Long-term effects in progeny of paternal environment and of gamete/embryo cryopreservation

Maurice Auroux*

CHU de Bicêtre (Université Paris-Sud), Andrologie et Biologie de la Procréation, 94275 Le Kremlin-Bicêtre, France

In addition to gross malformations, many problems relating to the formation of gametes and embryos can generate, within a continuum of abnormalities, a number of problems that are less evident. On the basis of genetic and/or biochemical or cytological changes, these effects generally appear long after birth as functional difficulties that range from growth changes and altered endocrine functions and cancer to very late behavioural disorders. Such problems may have effects on males and females before conception, on the embryo during gestation, and may also impact on the success of assisted reproduction techniques. For this reason, we have examined the experimental and clinical data that indicate the long-term consequences, for progeny, of iatrogenic and toxic environmental factors on the male reproductive system, and in particular the effect that one specific condition—cryopreservation—may have on gametes and the conceptus. We then focus on the interpretation given to these data which, in general, emphasize the need not only for further experiments to help understand the mechanism of anomalies and increase the level of vigilance in humans, but also to extend follow-up investigations in children.

Key words: gamete and embryo cryopreservation/long-term effects/paternal exposures/progeny

TABLE OF CONTENTS

Introduction
Experimental and clinical data
Potential mechanisms
Conclusions
Acknowledgements
References

Introduction

During the 1990s, a number of conflicting articles discussed the reality of a steady reduction in both quantitative and qualitative sperm parameters (Carlsen et al., 1992; Auger et al., 1995; Bujan et al., 1996; Fisch et al., 1996; Becker and Berhane, 1997; Younglai et al., 1998). Although the demonstration of quantitative changes remains controversial, an important topic today—given the toxicity of our environment and the increase in the average life-span—is that of qualitative changes. Many investigations have shown that spermatogenesis and spermatozoa are susceptible to attack by a number of natural or artificial physical and chemical factors, including ageing, stress, ionizing radiation, anti-cancer therapy, and exposure to metal pollutants and solvents. Thus, fertility can be changed in populations at risk (Steeno and Pangkahila, 1984). Although the factors described have a clear negative effect on male fertility, the consequences of development of offspring following fertilization by impaired gametes is an additional problem that magnifies the importance of reduced fertility (for review, see Anderson, 1990).

In pathological development, malformations may occur of organs, cells, cell connections (e.g. synapses), and also of molecules (e.g. haemoglobin). In other words, there is a continuum between gross and more subtle malformations (Auroux, 1974, 1997). Beyond the many former difficulties relating to the formation of gametes, the embryo and fetus are indeed able to engender less obvious disturbances, that is, without changes in the apparent morphology. Such genetic, biochemical or cytological changes only appear long after birth, and may manifest as functional disorders such as growth retardation, delayed appearance of early post-natal developmental landmarks, altered endocrine functions, cancer, reproductive problems or behavioural disorders—so-called ‘long-term effects’ (Armitage, 1952; Auroux et al., 1967; Spergel et al., 1971; Auroux, 1974, 1997; Friedler, 1974; Bakke et al., 1976). In the USA, for example, 3% of all children were reported to have a major malformation that was detectable at birth; by comparison, developmental disorders are detected by 1 year of age in 6–7% of infants, and in 12–14% of older, school-age children (Kimmel et al., 1993). A separate survey suggested that ~17% of children have had one or more developmental disability up to the age of 17 years (Boyle et al., 1994), and that ~70% of these developmental disorders were of unknown aetiology.

It is as part of this continuum that we consider the experimental results, as well as clinical data, that indicate the long-term
consequences—for the progeny—of environmental effects on potential fathers on the one hand, and the effects of a specific condition, cryopreservation, on gametes and embryos on the other hand. We discuss the interpretation given, at the present time, to these data.

**Experimental and clinical data**

Until now, the most demonstrative data are notably of an experimental nature, as clinical enquiries encounter major problems with regard to feasibility, standardization of treatment and, occasionally, also ethics. The factors involved, whether iatrogenic or related to toxicity, form part of the individual environment. By the 1980s, approximately 50 000 artificial substances were currently in use by society, with an additional 200–500 new materials introduced each year (Johnson, 1980). It is possible that some of these products—which may have teratogenic properties—might lead to long-term adverse effects. In a recent review of the literature concerning paternal exposure to 232 different agents (medications, social drugs, radiations, pesticides, environmental chemicals and chemotherapeutics) in the context of apparent infertility or teratogenic effects, 10 compounds were found to have been the subject of enquiry on five or more occasions. These agents, and the frequency of reports were: cocaine (22), marijuana (14), methotrexate (10), prednisone (nine), agent orange (dioxin contaminant; 10), lysergic acid diethylamide (LSD; seven), xylene (five), fluoxetine (five), alcohol (five) and toluene (five) (Poynor et al., 1997). Subtle behavioural changes have been demonstrated, for instance, in the offspring of male rats exposed to cocaine (Abel et al., 1989).

Numerous factors responsible for these long-term disorders are now discussed.

**Iatrogenic agents**

**Biochemical agents**

Early studies have shown that thalidomide, methadone or morphine could, when administered to males, lead to disturbances in progeny; examples include increased fetal and neonatal mortality rates in rabbits treated with thalidomide (Lutwak-Mann et al., 1967), or birth weight decrease and change in emotive response in adult rats treated with morphine or methadone (Smith and Joffe, 1975; Soyka et al., 1978). These data have been confirmed in male mice, in which the administration of morphine or methadone led to problems in the offspring’s spontaneous activity and learning capacity, despite the reproductive indices being unaffected (Friedler and Wheeling, 1979; Friedler, 1985). Benzodiazepines pose a particular problem because they introduce adverse effects in spermatogenesis that are common to both father and progeny (War and Das, 1983); doubtless this question will be re-encountered with other, very different, substances such as lead or cannabinoids.

