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Temperature control of spermatogenesis and prospect of male contraception

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1. ABSTRACT

Artificial cryptorchidism or local testicular heat treatment could induce reversible oligospermia or azoospermia in monkeys via germ cell apoptosis. A single exposure of rat or monkey testes at 43 degrees centigrade resulted in selective and reversible damage to seminiferous epithelium**.** Local warming monkey testes at 43 degrees centigrade water for consecutive two days (30 minutes per day) showed that the sperm amount in the semen decreased up to 80% at 28 days and is completely reversible**.** Furthermore, the heat treatment in combination with testosterone, the sperm account reduced to zero in 2 month time. Withdrawing the testosterone implant, the density of semen sperms recovered to the normal levels after 2-3 months. Apoptosis induced by testosterone occurs mainly at stages 7-8, while testicular "heat stress" induces germ cell apoptosis mostly at stages 1- 4 and 10-12, an additive reversible germ cell apoptosis could be achieved. These findings have provided an important theoretical basis for designing combined male contraceptives. This present review summarized progress on the mechanism of heat-induced germ cell apoptosis.

2. INTRODUCTION

Spermatogenesis is precisely regulated processes of spermatogonia division and spermatocyte differentiation controlled mainly by FSH and testosterone, beginning with a mitotic proliferation of stem cells. Half of these mitoses then enter a lengthy meiotic phase as preleptotene spermatocytes. The latter cells complete the meiotic phase from spermatocytes to round spermatids that subsequently differentiate into elongated spermatids (1,2). Spermatogenesis is a dynamically and highly synchronized process of continuous germ cell maturation, including proliferation and differentiation of spermatogonial stem cells, meiotic cell division of differentiating spermatogenic cells, morphogenic maturation of spermatids and ultimately release of mature haploid spermatozoa into the tubule lumen. However, the molecular mechanism and signaling pathways of these events in primate are not completely understood.

Development of new prophylactics is closely related to the breach of theory obtained from basic research in reproductive medicine (3,4). Contraception, in fact, is to

disturb normal physiological rule of body with a 100% succeeding contraceptive rate without obvious side effect (4-8), and should be a life style of human being to pursue a long-term body and mind healthy on the earth. Therefore exploiting new contractive methods should be based on precisely understanding a certain key point that can be easily blocked and have no obvious side effect on human health (4). During the long process of spermatogenesis, specific interested target genes should exist, which either control the opening of blood-testis barrier, or the spermatocyte meiosis, or spermatid differentiation and maturation. Therefore it is possible to find out key gene (s) specifically expressed at a particular easily blocked weak step during spermatogenesis in testis, or germ cell maturation in epididymis for developing male prophylactics(3). Disturbance of testicular spermatogenesis locally will not affect human health and is the most ideal approach to developing safe and valid contraceptives. Unfortunately, no theoretical basis so far with the defined molecules from such studies has been directly used for developing male contraceptive. Further breakthrough of these findings to confirm their specific role in spermatogenesis or germ cell maturation would be possible to develop the most ideal new male contraceptive.

