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Hormonal Suppression for Fertility Preservation in Males and Females

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Abstract

Methods to restore fertility of men and women sterilized by medical treatments and environmental toxicant exposures are under investigation. Rendering spermatogenesis and ovarian follicular development kinetically quiescent by suppression of gonadotropins has been proposed to protect them from damage by cytotoxic therapy. Although the method fails to protect the fertility of male mice and monkeys, gonadotropin and testosterone suppression in rats before or after cytotoxic therapy does enhance the recovery of spermatogenesis. However the mechanism involves not the induction of quiescence but rather the reversal, by suppression of testosterone, of a block in differentiation of surviving spermatogonia caused by damage to the somatic environment. In men, only one of eight clinical trials was successful in protecting or restoring spermatogenesis after cytotoxic therapy. In females, protection of primordial follicles in several species from damage from cytotoxic agents using GnRH analogues has been claimed; however only two studies in mice appear convincing. The protection cannot involve induction of quiescence in the already dormant primordial follicle but may involve direct effects of GnRH analogues or indirect effects of gonadotropin suppression on the whole ovary. Although numerous studies in female patients undergoing chemotherapy indicate that GnRH analogues might be protective of ovarian function, none of the studies showing protection were prospective randomized clinical trials and thus are inconclusive. Considering interspecies differences and similarities in the gonadal sensitivity to cytotoxic agents and hormones, mechanistic studies are needed to identify the specific beneficial effects of hormonal suppression in select animal models that may be applicable to human.

I. Introduction

Medical treatments required for life-threatening diseases such as cancer or exposure to environmental toxicants may jeopardize the fertility of men and women of reproductive age. In men, such exposures can lead to effects ranging from temporary oligospermia to permanent azoospermia, and occasionally to androgen insufficiency. In women, such exposures can result in a range of effects from temporary amenorrhea to premature menopause and permanent amenorrhea, with the associated estrogen insufficiency. Whereas transient effects do affect the quality of life, the most serious effects of concern are the irreversible, permanent effects.

Methods to prevent these effects on fertility and to restore gonadal function after the toxic treatment are of great importance to men and women of child-bearing age. A variety of biochemical and biological approaches (thiol radioprotectors, prostaglandin analogues, growth factors, blockers of apoptotic pathways, and reduction in blood flow) have been tested to protect the testes in experimental animal model systems against radiation and chemotherapy (reviewed in (Meistrich *et al.*, 2007). However, the greatest research interest and nearly all clinical trials

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have involved hormonal modulation in attempts to prevent or reverse damage to the germline from radiotherapy and chemotherapy. We will discuss the current status of the knowledge on the hormonal suppression as a means to preserve fertility in men and women separately.

II. History and Hypotheses

The use of hormone suppression for protecting gonadal function after cytotoxic exposure is based on the observation that non-cycling cells are generally more resistant to killing by certain toxicants, particularly antineoplastic agents, than are rapidly proliferating cells. The greater sensitivity of cycling as compared to non-cycling cells is the basis for the antitumor action of many of these antineoplastic agents.

The mechanism originally proposed for protection of spermatogenesis was that interruption of the pituitary-gonadal axis would reduce the rate of spermatogenesis and render the resting testis more resistant to the effects of chemotherapy (Glode *et al.*, 1981). Although the Glode study claimed that pretreatment with gonadotropin-releasing hormone (GnRH) protected spermatogenesis in the mouse from damage from cyclophosphamide, attempts to repeat these original observations using more quantitative endpoints revealed that there was no protection (da Cunha *et al.*, 1987). Since suppression of gonadotropins and testosterone only blocks the completion of spermatogenesis but has no effect on the kinetics of the developing cells (Meistrich *et al.*, 1997), the premise on which the mechanism was based was incorrect and the negative outcome should be expected. Furthermore, the stem spermatogonia, which are more important targets than the differentiating germ cells for the long-term effects of cytotoxic damage, did not appear to be affected at all by hormonal suppression.

Despite the failure until now to observe protective effects in mice, it has been convincingly shown that suppression of gonadotropins and intratesticular testosterone levels prior to or during exposure of rats to chemotherapy or radiation enhances the subsequent recovery of spermatogenesis (Delic *et al.*, 1986). Thus other mechanisms must be involved and careful attention must be given to the species used.

Similarly suppression of gonadotropins with GnRH agonists or steroidal oral contraceptives has been proposed to suppress ovarian function, specifically to halt follicular development and follicular cell division, with the goal of protecting these now dormant ovarian follicles from destruction (Ataya *et al.*, 1985, Chapman & Sutcliffe, 1981). However, gonadotropins act primarily on the cyclic recruitment of antral follicles, while the initial recruitment of primordial follicles is not directly controlled by gonadotropins (McGee & Hsueh, 2000). It is these primordial follicles that provide the source for the long-term growing follicle and ova production and thus should be the target for protection. They lack gonadotropin receptors and besides are normally non-proliferating. Furthermore, although ovaries contain GnRH receptors, there is no evidence for the presence of GnRH receptors on primordial follicles (Danforth *et al.*, 2005).

