal and neonatal testes.^{121,125} By developing in vitro studies using mouse and rat Sertoli cell lines, we previously reported that a large number of toxic agents are able to affect Cx43 in mature Sertoli cells.^{127–132} There is now strong evidence that Cx43 is also able to regulate other components of the BTB and by this way to indirectly control spermatogenesis.^{67,68,75} Altogether, from these data, it is reasonable to speculate that, as for mature Sertoli cells, environmental toxicants affect Cx43 in the developing Sertoli cells. Subsequently, this defect could conduce to irreversible infertility of the male pups by altering BTB formation, reinforcing thus the significant role of Cx43 in the control of spermatogenesis.

Effects of toxicants on Cx43 in the developing testis: molecular mechanisms. The second exciting question is to know how these toxicants may affect Cx43 expression in the fetal and neonatal testis and conduce to infertility in male pups. Many environmental toxicants such as lindane, p,p'-dichlorodiphenyl dichloroethylene (p,p'-DDE), the major metabolite of DDT, diethyl maleate (DEM) and tert-butyl hydroperoxide (TBHP), BCA and flutamide induce oxydative stress and stimulate the expression of apoptosis-associated genes in the developing testis, leading to male infertility.^{112–115} The main effect of Reactive Oxygen Species (ROS), which are generated and upregulated by these chemicals, is to form DNA adducts and to damage DNA.¹⁶¹ In addition to nucleus alteration, it is recognized that an excess of ROS disrupts germ cell membrane.⁶ Other recent studies revealed that environmental toxicants which induced oxidative stress, may also impair Sertoli cell junctions by enhancing internalisation of tight and adherens proteins via activation of the phosphatidylinositol-3-kinase (PI3K)/c-Src/focal adhesion kinase (FAK) signaling pathway.^{7,119,126} Since there is evidence that proteins involved in cell adhesion or forming tight junctions and Cx are highly intermingled with each other to form intercellular junctional complexes in the testis, it is likely that Cx43 could be a target for environmental toxicants.

A plethora of mechanisms may contribute to the occurrence of Cx43 dislocation in response to toxicant exposure. Some of them have been described in Figure 5. The underlying mechanisms which mediate the downstream effect of a toxicant on Sertoli cell Cx43 may involve mitogen-activated protein kinases (MAPKs), such as Erk1/2.^{127,128} There is evidence that other toxicants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)¹²⁵ and γ -hexachlorocyclohexane (lindane or HCH),¹²⁸ can also be mediated through activation of c-Src kinase or, as recently suggested for BPA, via activation of the phosphatidylinositol-3-kinase (PI3K)/c-Src/focal adhesion kinase (FAK) signaling pathway.^{7,119} Activation of these signaling pathways by any disturbing agents may conduce to disruption of Cx43 at different levels.

Figure 5. Schematic drawing illustrating the potential molecular mechanisms involved in the effects of environmental pollutants on testicular Cx43 at the cellular level, during fetal and neonatal development. The subsequent adverse consequences on cell junctions between Sertoli cells and between Sertoli cells and gonocytes or primitive spermatogonia are indicated.

Mutation of Cx43 gene, resulting from an error in DNA repair or DNA replication in response to environmental toxicants, is also possible, but has never been described. In contrast, impaired Cx43 gene expression, at the transcriptional level, altered mRNA stability at translational or post-translational levels (tyrosine and serine phosphorylation of Cx43) has been currently reported for many toxicants (see above). Epigenetic inactivation of Cx43 gene through histone acetylation, DNA methylation and microRNA-related could be one mechanism by which many chemicals and biological toxic compounds exert their suppressing effect on Cx expression. Indeed, inactivation of the Cx26, Cx32 and Cx43 genes by hypermethylation of their promoter regions has been demonstrated.¹⁶²⁻¹⁶⁵ Epigenetic effect of toxicants has been well documented for hepatic Cx43,^{33,139} and could occur in the testis. This epigenetic effect could also be operative, during critical male programming windows of testis development, and lead to transgenerational reproductive dysfunction.^{16,166} Such hypothesis must be further investigated.

Tyrosine and serine phosphorylations of Cx43 play an essential role in protein maturation and trafficking from the Golgi to the membrane, its docking, and in the control of gap junction

coupling and degradation.^{27,167} Since most of the mitogen-activated protein kinase signaling pathways, which mediate the toxicant-induced hepatic¹³⁹ and testicular¹²⁶ injury, also control Cx43 phosphorylation, it is likely that toxicants, through these signaling pathways, affect Cx phosphorylation and consequently the role of Cx43 gap junctions between Sertoli cells and between Sertoli and germ cells. In agreement with this hypothesis, our laboratory demonstrated that many environmental toxicants (DDT, pentachlorophenol, dieldrin, dinitrobenzene, cadmium chloride, cisplatin, gossypol, bisphenol A and tert octylphenol) disrupt GJIC by reducing the amount of Cx43 and inducing aberrant intracellular localization of the protein in immature Sertoli cells.^{130,132}

In one of these works, we also reported that most of the environmental toxicants are also able to disrupt tight and adherens junctions either by reducing their amount or by inducing aberrant intracellular localization of membranous proteins, such as occludin, zonula occludens and N-cadherin.¹³² The possibility that this effect could be mediated through Cx43 has been postulated. Indeed, recent observations demonstrated that the loss of Cx43 gap junctions, in response to two toxicants (cadmium and