3. SPERMATOGENESIS AND REGULATION

Spermatogenesis shows a strict control of many specific molecular and cellular events, including Sertoli-germ cell interactions, a programmed change in chromatin structure, and a sequential specific gene expression (1,2,9). Spermatozoa in mammals must undergo serious modifications morphologically, physiologically and biochemically early in testis and later in epidydimis under control of pituitary FSH and LH regulated by hypothalamus gonadotrophin releasing hormone (GnRH) and testicular paracrine/autocrine factors (10-12). FSH is capable of amplifying the population of the differentiated spermatogonia (B1, B2, B3 and B4) and controls the rate of the spermatogonia production, and in synergy with testosterone regulating spermatogenesis (1,2). Pituitary FSH-beta expression is governed by a feedback of inhibin-beta, which is a major component of testicular negative feedback signals secreted by Sertoli cells, and inhibited by LH via testosterone secreted by Leydig cell. FSH is the major determinant of adult complement of Sertoli cells and is obligatory for initiation, maintenance and restoration of spermatogenesis (13). Spermatogenesis can be divided into three phases (14-16): 1) the mitosis phase: This phase takes place along basal lamina of seminiferous epithelium outside of blood-testis barrier. The immature type A spermatogonia proliferate and differentiate into type B spermatogonia, some of which in turn differentiate into preleptotene and leptotene spermatocytes. They migrate upwards away from base of seminiferous tubule and traverse blood-testis barrier entering into adluminal compartment. 2) the meiotic phase: This phase largely takes place behind the blood-testis barrier in adluminal compartment. The primary spermatocytes first undergo meiosis I to form secondary spermatocytes which then rapidly divide again by meiosis II to differentiate into haploid spermatids. This process is extended over a long period of time, so spermatocytes are found in every stage of spermatogenesis. 3) spermiogenesis: The haploid spermatids undergo dramatic morphogenesis into spermatozoa which is characterized by nucleus elongation and chromatin condensation, acrosome formation and elongation of flagella. The fully developed spermatozoa will be translocated to adluminal surface of seminiferous epithelium to be released into tubule lumen at spermiation. It takes about 70 days to finish these phases. During the long process sequential expression of a series of special genes and interaction of the expressed products are involved. Evidence has shown that only small numbers of spermatogonia may occasionally differentiate into primary spermatocytes in immature human testis, the vast majority of germ cells do not undergo meiosis until from puberty (17, 18). At puberty, there are remarkable structural and functional changes in testis, such as Sertoli cells ending mitotic divisions, Leydig cells differentiating and producing testosterone in response to LH, and germ cells proliferating and initiating meiosis (19,20). In the seminiferous epithelium, the association between Sertoli and germ cells throughout these three phases of development are arranged into defined stages of seminiferous epithelial cycle, each characterized by a well-defined cellular association with a characteristic duration and length (21-23). The number of stages differs between species but is constant for a given species, such as 12 stages defined in monkey, 12 stages in mice and 14 stages in rats (23,24). The production of a normal number of spermatozoa depends on highly specific regulation of gene expression in germ cells, and paracrine and hormonal control of germ cell proliferation, differentiation, and survival or apoptosis. And this process is also aided by adjacent Sertoli cells which create a proper environment by providing structure support, nutrition, certain hormones, and other signaling molecules throughout spermatogenesis for maturation of developing germ cells in testes (1,25-28). Thus, a fine architecture of seminiferous epithelium and interactions between different types of cells in the seminiferous epithelium are crucial for spermatogenesis.

The concerted action of FSH, testosterone, and local testicular paracrine factor such as inhibin is most important in spermatogenesis (12,29,30). We have demonstrated presence of inhibin subunit-alfa and-beta in monkey Setoli cells (31). Ramaswamy and Weinbauer at al (29, 30) showed that the testicular inhibin B in monkey is stimulated by FSH, and inhibited by LH indirectly through testosterone produced by leydig cells. Since male germ cells possess neither FSH nor androgen receptors, the action of FSH and testosterone should occur through Sertoli cells. FSH is required for determination of Sertoli cell number and for maintenance of normal sperm production. Full Sertoli proliferation can be accomplished by FSH activity without LH requirement (32), while postnatal mitotic and meiotic germ cell development can be promoted by FSH alone, LH-mediated effects remain a critical determinant for initiating the full complete of germ cells and final stages of post-meitic development (32,33). Immunization of male bonnet monkeys with recombinant FSH receptor preparation induced sexual dysfunction (34), serum testosterone levels and LH receptor function

following immunization remained unchanged. The immunized monkeys showed 50% reduction in transformation of spermatogonia (2C) to primary spermatocytes (4C) with reduction in fertility index. Furthermore, FSH receptor knockout young mice have underdeveloped testis and 50% reduction in Sertoli cells. These changes are also experimentally inducible in FSH- and/or FSHR-immunized male monkeys, creating a state of infertility (35,36). Ramaswamy et al (37,38) have demonstrated a FSH—inbihin B feedback loop to control spermatogenesis in adult male rhesus monkey. They found that orchidectomy was associated with a marked increase in the number of all germ cells more mature than undifferentiated spermatogonia in the remaining testis, spermatogenesis does not normally operate at ceiling, while Sertoli cell number was not changed. They suggest that FSH secretion controlled by the feedback of inhibin beta is insufficient to stimulate spermatogenesis at the early stage in the orchidectomy monkeys, the elevated secretion of FSH appears to result in sperm output by the remaining testis (38). Based on their finding in the irradiated non-human primate model, Foppiani et al (39) further suggested that inhibin B may be regarded as a more sensitive marker of premeiotic germ cell proliferation than FSH. Differences among species may be present regarding specific steps and regulation of spermatogenesis (40-42). In rat, testosterone is an absolute requirement for spermatogenesis to promote adhesion of round spermatids to Sertoli cells, without which they are sloughed from epithelium and spermatid elongation fails. Release of mature elongated spermatids from Sertoli cells (spermiation) is also under FSH/testosterone control in rat. Data from monkey and men indicate that impairment of spermiation is a key to achieving azoospermia (42).