Further research, based on the effects of some anti-mitotic drugs on the offspring of fathers exposed to them, have revealed clear effects in animals, while investigations in humans remain confused and controversial because of the heterogeneity of treatments and the methods used. In humans (for review, see Marmor, 1999), some large cooperative enquiries, involving fathers or mothers, and 5000 to 50 000 births, have not reported any significant increase in gross malformations at birth (Li et al., 1987; Mulvihill et al., 1987; Dodds et al., 1993; Green et al., 1997), although these studies only used questionnaires to obtain information. Children were neither examined nor followed-up, and long-term effects were not sought. In contrast, some smaller studies showed an increase in early miscarriages or congenital malformations (Russel et al., 1976; Holmes and Holmes, 1978). Nonetheless, the existence, in men, of sperm chromosomal aberrations that were shown to occur during and after chemotherapy or radiotherapy (Brewen and Preston, 1975; Genesca et al., 1990; Martin, 1993; Monteil et al., 1997; Robbins et al., 1997) suggests that female contraception during treatment and in the following months is recommended. Similarly, cryopreservation of spermatozoa is advised against during these periods.

Experimental data are better established. Anti-mitotic drugs, when administered to males, may lead to problems in the progeny. For example, procarbazine causes a significant increase in post-implantation losses (Velez de la Calle and Jegou, 1990); cyclophosphamide causes malformations when matings are carried out during or at the end of treatment (Trasler et al., 1985), in addition to functional problems when mating is delayed after treatment. Functional problems relate to either behaviour in prepubescent rats (Adams et al., 1981) or to an increase of post-natal mortality without malformation or problems of growth, and a reduction in learning capacity in adult rats (Auroux and Duhouet, 1985; for review, see Auroux et al., 1990a) (Figure 1). These changes were accompanied by alterations in cerebral biochemical factors, such as hippocampal choline acetyltransferase activity and frontoparietal cortex noradrenaline, involved in memory function (Auroux et al., 1990b). Analogous results had been reported which relate to choline acetyltransferase and other enzymes (acyethylcholinesterase and glutamic acid decarboxylase) (Hsu et al., 1987). These problems may be transmitted to the next generation; indeed, they have been observed up to the third generation (Auroux et al., 1990a). They present the characteristics of dominant mutations, which clearly raises a serious problem, as underlined by other authors (Nomura, 1982; Adams et al., 1984; Davies et al., 1992; Colie, 1993; Friedler, 1993; Olshan and Faustman, 1993; Hales and Robaire, 1994). The anomalies we have observed in the third generation present some peculiarities, probably due to complex genetic rearrangements. For example, an acceleration of weight gain in males, commencing in adults and increasing further in senescence. Thus, some anomalies might be expressed long after birth, a situation which is reminiscent of human genetic pathologies such as Huntington’s chorea syndrome.

An important point is that the perturbations vary as a function of delay in recovery on cessation of treatment. If this delay is long (e.g. 100 days—about two spermatogenic cycles), the percentages of successful rats do not differ between controls and experimental animals, although success requires more attempts by the latter. Moreover, males are more affected than females—a vulnerability previously noted in progeny of parents affected by opioids and alcohol (Friedler, 1985; Abel and Lee, 1988; Abel, 1989a; Cicero et al., 1991). If the delay is short (e.g. 60 days—slightly more than one spermatogenic cycle), the percentage success is lower in experimental animals than in controls, again with those succeeding requiring more attempts than the controls. Once again, males are more affected than females, but spontaneous activity is
was that of increasing sterility in male F1 progeny (Ford et al., 1969). Other phenomena, such as increased post-implantation losses (Schröder and Hug, 1971; Jegou et al., 1991) and various malformations (cleft palate, open eyelid, tail anomalies and exencephalus) (Nomura, 1988) were also observed. We therefore considered whether repeated low-level medical radiations were also toxic. A recent publication showed that, among 7678 births, the mean birth weight of babies of radiation-exposed fathers was lower than in an unexposed group. A similar difference was noted for intra-uterine growth. The downward trend in birth weight (adjusted for gestational age) and fetal growth persisted, despite controls covering infants’ sex and parental variables such age, height, race, education, occupational exposure, parity and maternal smoking (Shea and Little, 1997). We will see from further discussion that the problem concerning occupational and environmental exposure is intricate. However, since medical X-rays are the largest controllable source of man-made ionizing radiation, more detailed studies of the potential effects on progeny of the paternal X-irradiation seem justified, in particular with regard to long-term effects, and especially as the genetic origins of some mental deficiencies are known (Flint et al., 1995).

**Toxic agents**

**Biochemical agents: occupational and environmental exposure**

Many agents that may adversely affect males before mating can lead to problems in progeny which eventually appear after birth, and are related to different functions.

Ethylmethane sulphonate is used in industrial syntheses. It is a mutagen which, when administered to male mice, leads to total or partial sterility in viable pups of the F1 generation, the condition being related to multiple translocations in spermatocytes (Cacheiro et al., 1974). Analogous effects have been shown with lead (Stowe and Goyer, 1971; Lorenzo et al., 1978; Pinon-Lataillade et al., 1993; Wyrobek, 1993), which causes problems in spermatogenesis that are common to the father and progeny. In addition, the offspring of lead-exposed males present, at 30 days of age, a reduction in learning capacity (Brady et al., 1975). More recently, male-mediated developmental disorders have been reported in rats, including lead-induced changes in early gene expression in early embryos (Shelby et al., 1994), as well as changes in hippocampal development of late fetal and neonatal rats, but apparently without functional problems (Gandley and Silbergeld, 1994).