Although several early studies concluded that such suppressive treatments could protect rat ovarian follicles against the damaging effects of chemotherapy (Ataya *et al.*, 1985, Bokser *et al.*, 1990), these results did not show that primordial follicles were protected (Meistrich, 1994). Recent studies using a GnRH agonist (Danforth *et al.*, 2005) or an antagonist (Meirow *et al.*, 2004) do, however, show protection of primordial follicles in mice from the damaging effects of cyclophosphamide. However, the originally proposed mechanism does not appear to be valid.

III. Experimental Studies - Males

The generally accepted model for the male gonadal toxicity from antineoplastic agents is that actively dividing differentiated spermatogonia are most sensitive to these agents, which will lead to a reduction in the sperm count. The reduction should be temporary provided the stem spermatogonia survive. However, stem spermatogonia are killed by some of these agents at varying degrees and recover only gradually, resulting in prolonged reductions of sperm count; in the mouse this reduction is directly related to stem cell killing (Meistrich, 1982). It is rare that surviving stem spermatogonia fail to differentiate in mouse testes (Kangasniemi *et al.*, 1996b). In contrast, after exposure of rats to several antineoplastic agents (Kangasniemi *et al.*, 1996a) and other toxicants (Boekelheide & Hall, 1991), the stem spermatogonia that survive are blocked from differentiating and their progeny undergo apoptosis instead (Meistrich & Shetty, 2003). This block has been shown to be a result of damage to the somatic environment within the testis, not to the spermatogonia (Zhang *et al.*, 2007). There is, however, no evidence of a similar spermatogonial block in monkeys (Boekelheide *et al.*, 2005).

Several studies further support the conclusion that gonadotropin suppression does not protect spermatogenesis in mice from damage (Crawford *et al.*, 1998, Kangasniemi *et al.*, 1996b, Nonomura *et al.*, 1991). In contrast numerous reports suggest that hormone suppression protects rat testes from damage due to irradiation, procarbazine, doxorubicin, an indenopyridine compound, and heating (Delic *et al.*, 1986, Hild *et al.*, 2001, Jégou *et al.*, 1991, Kangasniemi *et al.*, 1995, Manabe *et al.*, 1997, Morris & Shalet, 1990, Parchuri *et al.*, 1993, Setchell *et al.*, 2002, Weissenberg *et al.*, 1995) and enhances future fertility in the face of these toxicants. In addition to direct suppression of gonadotropins with GnRH agonists or antagonists, which also results in both direct and secondary suppression of intratesticular testosterone, these studies also utilized combinations of GnRH analogues with antiandrogens, systemic physiological doses of testosterone (which suppresses gonadotropins and results in reduced intratesticular testosterone levels), progestins (which are very effective at suppressing gonadotropins but have weak androgenic activity), and estrogens (which both suppress gonadotropins and inhibit testosterone synthesis) It should be noted that in all these studies protection was not assessed directly at the time of cytotoxic exposure, but rather by the enhanced ability of spermatogenesis to recover from surviving stem cells (Meistrich *et al.*, 2000), which is actually the most relevant endpoint for future fertility.

Attempts to protect spermatogenesis in other animal species (dog, monkey) have not yielded any reproducibly positive results. Although one group reported that GnRH-agonist shortened the time to recovery of spermatogenesis after treatment of dogs with cyclophosphamide, cisplatin, or radiation (Nseyo *et al.*, 1985), another study reported potentiation of the damage (Goodpasture *et al.*, 1988). Similarly, one preliminary report based on an extremely small number of baboons suggested that GnRH-agonists might decrease the gonadal damage from cyclophosphamide (Lewis *et al.*, 1985), while larger studies showed neither protection nor stimulation of recovery of spermatogenesis in macaques from radiation damage by GnRH-antagonist treatment (Boekelheide *et al.*, 2005, Kamischke *et al.*, 2003).

We proposed that prevention of the pronounced block in differentiation of surviving stem spermatogonia in rat testes after exposure to cytotoxic agents (Fig. 1A,B) is the mechanism by which hormone suppression appears to protect spermatogenesis from toxicant exposure (Meistrich *et al.*, 2000). It is important to note that many of the studies showing protection involved subchronic exposure to the cytotoxic agent, so the hormonal suppression was given after the initial exposures, and in some cases even extended beyond the last exposure (Pogach *et al.*, 1988). Furthermore, when the hormonal suppression was administered to the rats only after the cytotoxic insult, either immediately or after a delay (Fig. 1C), the numbers of differentiated germ cells still dramatically increased (Meistrich & Kangasniemi, 1997).