The local factor, SCF/c-kit system in testis controlled by FSH is believed to regulate germ cell proliferation and apoptosis. SCF induces type A spermatogonia DNA synthesis and proliferation. SCF/c-kit up-regulated cyclin D3 and promoted cell cycle progression via phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia (43, 44). Estradiol should be also considered as a "male hormone", and appears to be a potential germ cell survival factor in primate (45,46). Aquila *et al* demonstrated ER-alpha and ER-beta concomitant expression in human ejaculated spermatozoa, and suggested that estrogen may be able to influence both capacitation and acrosome reaction (45). Pentikainen *et al* reported that estrogen receptors for both the beta and alpha were present in the early meiotic spermatocytes and elongated spermatids of human testis and found that low dose of 17 beta-estradiol was capable of inhibiting germ cell apoptosis (46). The localization of estrogen receptor beta and aromatases in pachytene spermatocytes was also demonstrated, indicating a role of estrogen in this step of spermatogenesis. Tirado *et al* suggested that cytoplasmic ER-beta participates in the apoptotic process of pachytene spermatocytes induced by MAA (47).

4. FUNCTION OF SERTOLI CELLS IN SPERMATOGENESIS

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Spermatogenesis is dependent upon function of Sertoli cells. They are the only somatic cells in seminiferous epithelium. Number of Sertoli cells determines germ cell numbers and each Sertoli cell can interact with 30~50 different germ cells at each stage of the spermatogenic cycle. Germ cells rely heavily on Sertoli cells for structural and nutritional support. Firstly, testosterone (T) and FSH action on spermatogenesis is regulated via Sertoli cells because of germ cells lacking androgen and FSH receptors (48); Secondly, existence of a well-organized system of cell-cell adhesion junctions in epithelium is a prerequisite for spermatogenesis. Adjacent Sertoli cells form tight junctions (TJs) constituting blood-testis barrier (BTB), which provides a specialized and protected environment for germ cell development. Thus germ cells are only dependent on Sertoli cells to supply nutrients and growth factors. Successful migration of developing germ cells across seminiferous tubules is dependent on extensive reassembly of TJs, cell-cell actin-based adheren junctions (AJs) and cell-cell intermediate filament-based desmosome-like junctions, which are located in adjacent Sertoli cells, and between Sertoli and germ cells. Disruption of these junctions leads to failure of spermatogenesis (49). *In vivo* and *in vitro* experiments showed that T is important for adhesion of spermatids to Sertoli cells and essential for TJs development (50-52). T could cause an early and dose-dependent increase in trans-epithelial electrical resistance (TER) *in vitro* (53). Fetal exposure to the androgen antagonist, flutamide, affected cluadin-11 expression in prepubertal rat when BTB was being formed (54). T was shown to stimulate claudin-11 and claudin-3 expression in cultured Sertoli cells through its receptor AR. Sertoli cell-specific ablation of AR resulted in an increased permeability of the BTB (54), vimentin expression was significantly increased, while claudin-11, occludin, laminin alpha 5 and gelsolin were significantly decreased (55). Besides AR, wilms' tumour gene 1 (WT1), which has bi-functional role in embryonic gonad formation (56) and spermatogenesis in adult, is also necessary for cell adhesion in Sertoli cells. WT1 knockdown specifically in mouse Sertoli cells led to disregulation of AJ-associated genes, AJs loss and increased germ cell apoptosis (57).

The cadherin/catenin complex represents the major structural and functional unit of AJs. The extracellular domains of two cadherins in the adjacent cells interact homo-typically, and the intracellular domains of the cadherins associate with β- or γ-catenins forming the cadherin/catenin complex. The complex in turn interacts with actin filament bundles via its interaction with vinculin and α-catenin. N-cadherin interacts with β-catenin at a ratio of almost 1:1 in the testis (58). N-cadherin and β-catenin may be used as good marker molecules of AJs in experiment. ZO-1 is the first TJ-associated cytoplasmic protein subjected to extensive investigation, which is a linker protein coupling the transmembrane TJ protein such as occludins, claudins and JAM, to the cytoskeleton. ZO-1 associates with occludin at a stoichiometric ratio of 1:1 (59). For this reason ZO-1 may be also chosen for a good marker molecule of TJs.