Triethylenemelamine (TEM) is an industrial, anti-neoplastic and mutagenic chemical which, when administered to male mice before maturity, results on the one hand in neurological deficits in offspring that are expressed by an abnormal swimming pattern and on the other hand an inherited TEM-induced chromosomal translocation (Rutledge et al., 1986). Another industrial chemical, ethylene dibromide, leads to alterations in both behaviour and neurotransmitter levels in the offspring of male rats mated at 4 weeks after exposure, this interval indicating that the effect on spermatogenesis occurred during the pre-meiotic stages (Fanini et al., 1984; Hsu et al., 1985). Similar effects on the offspring’s brain neurotransmitters, but without behavioural changes, occurred after inhalation exposure of fathers to an industrial solvent, 2-methoxyethanol (Nelson et al., 1984). Another study showed that the birth weight of offspring born after the fathers had been exposed to industrial solvent was lower than that of control children (i.e. offspring of non-exposed fathers) (Daniell and Vaughan, 1988). Other studies showed that paternal exposure to these solvents was associated with an increased relative risk of spontaneous abortion and congenital malformation, but these data need to be confirmed (Lindbohm, 1995).

**Physical agents: radiation**

It was shown long ago that when male mice were irradiated with X-rays and mated with unexposed females, the long-term effect was that of increasing sterility in male F1 progeny (Ford et al., 1969). Other phenomena, such as increased post-implantation losses (Schröder and Hug, 1971; Jegou et al., 1991) and various malformations (cleft palate, open eyelid, tail anomalies and exencephalus) (Nomura, 1988) were also observed. We therefore considered whether repeated low-level medical radiations were also toxic. A recent publication showed that, among 7678 births, the percentage of successful animals decreased in male and female offspring from treated rats. These anomalies were observed up to the third generation, thus having the characteristics of dominant mutations. These behavioural changes were accompanied by alterations in cerebral biochemical factors (Auroux et al., 1990a,b).
Anaesthetics: The effects of anaesthetics were first investigated in humans more than 25 years ago (Cohen et al., 1974). A retrospective epidemiological study reported an increased risk for both spontaneous abortions and congenital malformations in offspring after chronic paternal or maternal exposure to nitrous oxide (Guirgis et al., 1990). However, in this study both the level and duration of exposure were higher than in prior investigations where no significant effects were detected (McDiarmid, 1993). These results suggested an inadequate scavenging of unused anaesthetic gases (Savitz et al., 1994). It must be emphasized that the long-term effects have not, as yet, been explored. In mice, paternal inhalation exposure to nitrous oxide resulted in a decreased birth weight of male pups, delayed onset of eye opening, and an attenuated hypothermic response to morphine administration. No other alterations were detected (Friedler, 1985).

Biochemical agents: social drugs (alcohol, tobacco, drugs)

Alcohol: When the now so-called ‘fetal alcohol syndrome’ was first described, and the role of maternal alcoholism was recognized (Lemoine et al., 1968), no such similar concern was expressed about the possible influence of paternal alcoholism on developmental outcome. However, several experimental studies later examined the male-mediated effects of oral alcohol administration. In rats, chronic administration resulted in increased resorption and post-natal mortality, and lower fetal weight (Klassen and Persaud, 1976; Mankes et al., 1982); a similar treatment did not induce such effects in mice, however (Randall et al., 1982). A significant reduction in fertility, viability and litter size was reported in offspring of male rats mated 24h after a single large injection of alcohol (Cicero et al., 1994). The mode of alcohol administration seemed to play an important role in these experiments. For example, in mice a marked decrease in birth weight and fertility was observed only when matings were within 5 days of acute (4 weeks) paternal exposure to alcohol (Anderson et al., 1981). In contrast, investigations in rats and mice found that paternal chronic (8–9 weeks) oral alcohol treatment did not affect birth weight or fertility in offspring when mating immediately followed alcohol administration (Abel and Lee, 1988; Abel, 1993). With regard to the long-term effects, several studies showed that a number of functional parameters were altered in rodent offspring by paternal exposure, including thermoregulation (Friedler, 1988), immunological competence (Berk et al., 1989; Gottesfeld and Abel, 1991), various behaviours (Abel and Lee, 1988; Friedler, 1988; Abel, 1989a; Wosniak et al., 1991) and neuroendocrine functions. With regard to the latter point, testosterone and hypothalamic beta-endorphin concentrations were significantly reduced in the offspring of alcohol-treated fathers, and there was also a reduction in seminal vesicle weight (Cicero et al., 1990). Behavioural problems (i.e. changes in spontaneous activity and impairment of learning capacity) in offspring following paternal oral exposure to alcohol varied with species, strain and test parameters (Abel and Lee, 1988; Friedler, 1988; Abel, 1989a; Wosniak et al., 1991). No behavioural effects were found in rat offspring of fathers exposed to ethanol by inhalation, but changes in concentrations of brain noradrenaline, 5-hydroxytryptamine and metenkephalin were detected (Nelson et al., 1988). In general, these results suggest that alcohol might have a mutagenic effect, though other possible explanations will be discussed later.

In humans, a positive association only between excessive paternal drinking and infant low birth weight has been reported (Little and Singh, 1985), but this finding remains the subject of debate (Savitz et al., 1992).

Tobacco: Although the consequences of maternal smoking for progeny have been studied, those of paternal smoking have received much less attention, and studies have essentially been concerned with smoking by both parents (Savitz and Chen, 1990; John et al., 1991; Roeleveld et al., 1992). The specific role of paternal smoking before conception is difficult to establish, and very few experimental data exist. Data concerning the toxicity of tobacco with regard to spermatogenesis are vague, and sometimes contradictory. However, at <35 years of age, the proportion of male smokers would be significantly higher in fertile subjects than in fertile ones (Spira et al., 1981), and the progeny of exposed males seem themselves to be exposed to a risk of cancer as will be seen later.