However, because testosterone, which is required for spermatid differentiation, was suppressed, spermatogenesis proceeded only to the round spermatid stage: no sperm were produced. Nevertheless, when additional time without further suppressive treatment was allowed before the rats were killed, all tubules showed almost complete spermatogenic recovery, sperm counts increased, and the fertility of the rats significantly increased (Meistrich *et al.*, 2001). This phenomenon appears to be quite general: post-treatment with GnRH agonists or antagonists, with or without antiandrogen, low-dose systemic testosterone, estradiol, or hypophysectomy are all effective at stimulating recovery (Shetty *et al.*, 2002, Shetty *et al.*, 2006), and recovery has been stimulated following gonadal toxicity from anticancer agents such as radiation, procarbazine (Meistrich *et al.*, 1999), or busulfan (Udagawa *et al.*, 2001), environmental toxicants such as hexanedione (Blanchard *et al.*, 1998) or dibromochloropropane (Meistrich *et al.*, 2003), an indenopyridine compound (Hild *et al.*, 2001), or heat treatment (Setchell *et al.*, 2001). The endogenous hormone primarily responsible for the inhibition of spermatogonial differentiation in toxicant-treated rats was testosterone, although FSH also had a minor inhibitory effect (Shetty *et al.*, 2006), and other exogenously administered androgens were also inhibitory (Shetty *et al.*, 2002). We have also observed that hormonal suppression after irradiation of mouse testes modestly but highly significantly increased the percentage of tubules in which differentiation of surviving spermatogonia occurred (G. Wang and M. L. Meistrich, unpublished observations).

Hormonal suppression with GnRH analogues or hypophysectomy has also been shown to promote the survival and differentiation of spermatogonia that are transplanted into testes of animals that were depleted of endogenous stem cells. Although the effects were most dramatic when rat testes depleted by cytotoxic treatments were used as recipients (Ogawa *et al.*, 1999, Zhang *et al.*, 2007), enhanced proliferation and differentiation of transplanted spermatogonia were also observed when mouse testes were used as recipients (Dobrinski *et al.*, 2001, Kanatsu-Shinohara *et al.*, 2004, Ohmura *et al.*, 2003, G. Wang and M. L. Meistrich, unpublished observations).

The mechanism by which somatic cells in rat testis are protected from damage if testosterone is suppressed at the time of cytotoxic exposure is not known. Neither is the mechanism by which testosterone suppression after cytotoxic exposure enhances the ability of the somatic elements of the testis to maintain the differentiation of spermatogonia. We had previously ruled out the possibility that the protective effect of hormonal suppression given before cytotoxic drug exposure was due to reduced delivery to the tissue or altered metabolism of the drug (Meistrich *et al.*, 1994). We have recently shown that the block in spermatogonial differentiation in rats treated with cytotoxic agents is associated with the increased levels of interstitial edema in the testes (Porter *et al.*, 2006). Based on data indicating that more rapid stimulation of recovery of spermatogonial differentiation in irradiated rats was achieved by elimination of Leydig cells with ethane dimethane sulfonate than with total androgen ablation (G. Shetty and M.L. Meistrich, unpublished data), we are analyzing the role of Leydig cells as targets for the testosterone-induced inhibition of spermatogonial differentiation in toxicant-treated rats.

IV. Experimental Studies - Females

Studies have utilized mice, rats, and monkeys to investigate protection against cyclophosphamide and radiation-induced ovarian damage. In these studies, the important target for which protection should be demonstrated was the primordial follicle, since it provides the reserve for production of growing follicles and ova over a prolonged period of time. Protection of developing follicles would result only in a short term enhancement of ovarian function, since these follicles are either recruited in a cyclic manner to undergo further development and ovulation or otherwise undergo atresia (McGee & Hsueh, 2000).

The mouse appears to be a good model to study the protective effects of various agents against cyclophosphamide damage since the primordial follicles in mouse are moderately sensitive to killing by cyclophosphamide (Plowchalk & Mattison, 1991). An initial study claimed protection of mouse ovaries against cyclophosphamide with the synthetic steroid danazol, but that study examined only developing follicles, so the results do not bear on long-term protection of ovarian function (Budell *et al.*, 1988). Protection of mouse primordial follicles from the damaging effects of cyclophosphamide was first reported in a study using a GnRH antagonist (Meirow *et al.*, 2004). Although one study claimed protection using a GnRH agonist based on marginal statistical significance (Yuce *et al.*, 2004), a subsequent study did show significant protection (Danforth *et al.*, 2005). However, that study made the surprising observation that GnRH antagonists, even given without cyclophosphamide, had deleterious effects and depleted primordial follicles, which needs to be further investigated and confirmed (Gupta & Flaws, 2005).

In contrast, studies using rats for assessing the protection of ovarian function from cytotoxic agents such as cyclophosphamide did not yield any information on the long-term effects because rat primordial follicles are not sensitive to killing by cyclophosphamide (Ataya *et al.*, 1985). The ability of GnRH-agonist treatment to maintain the numbers of small follicles and fertility in cyclophosphamide-treated rats (Ataya & Ramahi-Ataya, 1993, Ataya *et al.*, 1985) is therefore most likely a result of inhibition either of initial recruitment or of physiological loss of primordial follicles (Ataya *et al.*, 1989). This observation that GnRH-agonist treatment maintains the number of primordial follicles was surprising, since these follicles should be unaffected by gonadotropins, and other studies have failed to reproduce this observation (Bokser *et al.*, 1990, Jarrell *et al.*, 1987).