Increasing evidence suggests that local fibrinolysis generated by plasminogen activator (PA) and modulated by plasminogen activator inhibitor type-1 (PAI-

1) is essential for mammalian spermatogenesis (31,60), but no available derect evidence has shown involvement of the PA/PAI-1 system in regulation of AJs and Tjs molecule expression. Tissue type PA (tPA) and urokinase type PA (uPA) are secreted and regulated by FSH, forskolin, cAMP and GnRH in a stage-dependent manner (31, 60-63). Urokinase PA receptor (uPAR) and PAI-1 have been found binding on the sperm membrane surface and expressed during spermatogenesis (31). Evidence has been reported that germ-somatic cell interaction in the seminiferous epithelium is essential in spermatogenesis. To study if germ-somatic cell interaction could alter the PA/PAI-1 activities in the testicular cells, we have established a co-culture system of germ cells with the somatic cells, Sertoli cells or Leydig cells. tPA activity in the conditioned media of rat Sertoli cells in the presence of germ cells was negligible, and the PA activity was not regulated by FSH, hCG or forskolin. However, tPA (but not uPA) activity in the media obtained from the co-cultures of Sertoli cells with Leydig cells in the presence of germ cells was dramatically stimulated by these hormones (60,64), suggesting that factor(s) produced by Leydig cells may be important in enhancing the tPA expression in the Sertoli cells. It is also interesting that the Sertoli-germ cell conditioned medium contained considerable amount PAI-1 activity, down-regulated by the hormones. These data suggest the importance of the interaction between the germ cells and the somatic cells for the counterbalance regulation of tPA activity by its inhibitor PAI-1 which may be expressed mainly by the germ cells and essential for successful spermatogenesis in the seminiferous epithelium. Our evidence also showed that the tPA activity in the Sertoli cells strongly increased at stages 7-8 when germ cell meiosis took place, and at the stage 9-12 when spermiation occurred in the Sertoli cells (63,65), suggesting that plasminogen activators regulated by FSH may be involved in the processes of spermatogenesis and spermiation in the Sertoli cells (66-68). The requirement for FSH in promoting fertility in the male monkey is reasonably well established, however the evidence is still circumstantial and more work needs to be down to establish the hypothesis beyond any doubt in human (13, 68).

AR and WT1 expressed in Sertoli cells are indispensable for spermatogenesis (69-71), and crucial in regulating cell junctions in testis. AR expression appears before Sertoli cell maturation (72). Its expression is stage-specific and highest during stages 7 to 8, when new TJs form and premeiotic cells move through the BTB (73). AR is important in regulation of both AJs and TJs. In Sertoli cell-specific AR knock-out mouse, expression level of junction-associated molecules significantly decreased, while vimentin expression increased (59). This change is similar to that observed in our study (74). WT1 is a key regulatory factor controlling development of genitourinary system, and its expression is maintained in Sertoli cells during embryo development as well as in adults (75). WT1 knockout in mice before sex determination results in apoptosis of the genital ridge; Ablation WT1 function specifically in Sertoli cells after sex determination leads to the disruption of developing seminiferous tubules and subsequent progressive loss of germ cells (76); After birth, knockdown of WT1 in Sertoli cells led to loss of AJs, disregulation of AJ-associated genes and increased germ cell apoptosis (57). Consequently, WT1 is essential for spermatogenesis.

It has been reported that AR in Sertoli cells first appears before the cell final maturation and suggested to be a marker of mature Sertoli cells. On the other hand, WT1 maintains its expression in the Sertoli cells throughout embryo and life development. After heat treatment the loss of both AR and WT1 expression suggests a dramatic change in Sertoli cell function (74), the mechanism by which is complex and unclear. In human cryptorchid testis, germ cell development and maintenance of spermatogenesis correlated well with local AR expression in Sertoli cells. Absence of AR expression in dysgenetic Sertoli cells precisely correlated with a lack of local spermatogenesis in the tubules. Considering the importance of AR and WT1 in spermatogenesis, we suppose that loss of AR and WT1 expression in Sertoli cells in response to heat treatment may be one of the most important causes that lead to the cell dedifferentiation and germ cell apoptosis (74).

In addition to AR and WT1, TGF-β3 and LRH-1 may be also involved in the regulation of junctional complexes expression after the heat treatment (77). TGF-β3 has been found to regulate TJs and AJs dynamics via different signal pathways *in vivo* and *in vitro* (78-80).