Drugs: Paternal exposure of mice to morphine or to methadone can produce behavioural deficits in the offspring, in particular a reduction in learning capacity (Friedler, 1985). Morphine exposure of male rats during puberty and adolescence led, in their 2-month-old male offspring, to a reduction in serum LH and testosterone concentrations, accompanied by altered organ weights (seminal vesicles, testes, adrenals). Female offspring also had some endocrinological deviations, albeit different from those in males (Cicero et al., 1991). Finally, the exposure of male mice to cannabinoids can lead to an increase in pre- and post-natal deaths in progeny and, among survivors, to changes in spermatogenesis similar to those of the fathers, i.e. translocations in spermatocytes, which suggest the existence of mutations (Dalterio et al., 1982), as seen for exposure to lead or benzodiazepines. Moreover, in rats, cocaine-exposed males produce offspring with changes of spontaneous activity during the prepubescent period (Abel, 1989b).

Thus, in general these results suggest that drugs may produce adverse spermatogenic, endocrinological and neurodevelopmental effects in the offspring of experimental animals. This emphasizes the need for epidemiological research and the follow-up of children born under these circumstances. All the more so that, beyond the identification of drugs of abuse in human semen (Gerber and Lynn, 1976; Mann and Lutwak-Mann, 1982) or bound to spermatozoa (Yazigi et al., 1991), no information is available at present on the potential male-mediated impact on human progeny of any drug of abuse.

Physical agents

Radiations: In 1990, a controversial epidemiological investigation of long-term effects showed that children of fathers exposed to low-level ionizing radiation at the Sellafield nuclear plant in England presented a six- to eight-fold increase in leukaemia (Gardner et al., 1990). This study conflicted with reports on atomic bomb survivors, which concluded that the risk for progeny was not significantly higher than in cases with non-irradiated parents (Schull et al., 1981), in particular with regard to cancer (Sankaranarayanan, 1999). However, other authors are convinced of the need to study additional groups exposed occupationally to ionizing radiation and, in particular, to study the second
generation descended from exposed subjects (Sever, 1991). Indeed, some research investigations have shown that irradiated male mice could produce male pups with translocations in spermatocytes accompanied by sterility following irradiation with X-rays (Ford et al., 1969; Cacheiro et al., 1974), and dominant cataract mutations following irradiation with gamma-rays (Ehling et al., 1982). Animal experiments showed spermatogenic anomalies and, in offspring, an increase of post-implantation losses or malformations, as mentioned previously (Nomura, 1988; Jegou et al., 1991). Finally, a conclusive long-term effect after gamma-irradiation of paternal gametes was revealed by an increase in aggression of F₁ male mice (Schröder, 1980) and varying behavioural disorders in F₁ rats (Lowery et al., 1990). Therefore, these experimental results justify the institution of human investigations.

Heat: In rams, epididymal spermatozoa which undergo a slight increase of temperature are able to fertilize, but the rate of the resultant live embryos is diminished (Mieusset et al., 1992). The question of early miscarriage in women of heat-exposed males should therefore be examined, at first experimentally and, if necessary, clinically.

A specific long-term effect: transgenerational carcinogenesis

A number of studies in humans have investigated the cancer rate in progeny from males exposed to a harmful environment, including mutagenic factors and toxic occupational or social substances. Some studies have appeared reassuring, but have included only very young children, in whom the frequency of cancer is very low (Li et al., 1987; Green et al., 1997). It is notable that some cancers of the young or of the adolescent (e.g. osteosarcoma) might be due to a new mutation in the paternal tumour suppressor Rb gene (Toguchida et al., 1989), in which case paternal exposure to mutagenic factors would play an essential role.

A review concerning the consequences of cancer chemical treatments on the progeny of exposed parents is rather reassuring because no increase of cancer in children has been demonstrated (Draper, 1989). A recent study has shown that, in the F₁ generation born of about 300 exposed parents (chemotherapy and/or radiotherapy), the rate of 15 genetic sentinel diseases was not increased (Byrne et al., 1998). However, these results conflict with those concerning the effects of occupational and experimental agents.

Occupational and experimental agents

The relationship between fathers’ employment and childhood cancer was first suggested in 1974 (Fabia and Thuy, 1974). Shortly thereafter, a positive association was identified between the exposure of fathers to solvents and brain tumours in their children (Peters et al., 1981). Chlorinated hydrocarbon solvents, paints, petroleum products, pesticides and metals have been most strongly implicated, but specific aetiological agents have not been identified (Savitz and Chen, 1990; O’Leary et al., 1991; Frangos and Peters, 1993). Moreover, as others have recently emphasized (Yu et al., 1999), numerous epidemiological studies suggest significant correlations between paternal exposure to radiations or chemical carcinogens (particularly metals), and the incidence of childhood cancers, including brain tumours, Wilms’ tumours, hepatoblastomas, retinoblastomas, sarcomas and, as mentioned previously, leukaemias (Gardner et al., 1990; Savitz and Chen, 1990; Olsen et al., 1991; Bunin et al., 1992; Tomatis et al., 1992; Yamasaki et al., 1992; Bhatia and Neglia, 1995).

Numerous experimental studies involving chemicals or radiations have confirmed the findings of the epidemiological studies. Exposure of male mice to X-rays and urethane at different stages of spermatogenesis induces a significant increase in offspring lung-heritable tumours, particularly at post-meiotic stages. These are inherited up to F₃ generation, as if they were dominant