BPA), is associated with a redistribution of anchoring and tight junction proteins from the Sertoli/Sertoli interface to the expression through phosphorylation cytosol resulting from exaggerated endocytosis.^{53,67,126} These data are supported by our findings demonstrating that the levels of N-cadherin, β -catenin and occludin were markedly enhanced in the testis of Sertoli cell specific Cx43 KO transgenic mice.⁶⁴ Thus, downregulation of Cx43 in transgenic mice or aberrant intracytoplasmic Cx43 localization in the toxicant-induced testicular injury may disrupt cell-cell junction in the developing testis, leading to impairment of fertility in the male pups. Altogether, these data support the hypothesis that Cx43 could be one of the main targets for toxicants and that impaired Cx43 expression hampers other proteins of the BTB as a cascade-like system. As we recently reported for DDT, it is obvious that toxicants may control many protein component of the BTB in two different ways: by reducing their membranous levels and/or through redistribution of proteins from the Sertoli/Sertoli interface to the cytosol through an exaggerated endocytosis.¹³⁰ To date, the mechanisms by which environmental toxicants induce delocalization of BTB membranous proteins are not clearly established.

During their life cycle, Cx associate with numerous proteins partners including cytoskeletal components (tubulin, actin and actin-binding proteins), junctional adherens (cadherins, α - and β -catenin), members of the membrane associated guanylate kinase (MAGUK) protein family (ZO-1 and ZO-2), kinases and phosphatases involved in the control of trafficking, assembly, function, internalization and degradation.³¹ At the cell membrane, all these proteins organized around Cx, which appears as a keystone in the structured nexus. We recently demonstrated that the disruption of Cx43 gap junctions, in Sertoli cells exposed to γ -hexachlorocyclohexane, is associated with a remodelling of Cx43 protein partners.^{128,168} In fact, phosphorylation of tyrosine and serine residues, in the Cx43 C-terminal region, induces recruitment of new partners involved in endocytosis leading to aberrant Cx43 internalization into the cytoplasm. Thus, from these data, one could postulate that toxicants, by altering interaction of Cx43 with its partners, disrupt the entire nexus structure organization. Further studies are needed to explore this eventuality. It is likely that this alteration of Cx43 further impedes the cross-talk between adherens, tight and gap junctions, leading to destabilization of the BTB as recently suggested.^{64,67,68,126} By developing molecular modeling analysis, it has been recently assessed that a toxicant such as BPA could disturb BTB integrity by perturbing the gap junction structure.¹²⁶ Altogether, these data strongly support the key role played by Cx43 in the adverse effect of environmental toxicants. The deleterious effect on Cx43 function could further disturb BTB integrity in the adult or BTB development in the perinatal testis.

Concluding Remarks and Perspectives

Over the past 20 y, the importance of Cx signaling in human physiology has been highlighted by the discovery of many Cx gene mutations in a wide range of tissues and systems.¹⁶⁹ Beyond

Cx mutations themselves, alterations of Cx expression, including impaired phosphorylation, trafficking and degradation, have been associated with pathological features and cancer,⁷⁹ which is often the consequence of environmental toxicant exposure.¹⁷ In the testis, there is now compelling evidence that Cx43, between Sertoli cells and between Sertoli and germ cells, is essential for Sertoli and germ cell proliferation, differentiation and survival.⁵⁵⁻⁵⁷ In addition to these features, recent data suggested that Cx43-based gap junctions are also involved in the control of BTB integrity.^{64,67,68,126}

The current review of Cx dysfunction, after fetal and neonatal testis exposure to environmental toxicants, specifically points out Cx43 as a pivotal protein in male reproductive function and a precocious target of environmental toxicants in the developing gonad. Exposure to these chemicals in the course of critical periods of testis development, during which Leydig and Sertoli cells differentiate into cells that acquire mature functions, appears highly susceptible for fertility of male pups. Since Cx43 regulates general cellular processes, such as proliferation, differentiation and apoptosis, and is a crucial spermatogenesis regulator, as a blood-testis barrier component, its perturbation by toxicants seems to be efficient for explaining alteration of testis development and spermatogenesis functions.

In most reported studies, the effect of testicular toxicants has been evaluated as a single molecule effect. Nonetheless, it is likely that wild-type animals and humans are exposed simultaneously to various environmental toxicants that can exert synergistic effect.¹⁷⁰ Then, further investigations are needed before a definitive conclusion can be drawn on Cx43 effect. In addition, the findings described herein, mostly in rodents, must also be interpreted with care since, to our knowledge, no study has been conducted on the becoming of gonadal Cx43 after in utero and lactational toxicant exposure in humans. Indeed, it is surprising that altered expression of Cx43 gene may affect testis function in mouse⁵²⁻⁵⁷ and in rat⁶¹ but simultaneously spare this function in humans. Furthermore, Cx43 mutations have been tightly associated with the oculodendrodigital dysplasia (ODDD) syndrome.⁸⁹ However, to date, phenotypic descriptions of ODDD patients have not concerned reproductive function, which has probably not been investigated in most cases, since these patients have not been reported to be infertile. In the human testis, it is possible that Cx43 dislocation is masked by the presence of another gap junction protein that could play such a role. It could also be hypothesized that a punctual mutation of Cx gene does not affect the functionality with the same efficiency in different tissues, since Cxs are known to form homotypic or heterotypic channels with variable functionalities. Further investigations in this direction are needed to explain these quite different results in rodents and humans. Moreover, it seems important to identify which Cx may play a similar role in the human testis in response to environmental toxicant exposure.

Another interesting question is to know if the observed effect of environmental toxicants on testicular Cx43 is directly mediated through or results from altered expression of one or several of specific protein partners that associate with Cx43 to control its expression.³¹ The fact that one Cx can regulate other proteins of

the BTB could be further examined after toxicant deposition to go further into the fine analysis of the role of one membranous protein vs. the others in spermatogenesis.

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