The one study in cyclophosphamide-treated monkeys showed that prolonged (1.5 years) GnRH-agonist treatment reduced the rate of loss of primordial follicles (Ataya *et al.*, 1995b). Although the results were interpreted as indicating the GnRH agonist can protect primate ovaries against cyclophosphamide-induced damage, there was no control group treated with GnRH agonist alone, so the result may also represent GnRH-agonist inhibition of recruitment of primordial follicles.

GnRH agonist has also been shown to prevent the doxorubicin-induced inhibition of estradiol production by granulosa cells *in vitro* (Imai *et al.*, 2007). However, this study utilized granulosa cells from mature follicles, whereas the important target, the primordial follicles, may not have GnRH receptors.

Even less success has been reported with respect to hormonal protection of ovarian function from radiation. Radiation kills primordial follicles in all mammals studied, but those of the mouse are exquisitely sensitive and those of the rat are moderately sensitive (Baker, 1978). In mice, gonadotropin reduction due to a hypogonadal mutation or GnRH-antagonist treatment failed to protect primordial follicles from radiation (Gosden *et al.*, 1997). Treatment with a GnRH agonist, but not with medroxyprogesterone acetate, partially protected against radiation-induced loss of primordial follicles in rats (Jarrell *et al.*, 1987, Jarrell *et al.*, 1989). No protection from radiation-induced loss of primordial follicles in monkeys was observed with GnRH-agonist treatment (Ataya *et al.*, 1995a).

In summary, very mixed results have been obtained in experimental studies regarding protection of primordial follicles from damage by cytotoxic agents by hormonal suppression. Some studies that claimed protection examined only growing follicles and thus could not support the notion that hormonal suppression protects the primordial follicles. Other studies showing that extended GnRH agonist treatment reduced the primordial follicle loss in animals treated with cytotoxic agents do not demonstrate that these follicles are protected but may be

interpreted as inhibition of the normal initial recruitment of primordial follicles. Such a mechanism would not benefit women receiving GnRH-agonist treatment during cytotoxic therapy; however, if the mechanism is valid, prolonged GnRH -agonist treatment would preserve surviving primordial follicles for a longer period of time if the women want to delay childbearing. In cases where protective effects have been observed against cyclophosphamide-induced depletion of primordial follicles in mice, the protection may involve direct effects of the GnRH analogues on the ovary or indirect effects of gonadotropin suppression such as reduced drug delivery due to a reduction of ovarian blood flow (Meirow *et al.*, 2004).

V. Clinical Trials - Males

Seven clinical trials have been performed in attempts to demonstrate protection of spermatogenesis in humans by hormone suppression treatment before and during cytotoxic therapy, but six indicated no protection (Table 1). Three of these trials involved patients treated for Hodgkin's lymphoma and three involved testicular cancer patients. Treatment with GnRH agonist resulted in only 20% of patients recovering sperm count after cessation of chemotherapy (Johnson *et al.*, 1985). However, no concurrent control group of patients receiving similar regimens of chemotherapy without GnRH agonist was enrolled in this study. In another study, hormone suppression with testosterone combined with GnRH agonist prior to and during chemotherapy was randomized with no hormonal suppression (Waxman *et al.*, 1987), but none of the patients from the control and treated groups showed evidence of recovery of spermatogenesis at 1 to 3 years after completion of therapy. Suppression of gonadotropins and intratesticular testosterone levels with testosterone injections alone during treatment (Redman & Bajorunas, 1987) also did not provide gonadal protection benefit: 70% of the patients in both the treated and control group showed spermatogenic recovery at 3 years. Suppression of gonadotropins with medroxyprogesterone acetate during chemotherapy combined with radiotherapy did not improve the recovery of sperm count or normalize FSH levels, which was used as a surrogate for sperm count in patients in whom sperm counts were unavailable; indeed, they appeared to be lower in the patients receiving concurrent treatment with hormonal suppression than in controls (Fossa *et al.*, 1988). Two more studies used GnRH agonist (Kreuser *et al.*, 1990) or GnRH agonist plus an antiandrogen (cyproterone acetate) (Brennemann *et al.*, 1994) prior to and for the duration of chemotherapy or radiation therapy, respectively. In these studies the chemotherapeutic regimen was only 2 courses of PVB and the gonadal dose of radiation was 0.2 Gy, which allowed spontaneous recovery of sperm counts in all the control patients within 2 years. The time course of recovery of spermatogenesis after chemotherapy was identical for the groups of patients with or without GnRH-agonist treatment. Although fluctuations in sperm counts made it difficult to determine whether the time course of recovery of spermatogenesis was affected by hormonal treatment, the time course of reduction of elevated FSH levels back to pretreatment values was similar in controls and in the patient groups treated with GnRH agonist and antiandrogen.