![Figure 2](image-url). Male-mediated offspring abnormalities. Frequency of tumours (~90% in the lung) in the adult (aged 8 months) progeny of male mice irradiated at various stages of spermatogenesis, plotted against X-ray doses. ●, acute doses; ○, fractionated doses. Tumour frequency was given by percentage of tumour-bearing progeny in survivors. Vertical bars indicate 90% binomial confidence limits. The dose–response data for radiation show a clear increase in tumour frequency with dose for treatment of post-meiotic stages, but less clear results for spermatogonial treatment. This suggests some repair at high X-ray doses (Nomura, 1982).
mutations with about 40% penetration (Nomura, 1982) (Figure 2). A recent report indicated that male mice treated with urethane, sired offspring with an increased incidence of liver tumours in F1 males, but not in females (Edwards et al., 1999). With regard to exposure to metals, pre-conception treatment of male mice with chromium(III) chloride led to phaeochromocytomas in male and female F1 offspring, and increased the incidence of male reproductive gland tumours and of renal non-neoplastic lesions, which often occur naturally during the ageing process (Yu et al., 1999). In the case of radiation, female offspring of male mice exposed to X-rays at 1 week before mating showed a trend towards a higher tumour incidence of the haematopoietic system than the F1 controls. In addition, a higher percentage of bronchoalveolar adenocarcinomas in male offspring born of irradiated fathers mated under the same conditions confirmed the increased sensitivity of post-meiotic germ cell stages towards transgenerational carcinogenic effects. In other respects, K-ras mutations increased during tumour progression from bronchoalveolar hyperplasia to adenoma (Mohr et al., 1999). Finally, it is interesting to note that preconception paternal irradiation with plutonium-239 results in an increase in the offsprings’ susceptibility to the induction of lymphohaematopoietic malignancy on encountering an irradiation or chemical secondary carcinogenic agent (Lord et al., 1998).

Social drugs: tobacco

Childhood cancers reported in the offspring of men who smoked prior to conception relate to either brain cancer (Preston-Martin et al., 1982) or rhabdomyosarcomas (Grufferman et al., 1982). Two recent studies have confirmed the risk of cancer in children (Ji et al., 1997; Sorahan et al., 1997), particularly for acute leukaemias and lymphomas (Ji et al., 1997). However, results from other studies which consider the association between smoking, alcohol consumption and use of recreational drugs, remain doubtful (Little and Vainio, 1994).

A specific condition: cryopreservation of gametes and embryos

For some years, assisted reproduction techniques have undergone rapid development, with some spectacular results. Gamete and embryo cryopreservation have contributed greatly to these successes. However, although until now no dramatic accident has occurred, consideration should be given as to whether—from the long-term viewpoint—this specific condition is either harmful or harmless with regard to the offspring.

Cryopreservation of gametes

Spermatozoa: Cryopreservation of spermatozoa is a well-proven technology, and many studies have been performed during the past 50 years, especially in the field of veterinary science. Clinical results do not reveal any dramatic biological hazards, and the thousands of children born of donor frozen–thawed spermatozoa appear to be of normal constitution. However, some reports have indicated that cryopreservation of spermatozoa might lead to a minor increase of aneuploidies (T21, T13) (Forse et al., 1985; Federation des CECOS et al., 1993), without the possibility of knowing the respective roles of paternal ageing and of cryopreservation. Moreover, the investigations of long-term effects are difficult and concern only some small population samples. In addition, those studies which have been carried out have not reported any significant data (Manuel et al., 1986).

Experimental results have shown that cryopreservation produces changes in DNA methylation (Ashwood-Smith, 1986) and chromatin stability of human spermatozoa (Royère et al., 1987). Cryopreservation also disturbs calcium transport through the cellular membrane (Bailey and Buhr, 1993). An increase in intracellular calcium can raise the frequency of nuclear DNA breaks through endonuclease activation (Epe, 1993), together with the production of free radicals, which are known to be harmful via lipid peroxidation (Leibovitz and Siegel, 1980; Rao and David, 1984). These changes in calcium metabolism may explain the reduced motility of some cryopreserved but apparently normal spermatozoa. It is interesting, in this regard, to note that the susceptibility of mitochondrial DNA to free radicals is greater than that of nuclear DNA (Wallace, 1992).

Despite these results, the experimental production of ‘normal’ mice from cryopreserved spermatozoa has been achieved (Yokoyama et al., 1990; Songsasen and Leibo, 1998; Wakayama et al., 1998), and some authors even conclude that motility and plasma membrane integrity are not essential for fertilization and the production of live offspring when nuclei of non-viable cryopreserved spermatozoa are injected into oocytes (Wakayama et al., 1998). Cryopreservation of precursor cells seems to give similar results, since mouse oocytes injected with cryopreserved round spermatids can develop into normal offspring (Ogura et al., 1996). In humans, the same result has been obtained recently, as frozen–thawed testicular round spermatids from a patient with a history of incomplete spermatogenesis were able to maintain their viability and capacity to fertilize, and to lead to full-term pregnancy (Gianaroli et al., 1999). Nevertheless, the only criteria used to qualify the resultant progeny as ‘normal’ were the lack of malformation at birth and the capacity to fertilize. The long-term effects (as considered above) were not considered.

Oocytes: Cryopreservation of oocytes has proved difficult in mammals (Whittingham, 1977; Al-Hasani et al., 1986; Glenister et al., 1987; Parks and Ruffing, 1992; Wood et al., 1992), and only limited successes with births after IVF have been obtained in mice (Whittingham, 1977; Nakagata, 1993) and in bovine species (Otoi et al., 1995). In humans, some successes have also been obtained, with pregnancy or birth (Chen, 1986; Van Uem et al., 1987; Kuleshova et al., 1999; Porcu et al., 1999; Wurfel et al., 1999), even after such sophisticated technologies as transfer of ooplasm from cryopreserved–thawed donor oocytes into recipient oocytes (Lanzendorf et al., 1999) or cryopreservation of immature oocytes with subsequent in-vitro maturation (Tucker et al., 1998).