The one study that demonstrated hormonal treatment preservation of sperm production in men involved testosterone therapy of men who received cyclophosphamide as an immunosuppressive therapy for nephrotic syndrome (Masala *et al.*, 1997). During the treatment, the testosterone suppressed gonadotropin levels and suppressed the completion of spermatogenesis. All but one of the men who received cyclophosphamide alone remained azoospermic 6 months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all five men who received cyclophosphamide in combination with testosterone therapy.

The one attempt to restore spermatogenesis by steroid hormone suppression after cytotoxic therapy was also unsuccessful (Thomson *et al.*, 2002). Seven men with azoospermia secondary to high-dose chemo- and/or radiation therapy for leukemia or lymphoma in childhood were

treated with medroxyprogesterone acetate combined with testosterone to suppress gonadotropin and likely intratesticular testosterone levels many years after the anticancer treatment. None of the men recovered any sperm production during the 24-week follow-up after the end of hormonal treatment.

Even if the hormonal suppressive treatments that were successful in protecting and stimulating spermatogenic recovery in rats are applicable to human males, there may be many reasons for the unsuccessful outcomes of the aforementioned clinical trials. The use of testosterone or medroxyprogesterone either alone (Fossa *et al.*, 1988, Redman & Bajorunas, 1987, Thomson *et al.*, 2002) or combined with a GnRH analogue (Waxman *et al.*, 1987) is suboptimal given that, in animal studies, both of these steroids act directly on the testis to reduce the stimulatory effects of GnRH analogues on the recovery of spermatogenesis after cytotoxic damage (Shetty *et al.*, 2002, Shetty *et al.*, 2004); however, this might not be as significant in humans, which have higher intratesticular androgen levels (Jarow *et al.*, 2001). The number of patients and controls studied was small (Johnson *et al.*, 1985) and the cancer therapies variable. Some treatment regimens were not sufficiently gonadotoxic to cause sterility (Brennemann *et al.*, 1994, Kreuser *et al.*, 1990); conversely some regimens may have delivered doses well above that needed to ablate all spermatogonial stem cells, since no evidence of spermatogenesis was observed in almost all patients even after many years (Johnson *et al.*, 1985, Thomson *et al.*, 2002, Waxman *et al.*, 1987). Thus the application of these procedures to humans remains uncertain.

VI. Clinical Trials - Females

The greater proportion of prepubertal than of postpubertal women who maintain normal ovarian function after chemotherapy or radiotherapy (Horning *et al.*, 1981) has been used to promote the concept that the ovary that is not stimulated by gonadotropins has a greater tolerance for these cytotoxic treatments. However, the larger store of follicles at the younger ages is an alternative explanation (Faddy *et al.*, 1992). This alternate explanation is supported by the greater resistance of 20-year-old women than of 35-year old women to induction of permanent ovarian failure from chemotherapy or radiation therapy (Meistrich *et al.*, 2005).

Several studies reported by Blumenfeld's group (reviewed in Blumenfeld, 2007, Blumenfeld & Eckman, 2005), including a recent update of patients treated for Hodgkin lymphoma (Blumenfeld *et al.*, 2008), showed that 7% of ~125 women treated with a GnRH agonist during chemotherapy exhibited premature ovarian failure characterized by hypergonadotropic amenorrhea, whereas 53% of ~125 women in the control group treated with chemotherapy but not GnRH agonist (some concurrent with the GnRH-treated group and some historical controls) suffered premature ovarian failure. None of these studies were randomized prospective trials, although attempts were made to demonstrate similar age ranges and chemotherapy and radiation doses in the GnRH-agonist and control groups. In another study, adolescent girls (9 patients) received high-dose chemotherapy for bone marrow transplantation; those receiving GnRH agonist resumed menstrual cycles whereas those who were not treated with GnRH agonist had secondary amenorrhea (Pereyra Pacheco *et al.*, 2001). In a recent study 56 women were treated with a GnRH agonist and a synthetic estrogen; a control group did not take the treatment. The study reported that 90% of the gonadotropin-suppressed women recovered ovulatory and menstrual function, versus 23% of those not treated (Castelo-Branco *et al.*, 2007). The interpretation that protection occurred is, however, weakened by the fact that different treatments were given and age distributions in the control and treated groups were not comparable. Two phase II studies with adult breast cancer patients (100 patients in one and 29 patients in the other) suggested that a GnRH-agonist treatment protected ovarian function, but the claims were based only on historical data in the literature and not a control group (Del Mastro *et al.*, 2006, Recchia *et al.*, 2006). In addition, an observational study of 145 patients

reported that the patients taking oral contraceptives had only a 10% incidence of post-chemotherapy amenorrhea compared to 44% in the group not taking oral contraceptives (Behringer *et al.*, 2005). One study utilized treatment with a GnRH antagonist, in addition to an agonist, during chemotherapy and reported good short-term recovery of menstrual cycles, but the study did not have a control group (Potolog-Nahari *et al.*, 2007).

Although these studies indicate that suppression of gonadotropins and estrogen might be protective of ovarian function, none of them were prospective, randomized clinical trials, and hence must be considered inconclusive (Lee *et al.*, 2006). There was only one randomized study of the effects of GnRH agonist on chemotherapy-induced sterility that involved 18 patients, and there was no protective effect (Waxman *et al.*, 1987). However the more recent suggestive evidence discussed above indicates that randomized clinical trials, several of which are in progress, need to be pursued.

VIII. Relationship of Experimental to Human Studies

The results of studies of protection of long-term gonadal function by hormonal suppression in experimental animals and humans are summarized in Table 2. In males, hormonal suppression reliably stimulated recovery of spermatogenesis from surviving stem cells in rats but did not influence the survival of spermatogonial stem cells. In humans, only one of eight clinical trials showed that hormonal suppression enhanced subsequent gonadal function. In females, hormonal suppression provided mixed results on protection of mouse primordial follicles from damage due to cyclophosphamide and of rat primordial follicles from damage due to radiation. Several human clinical studies showed that GnRH-agonist treatment produced good prolonged maintenance of ovarian function after chemotherapy, but this conclusion must be viewed with caution, as none of these studies were randomized clinical trials.

Experimental studies, particularly in rodents, are of great value in that they may be highly controlled, have larger sample sizes, and can be used to optimize treatments and to elucidate mechanisms. Primate studies have greater variability and uncertainties and sample sizes are limited. The main question is what aspects of the rodent studies are applicable to the human and what aspects are not. Since primates and rodents diverged 66 million years ago (Mya) there will be differences. It is also noteworthy that mouse and rat diverged 41 Mya, whereas humans and macaques diverged 23 Mya. Thus, significant differences are expected among rodents and between rodents and different primates. It is important to understand the mechanism of protection or stimulation of recovery by hormonal suppression in order to determine which individual steps in the process will be similar or different between rodents and primates.

Cytotoxic agents to which rodents and primates have similar sensitivities with respect to their effects on spermatogenesis are appropriate to utilize in experimental studies for extrapolation to men. Mouse and human spermatogenesis are both sensitive to certain alkylating agents (procarbazine, chlorambucil, busulfan) and radiation as measured by stem cell killing and prolonged azoospermia, respectively (Meistrich, 1993). However there are differences as spermatogonial stem cells in mice are sensitive to killing by doxorubicin (Adriamycin) but not cyclophosphamide, whereas cyclophosphamide, but not doxorubicin, strongly induces long-term azoospermia in humans. In rats, both radiation and procarbazine (Meistrich *et al.*, 1999), but not cyclophosphamide (Meistrich *et al.*, 1995), produce a prolonged block to spermatogonial differentiation.

In rats exposed to moderate doses of cytotoxic agents, the induction of a block in spermatogonial differentiation is a much more likely cause of prolonged azoospermia than is spermatogonial stem cell killing. The reversal of this block in spermatogonial differentiation appears to be the mechanism by which hormone suppression protects or restores spermatogenesis in toxicant-exposed rats (Meistrich *et al.*, 2000) and the occurrence of such

a block should indicate whether hormonal suppression might stimulate spermatogenic recovery in another species. Although in many cases the seminiferous tubules in testicular biopsies taken from men with chemotherapy- or radiotherapy-induced azoospermia contain only Sertoli cells and no spermatogonia (Van Thiel *et al.*, 1972), occasionally the presence of isolated spermatogonia have been observed at relatively long times after chemotherapy treatment (Kreuser *et al.*, 1989). In addition, spontaneous recovery of spermatogenesis in some men more than 1 year after radiation (Hahn *et al.*, 1982) or chemotherapy (Drasga *et al.*, 1983) also implies a block in the differentiation of spermatogonia that survive these cytotoxic exposures. These results suggest that after some cytotoxic therapy regimens, there is a potentially reversible block to spermatogonial differentiation in men. It should be noted that there is no evidence of a similar spermatogonial block in monkeys (Boekelheide *et al.*, 2005). The human data emphasize that the cytotoxic therapy regimens need to be carefully selected in these clinical trials from a range of regimens. To have a chance of success, doses of cytotoxic therapies should be chosen at which there is an appreciable block to spermatogonial differentiation but not killing of all stem spermatogonia.

Since many chemotherapeutic and radiotherapeutic regimens may result in complete killing of the stem spermatogonia and the hormonal methods do not protect these cells from cytotoxicity, consideration should be given to the applications of hormonal suppression in combination with spermatogonial transplantation. Cryopreservation of spermatogonia and autologous transplantation is considered a potential method for restoring spermatogenesis and possibly rescuing fertility after chemo- or radiotherapy (Orwig & Schlatt, 2005). Hormonal suppression could restore the somatic environment in human testes sufficiently to promote the ability of transplanted stem spermatogonia to develop, as was the case with rat testes (Zhang *et al.*, 2007).