In mice, cryopreservation has been shown to induce an increase in morphological abnormalities in oocytes, a reduced fertility after IVF, and an increase of polyploid embryos, particularly digynic (Glenister et al., 1987; Carroll et al., 1989; Bouquet et al., 1992) which would be (paradoxically considering the births obtained) greater still in humans (Mandelbaum et al., 1987). This polyploidy could correspond to disruption occurring during the freezing–thawing process. The cryopreservation of oocytes involves not only a freezing–thawing cycle but also pre- and post-freezing manipulations where the oocytes are exposed to...
cryoprotectants, in particular dimethyl sulphoxide (DMSO), at
0°C. Cooling to 4°C seems to impair the meiotic spindle, possibly
leading to chromosomal dispersion (Magistrini and Szőlősi,
1980; Pickering and Johnson, 1987). On the other hand, according
to others (Vincent et al., 1990, 1991), the exposure of oocytes to
DMSO can increase the zona resistance to the entry of spermatozoia and disrupt the organization of the cytoskeleton
and the metaphasic plate. Our own study confirmed these results.
Indeed, IVF assays showed that DMSO reduced the fertility of
oocytes, whereas cooling to 0°C had no effect. DMSO, when used
at 0°C, was less deleterious for oocytes. Thus, the pre-freezing
manipulations seem to be important for the fertility of oocytes
(Bouquet et al., 1995). We have also shown that mouse embryos
obtained from frozen oocytes or oocytes exposed to pre-freezing
manipulations presented an increase in the frequency of sister
chromatid exchanges (Bouquet et al., 1993). Since the estimation
of sister chromatid exchange is a sensitive test of mutagenicity,
this suggests that the complete cycle of cryopreservation might
alter the oocyte and, more particularly, induce DNA damage.

Another technique of oocyte cryopreservation—vitrification—
has been used in man and in mice. Vitrification is a method of
directly transferring oocytes into liquid nitrogen after exposure to
a high concentration of cryoprotectant solution (Rall and Fahy,
1985). In man (Feichtinger et al., 1987), cryopreserved oocytes
were fertilized in vitro and transferred into patients, but pregnancies were not obtained. In the mice (Kola et al., 1988),
the number of IVF viable fetuses was widely diminished, and the
incidence of chromosomally aneuploid zygotes was increased,
most likely as a result of disruption of the meiotic spindle.
Moreover, fetal malformations, which were predominantly central
nervous system-type defects, were significantly increased.
Consequently, the authors consider that vitrification should be
used with caution in humans. Thus, oocyte cryopreservation—
whichever method is used—merits further experimental and long-
term clinical studies in young and adults born following the use of
these techniques.

Cryopreservation of embryos

Embryo freezing is common practice in several species, including
humans. This technique can be lethal to some embryos, but is not
considered to have any delayed effects in survivors. In humans, a
first study had shown that in mothers, human chorionic
gonadotrophin and 17 β-oestradiol concentrations were, in the
peri-implantation phase, higher in cases of fresh embryo transfers
than in cases of cryopreserved ones. This difference might be
explained by either the higher number of fresh embryos replaced,
or by the fact that the number of blastomeres and their metabolic
activity may be reduced after freezing and thawing (Salat-Baroux
et al., 1992). This is comparable with the findings that the
metabolism of spermatozoa might be altered by cryopreservation.

Experimentally, in a long-term study including pre-weaning
development, adulthood and senescence, we compared cryopre-
served and control embryos from mice of two inbred different
strains for several quantitative traits. Significant differences were
seen in morphophysiological, sensorimotor and behavioural
features, including body weight, pre-weaning development,
learning capacity (Figure 3) and mandible morphometry, some
of these features appearing in elderly subjects. Observed changes
varied as a function of strain, sex and age (Dulioust et al., 1995).

Other studies have suggested the possibility of detrimental effects
beyond implantation, as shown by increased rates of post-
implantation losses (Rall et al., 1987; Trounson et al., 1988;
Wilson and Quinn, 1989; Liu et al., 1993) or by reduced fetal
weight (Shaw and Trounson, 1989). As part of the continuum that
we have already envisaged, the long-term effects we have shown
are not surprising.

Since our report, a number of studies on children born from
cryopreserved embryos have been published (Sutcliffe et al.,
1995a,b; Olivennes et al., 1996; Wennerholm et al., 1997, 1998;
Aytoz et al., 1999). These authors all conclude that cryopreserva-
tion induced no major pathological features. However, some
differences were found compared with children born after either
IVF without cryopreservation or natural conception, for example
evaluating mental age in children aged about 2 years
(Sutcliffe et al., 1995a), birth weight of girls, and head

![Figure 3. Embryo cryopreservation. Learning capacities (LC) of adult F1
 generation from cryopreserved and control mouse embryos in two inbred
 strains (C3D2 and B6CBA). The Krushinsky test evaluates the ability of an
 animal to find, without previous experience, a dietary stimulus (milk) that has
 been presented and then removed from the animal’s visual fields, once each
 minute, ten times consecutively. The entire test included six sessions of 10 min
each over three consecutive days. ● control females; □, cryopreserved
 females; ▼, control males; △, cryopreserved males. A decreased LC is
observed in cryopreserved B6CBA females (*, P < 0.001). Other parameters
such as pre-weaning development, body weight and mandible morphometry
were changed as a function of strain, sex and age (Dulioust et al., 1995).](image-url)
circumference in singleton girls and twin boys at 1 year of age (Wennerholm et al., 1998). Do these findings suggest the possibility of long-term effects?

On the subject of vitrification, a recent study (Uechi et al., 1999) showed that the percentage of vitrified 2-cell mouse embryos which developed into blastocysts was significantly lower than that of fresh and slow, controlled-rate frozen embryos. Moreover, morphologically normal blastocysts showed a significant decrease in their glucose incorporation activity, and a significant diminution in their implantation rate. Consequently, these authors conclude that ‘more attention should be paid to its safety before vitrification is used routinely in a clinical programme’. We consider that, beyond embryonic, fetal and neonatal stages, long-term studies should be systematically included in the exploration of corresponding progenies.