It is also important to consider whether the molecular and cellular effects of hormonal suppression are similar in humans and in rats. Although the basic processes by which GnRH analogues suppress gonadotropin and testosterone levels and induce a block in the completion of spermatogenesis in normal adult males are similar in rodents and primates, there are quantitative differences. Whereas in rats and humans, GnRH antagonist reduced intratesticular testosterone concentrations to about 2% of that observed in controls (Matthiesson *et al.*, 2005, Shuttlesworth *et al.*, 2000), in macaques it only reduced intratesticular testosterone concentrations to 28% of control (Zhengwei *et al.*, 1998). Despite the less marked reduction in intratesticular testosterone levels, spermatogenesis was blocked at the B spermatogonial stage by GnRH antagonist treatment of the primates (Zhengwei *et al.*, 1998) compared to the round spermatid stage in rats (Kangasniemi *et al.*, 1996b). In humans, the block in spermatogenesis was also largely at the B spermatogonial levels, but later germ cells to the round spermatid stage were still produced at 20% of control levels (Matthiesson *et al.*, 2005). The restimulation of spermatogonial differentiation by hormonal suppression in rats may be dependent upon ability of germ cells to develop to the spermatocyte stage during the testosterone suppression, and hence might occur in men. Further germ cell differentiation in the presence of suppressed testosterone can be induced in human testes by treatment with FSH (Matthiesson *et al.*, 2006).

To extrapolate results of studies of protection of ovarian function from rodent systems to women, it is important that the cytotoxic mechanism of chemotherapy action on the follicles in the experimental model be similar to that in women. The mouse model seems to be appropriate for investigation of the protection against cyclophosphamide, as primordial follicles in the mouse and in women are moderately sensitive and show a dose-responsive loss (Plowchalk & Mattison, 1991) or a dose-dependent increase in sustained amenorrhea (Boumpas *et al.*, 1993), respectively, after exposure to increasing doses of cyclophosphamide. Although primordial follicles in both rodents and humans are killed by radiation, the exquisite

sensitivity of murine primordial follicles to death by apoptosis and the relative radioresistance of human primordial follicles makes this a less comparable model system (Baker, 1978).

It is also important to elucidate the mechanisms by which hormonal suppression protects primordial follicles in female mice from cytotoxic therapy in order to better determine how to apply the results to women and select an appropriate hormonal treatment for maximal protection.

It needs to be determined whether or not suppression of gonadotropins is involved in the protective mechanism. All successful experimental and clinical trials utilized GnRH analogues, and almost exclusively agonists rather than antagonists. GnRH receptors are present in the ovary so that the action of GnRH might be at the local level, rather than in the pituitary. Possible differential effect of GnRH agonists and antagonists on oocytes and granulosa cells also need to be investigated (Yano *et al.*, 1997).

Since currently there is no evidence that the primordial follicle is a direct target for action of gonadotropins or GnRH analogues, effects that are mediated at the level of the whole ovary should be considered. The possibility that the protective effect on mouse primordial follicle against chemotherapy could result in a decline in ovarian blood flow during GnRH-analogue treatment-induced ovarian quiescence (Meirow *et al.*, 2004), as had been observed in human females (Dada *et al.*, 2001), needs further investigation.

Another step that needs further study is the mechanism of death of the follicle after chemotherapy or radiation, which in the mouse is a result of apoptosis (Morita *et al.*, 2000). It is important to determine whether or not GnRH analogue treatment affects the apoptotic machinery in oocytes and/or granulosa cells, particularly the pregranulosa cells in primordial follicles, and whether it renders them less sensitive to the induction of apoptosis, and if it does, that it does not also protect the tumor cells from apoptotic death.

Based on recent reports that the primordial follicle pool may be dynamic and that there is a mechanism for replenishment of primordial follicles in adult mice (Johnson *et al.*, 2004), the replenishment of the primordial follicle pool may have to be considered, in addition to its maintenance. Although this work remains controversial and, if valid, its extension to human is uncertain, this phenomenon should be considered when trying to explain the effects of hormone suppression on the numbers of primordial follicles after exposure to cytotoxic cancer therapies.

In conclusion, the most immediate need is for randomized prospective clinical trials to test whether the promising results of previous non-randomized clinical studies on the use of GnRH-agonist treatment to protect ovarian function during cancer chemotherapy can be supported by a rigorous test. In addition, mechanistic studies in appropriate animal models are needed to determine which aspects of the beneficial effects of hormonal suppression on maintenance of both male and female fertility in select animal models may be applicable to human and to predict hormonal regimens that may offer maximal protection.

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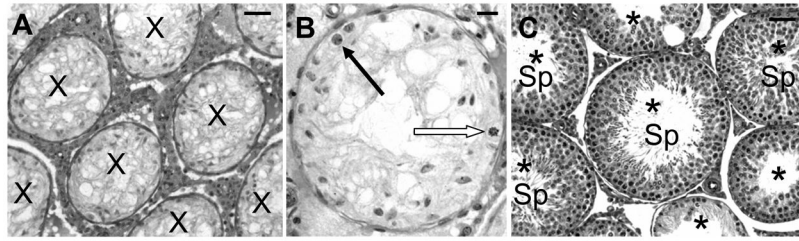


FIG 1. Photomicrographs of LBNF1 rat testes showing the radiation-induced block in the differentiation of spermatogonia (A & B) and the reversal of this block by GnRH-antagonist treatment. The testes were harvested 13 weeks after 5 Gy irradiation with or without GnRH antagonist treatment during wks 3 to 7 after irradiation. Note that with no GnRH antagonist treatment all tubules are atrophic (X) and contain only Sertoli cells and type A spermatogonia (A), with normal (filled arrow) and dividing (open arrow) spermatogonia shown at higher magnification (B). With GnRH antagonist treatment all tubules are repopulating (*) with mature spermatids in many of them (Sp) (C). Bars = 50 μ m (A and C) and 20 μ m (B).

Results of hormone suppression treatments given before and during cytotoxic therapy on spermatogenic recovery in men.

Table 1

Study	Disease	Cytotoxic therapy	Hormone treatment	Hormone-treated	Recovery ^b	Controls
Johnson et al., 1985	Hodgkin's	MOPP 3-6 cycles	GnRH agonist	1 of 5	No controls	No controls
Waxman et al., 1987	Hodgkin's	MVPP, ChlVPP	GnRH agonist + testosterone	0 of 20	0 of 10	0 of 10
Redman & Bajornas, 1987	Hodgkin's	MOPP ~4 cycles	Testosterone	≈70% of 23 ^c	≈70% of 22 ^c	≈70% of 22 ^c
Fossa et al., 1988	Testis Ca	PVB, ADR/CY, Radiation	Medroxy-progesterone	0 of 4 (2 of 12) ^d	2 of 3 (7 of 13) ^d	2 of 3 (7 of 13) ^d
Kreuser et al., 1990	Testis Ca	PVB	GnRH agonist	6 of 6	6 of 6	8 of 8
Brennemann et al., 1994	Testis Ca (Seminoma)	Radiation	GnRH agonist + antiandrogen	12 of 12	8 of 8	8 of 8
Masala et al., 1997	Nephritis	Cyclophosphamide	Testosterone	5 of 5	5 of 5	1 of 5

^a Chemotherapy regimens are as follows: MOPP, mechlorethamine, vincristine, procarbazine; and prednisone; MVPP, mechlorethamine, vinblastine, procarbazine, and prednisone; ChlVPP, chlorambucil, vinblastine, procarbazine, and prednisone; PVB: cisplatin, vinblastine, bleomycin; ADR/CY: Adriamycin, cyclophosphamide.

^b Fraction of men recovering testicular function as assessed by restoration of sperm counts to normospermic levels unless otherwise noted.

^c Actuarial recovery calculated by Kaplan-Meier analysis

^d Recovery assessed by restoration of FSH levels to within the normal control range.

Table 2

Summary of Effects of Hormonal Suppression on Protection and Stimulation of Gonadal Functions

Species	Effects of Hormonal Suppression in Males	Effects of Hormonal Suppression in Females
Mouse	Pretreatment suppression does not protect endogenous spermatogenesis. Suppression moderately enhances spermatogenesis from transplanted spermatogonia. Posttreatment suppression slightly stimulates recovery from surviving stem cells	Mixed results on protection of primordial follicles from cyclophosphamide. No protection of primordial follicles from radiation.
Rat	Pretreatment and posttreatment suppression markedly stimulate spermatogenic recovery from stem cells. Suppression markedly enhances spermatogenesis from transplanted spermatogonia	Mixed results on maintenance of primordial follicle number during prolong GnRH agonist treatment (independent of cytotoxic exposure). GnRH agonist, but not progestin, partially protects primordial follicles from irradiation damage. Prolonged GnRH agonist treatment maintains primordial follicle numbers during cyclophosphamide treatment but no proof of protection against cyclophosphamide-induced damage.
Non-human primate	Neither pretreatment nor posttreatment suppression enhance recovery of spermatogenesis after irradiation.	Suppression offers no protection from radiation-induced loss of primordial follicles.
Human	Suppression before and during therapy fails to protect spermatogenesis from damage from cancer chemotherapy or radiotherapy (6 studies). Suppression with testosterone before and during therapy protected spermatogenesis from damage from cyclophosphamide (1 study). Delayed posttreatment suppression failed to restore spermatogenesis.	Several non-randomized studies (some with concurrent controls) indicate that suppression markedly protects against premature ovarian failure. One small randomized study showed no protective effect of suppression.