Potential mechanisms

With regard to the male-mediated effect on progeny, xenobiotic agents can theoretically: (i) change spermatozoa genetic or epigenetic information at various stages of spermatogenesis (Adams et al., 1984; Auroux et al., 1990a; Hales and Robaire, 1994); (ii) be transported by contaminated spermatozoa into the female and/or the oocyte (Yazigi et al., 1991; Yazigi and Polakoski, 1992); and (iii) be carried by seminal fluid into the female, or impair the seminal fluid composition, which may be harmful for spermatozoa and/or the female and the embryo (Hales et al., 1986).

These different modes can of course be associated one with another and, given the varying profile of agents which have been implicated and the diversity of deficits reported, it is likely that such associations are frequent (Wyrobek, 1993; Friedler, 1996). However, among these possibilities the first is the best documented, particularly in relation to the long-term effects.

Germ cell injury: changes in genetic or epigenetic mechanisms

Mutations

Given that the duration of each stage of spermatogenesis, spermiogenesis and storage in the epididymis prior to release is known for man and several other species, it is experimentally possible—by controlling the duration of exposure to toxic agents before mating—to determine not only their stage-specific effects but also the time necessary for recovery (Zenick et al., 1994). Thus, we have seen that the effects of a well-known mutagen agent, cyclophosphamide, progressively decreased, from post-implantation losses to behavioural disorders, as a function of the time interval between exposure and mating (Trasler et al., 1985; Auroux et al., 1990a).

Heritable genetic defects in spermatozoa may be due to either mutations (Adams et al., 1984; Auroux et al., 1990a; Davies et al., 1992; Colie, 1993; Friedler, 1993; Olshan and Faustman, 1993; Hales and Robaire, 1994) or chromosomal abnormalities (Ford et al., 1969; Léonard et al., 1979; Kleiman et al., 1999). Relevant exposures may occur at any time between conception of either parent and the production of their gametes. This includes each parent’s development in utero, during childhood and at puberty. In addition, manifestation of sperm-induced lesions depends upon the repairability of the ovum (Wyrobek, 1993). In order to explain long-term, male-mediated effects, it has been proposed that some paternal exposures could lead to the selection of a particular population of gametes in the course of the fertilization process (Soyka et al., 1978; Friedler, 1993). From this aspect, active participation of the female in the transport of spermatozoa with particular functional characteristics might also play a role in this process (Overstreet and Katz, 1977). Nonetheless, the various experiments that we have reported—and especially those involving mutagenic agents and resulting in transgenerational anomalies—demonstrate the sperm-induced mutations.

In respect of subtle behavioural disorders, the invalidation of some genes in mice reveals the genetic nature of some behavioural phenomena. Indeed, it has been shown that invalidation of the fyn gene leads to an anomaly of the olfactory bulb, leading to behavioural disorders in the pups, which are unable to find their mother’s nipples (Yagi et al., 1993). In the same way, invalidation of the N-CAM gene leads to an another anomaly of the olfactory bulb and, furthermore, of an alteration of some neurones of the hippocampus accompanied by a reduction in learning capacity (Cremer et al., 1994). Thus, results obtained in the rat’s offspring following cyclophosphamide treatment of the father belong to well-known genetic behavioural disorders. There is at least one reason for such high genetic vulnerability in the brain in that it is the most polygenetically determined organ (Van Ness et al., 1979). Damage to polygenic groups is much more frequent than pinpoint mutations, and causes—as shown in Drosophila—some slight changes in the phenotype that alter one function or another (e.g. flying, through alteration of wings), but which are compatible with life (Ramel, 1983).

With regard to cancers, three types of genes may contribute to increase/decrease their incidence: (i) genes which code for DNA repair enzyme (XPA, AT1); (ii) proto-oncogenes (ras, myc); and (iii) tumour suppressor genes (p53, Rb1).

If the mutation involved is dominant, one mutation on one of the alleles is enough to increase the risk of cancer in the F1 generation. If it is recessive, another incident attacking the other allele is necessary to induce this risk (Vogel and Nivar, 1997). Consequently, the incidence of transgenerational cancer at once changes as a function of frequency of mutations in germinal cells and of a second mutation which, in particular, could depend upon environmental carcinogen exposure. In other respects, the susceptibility of germinal cells changes with their stage of development. Thus, the post-spermatogonia phases are more susceptible to mutations than stem cells (Russel, 1990), and the post-meiotic exposure is more grave than the pre-meiotic one (Nomura, 1982). These differences of susceptibility would be linked to the expression of some proto-oncogenes according to the maturation phase (Propst et al., 1988). They are also related to the cell DNA repair enzyme equipment. Indeed, late spermatids, immature and mature spermatozoa do not have a DNA repair system (Matsuda et al., 1989; Inoue et al., 1993). However, this question is very complicated, since the excision enzymes of some nucleotides would be well expressed in pachytene spermatoocytes and round spermatids, but weakly in leptotene and zygotene spermatoocytes and in spermatogonia. Moreover, in some species, oocytes would present a deficiency in these enzymes (Vogel and Nivar, 1997), and hence their spermatozoa-repairing capacity might be diminished. Under these conditions, the stage of sexual
maturation, the sex of an exposed parent, the acute or chronic mode of exposure and its level should play important roles in the induction of mutations.

For cryopreservation, the question has been posed whether a long freezing, which permits mutagenic factors such as natural radiations to accumulate their effects but does not permit repair systems to play their role, might be the cause of irreversible damage (Aschwong-Smith, 1986). As described earlier, it appears that cryopreservation could increase the production of free radicals (Rao and David, 1984), which in turn are able to alter DNA (Leibovitz and Siegel, 1980). Our own results on the long-term effects of embryo cryopreservation suggest that (beyond its immediate damage) embryo freezing, without being highly detrimental, may not be completely neutral (Dulioust et al., 1995, 1999). Similar doubts are emerging about other types of detrimental, may not be completely neutral (Dulioust et al., 1995, 1999). Similar doubts are emerging about other types of

Epigenetic factors

Epigenetic factors include genetic imprinting through DNA methylation. This is a type of epigenetic alteration in which, for some genes, only one allele (either paternal or maternal) is expressed during development (for a review, see Surani et al., 1990). Imprinting differences between male and female are complementary and settle during gametogenesis, but occur also in males during epididymal maturation (Ariel et al., 1994). Moreover, they are altered during embryogenesis (Monk and Grant, 1990; Richard-Chaillelet et al., 1991).

The paternal pronucleus is critical for the proliferation of cells of differentiated tissues (Surani et al., 1990). Thus, the loss of embryonic inner cell mass observed following paternal cyclophosphamide exposure suggests an effect on the male pronucleus (Kelly et al., 1992). In the same way, paternal administration of 5-azacytidine—a drug that is incorporated into DNA and blocks DNA methylation—could alter male germ cell development and function, resulting in alterations in fertilization and early embryo development (Doerksen and Trasler, 1996). For functional long-term effects, it has been shown in mice that some experimental uniparental disomies led to growth and behavioural disorders (Hall, 1990).

In the case of carcinogenesis, changes in genomic imprinting may be involved in the genesis of some childhood cancer, and this has been proposed as a mechanism for the paternal induction of Wilms’ tumour, which has been associated with a number of occupational exposures of the father (Reik and Surani, 1989; Olshan et al., 1990), and of chronic myeloid leukaemia (Hass et al., 1992). Indeed, numerous characteristics of transgenerational carcinogenesis suggest that this mechanism could not be initiated by gene mutation. These characteristics include the lack of Mendelian inheritance of effects with some animal models (Anderson et al., 1994), and the high incidence of transgenerational effects of parental treatments in the physiology of the offspring, which can reach 100% (Campbell and Perkins, 1988).

Thus, it is possible to postulate that the mechanism of pre-conception carcinogenesis involves imprinting changes, resulting in altered gene expression in the offspring (Anderson et al., 1994).

Finally, for assisted reproduction and cryopreservation, there are two well-founded remarks. In IVF, the use of immature imprinting spermatozoa (taken from the testis or from the initial portion of the epididymis) or of spermatids evokes the possibility of long-term effects. In cryopreservation, some cryoprotectants (e.g. DMSO) seem able to change imprinting (Aschwong-Smith, 1986), which leads to the same observation.

**Spermatozoa as carriers of exogenous agents**

It was demonstrated long ago that in rabbits, paternal thalidomide induced several congenital malformations, and that [14C]thalidomide remained bound firmly to spermatozoa (Lutwak-Mann, 1967). Accordingly, the hypothesis that a drug could bind to receptors on the plasma membrane of spermatozoa has raised the question that spermatozoa could transmit teratogens directly into an oocyte, or to its environment. The demonstration that cocaine could bind to an unspecified sperm surface receptor (Yazigi et al., 1991; Yazigi and Polakoski, 1992) and the behavioural changes in the offspring of male rats exposed to cocaine (Abel et al., 1989), supported this concept. However, although tetracycline is known to bind extensively to spermatozoa (Ericsson and Baker, 1967; Briggs, 1974), this antibiotic is not known to induce any paternally derived teratogenic effects, whereas the opposite is true when this drug is administered to the mother (Whalley et al., 1964). Thus, the problem remains to be solved.

In other respects, some viruses (e.g. hepatitis B) have been observed in spermatozoa (Hachouel et al., 1985). We have shown that HIV 1 was able to penetrate within the human spermatozoon (Dussaix et al., 1988, 1993), and subsequent transfer of HIV from the spermatozoa of patients to human oocytes has been observed (Baccet et al., 1994). Thus, viral contamination of the embryo seems possible, in which case the problem of long-term effects is once again posed in the case of insemination between an HIV-seropositive man and an HIV-seronegative woman.

**Changes in seminal fluid**

Many therapeutic and other exogenous chemicals have been identified in the fluids of all components of the male reproductive system in animals (Mann and Lutwak-Mann, 1982), as well as in
conclusions

The environment in which males exist, extending from industrial toxic agents to iatrogenic agents, and from alcohol to tobacco and drugs, can either attack the spermatozoon through mutations and epigenetic changes, or use it as a carrier. Either way, the situation may be harmful to the embryo. The argument has also been proposed that seminal fluid could also transport agents that might be absorbed by the vaginal epithelium.

All of these agents are able to injure the conceptus in an obvious manner, leading in turn to either post-implantation losses or malformations. As a part of the continuum that we described in the Introduction, these agents may also cause long-term effects that appear subtle, but are occasionally as serious as gross malformations. These effects include dysfunctions that manifest long after birth, as well as anomalies relating to growth, neuroendocrine function, behaviour, immunological competence and carcinogenesis. When mutations are involved, defects can be inherited. Furthermore, a particular environment, corresponding to gamete and embryo cryopreservation, can also lead to long-term effects in derived offspring. Moreover, as we have seen, these problems have been of major concern for some time. Finally, it is notable that certain natural factors not envisaged here, for example paternal age, might also have an influence on the long-term quality of progeny. In humans, some dominant autosomal diseases (for reviews, see Auroux, 1998; Tarín et al., 1998) on the one hand, and reduced longevity in daughters on the other hand (Gavrilov et al., 1997), seem linked to paternal ageing during the reproduction period. In addition, we have found comparable behavioural long-term deficits in rat, mouse and man, when young or senescent males were mated with age-constant mature females (for a review, see Auroux, 1998).

Many of the results reported here have been obtained from animal experimentation, and consequently it would be incorrect to extend them too rapidly to the human situation. Thus, at present we have two needs. First, to understand the mechanism of anomalies and increase the level of vigilance, and in this respect further experiments must be performed. Second, in order to explore the consequences of the different harmful agents in man, the follow-up of children (using an unobtrusive method) should be enlarged upon. From this viewpoint, thorough clinical and experimental investigations are not only a scientific necessity, but also an ethical obligation for public health.

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Paternal exposures cryopreservation and progeny