5. GERM CELL APOPTOSIS AND REGULATION

Apoptosis is a necessary process in body involved maintaining proper development and eliminating damaged or excess cells (81,82). This physiological process is apparent during proper development for tissues to eliminate unwanted cells. Apoptosis is an active form of programmed cell death, which is characterized by distinct biochemical and morphological changes, such as DNA fragmentation, plasma membrane blebbing and cell volume shrinkage. Of interest, germ cell death, in addition to its involvement in cellular proliferation and differentiation, is conspicuous during normal spermatogenesis of various mammalian species, and plays a critical role in limiting germ cell population and eliminating germ cells that are defective. Dysregulation of this physiological germ cell apoptosis can cause male infertility (83-86). During normal spermatogenesis in mammals, it has been estimated that up to 75% of differentiating spermatogenic cells died before they mature into spermatozoa (87).

Germ cell apoptosis occurs spontaneously in the testis during fetal and postnatal development in mammals at various phases of germ cell development. Since Sertoli cells are terminally differentiated cells and can only support a limited numbers of germ cells for differentiation and maturation. Cells beyond this number must be eliminated. In addition, apoptosis may also serve to eliminate germ cells with unrepaired DNA breaks that do not pass certain checkpoints (19). This suggests that mitotic and meiotic checkpoints may be present to help correcting for the number of germ cells relative to Sertoli cells, and account

for problems such as rearrangements during meiosis or unrepaired breaks in the DNA by inducing apoptosis of these damaged cells (28). The tumor suppressor gene, p53, mediates testicular spontaneous germ cell apoptosis and may act as a quality control mechanism to eliminate defective germ cells, thus failure to remove defective germ cells by this mechanism results in increased percentages of abnormal sperm germ cells and reduced fertility. P53-mediated germ cell apoptosis may be a result of cellular proofreading to maintain the cellular integrity of germ cells during spermatogenesis (88,89). The P53/Rb-mediated spermatogenesis and germ cell apoptosis are closely related to testicular orptan receptor (90,91). Orphan receptors belong to a category of the steroid/thyroid hormone receptor superfamily in which cognate ligands have not been identified. Some members of this superfamily have been reported to be involved in regulation of spermatogenesis. TR2 is specifically expressed in spermatocytes and elongated spermatids in testes of rat and rhesus monkey (90). In contrast, TR4 was dramatically increased in spermatocytes at meiotic prophase, reaching the highest level at this phase during first wave of spermatogenesis. Results from experimental cyptorchid monkey testis and *TR4*-knockout mice indicate that TR2 and TR4 may play an important role in late meiotic prophase and subsequent meiotic divisions (91,92), suggesting that the orphan receptors are essential for normal spermatogenesis. In the local heat treated monkey testis, the coincident expression of testicular TR3, TR4, and p53 mRNAs with spatio-temporal germ cell apoptosis suggest that the two orphan receptors may be also involved in the germ cell apoptosis (93). Hsp110 has been found specifically localized in cytoplasm of germ cells by forming a complex with p53 to prevent the potential induction of apoptosis by p53 and other stimuli (94).

Interestingly, In addition to physiologic germ cell spontaneous apoptosis, deprivation of gonadotropins or intratesticular testosterone by GnRH antagonist or by estradiol (95), exposure to local testicular heating (96-98), Sertoli cell toxicant (99, 100), chemotherapeutic agents (101) or radiation exposure (102, 103) could also induce germ cell apoptosis in mammals**.**

5.1. Heat induced germ cell apoptosis

In most mammals, including human, the testicular temperature is maintained constantly lower than the core body temperature to allow normal spermatogenesis. Exposure of testis to body temperature or above by local testicular hyperthermia, cryptorchidism, or varicocele results in increased death of germ cells (104-113). Evidence showed that elevation of testicular temperature by 1 degree centigrade suppresses spermatogenesis by 14% and thus decreases sperm output (111). Earlier studies using monkey as a model to test heat effect on spermatogenesis showed that the higher the temperature and the longer the duration of heat exposure, the more severe the germinal epithelium damage (112). Cryptorchidism is a common congenital anomaly usually leading to clinical infertility in man due to severe decrease in sperm production. Artificially induced cryptorchidism could decrease sperm production by germ cell apoptosis (113,114). In the artificial unilateral monkey cryptorchid testis, most of the germ cells underwent apoptosis on day 5 after induction of cryptorchidism. The apoptotic signal decreased with the depletion of most of the germ cells on day 10. With the recovery of spermatogenesis, almost no apoptotic signal was observed in the cryptorchid testis on day 30 and thereafter (115, 116) as shown in Figure 4.

Accumulated evidence indicates that a single exposure of rat 43 degrees centigrade for 15 min (or monkey 43 degrees centigrade for 30 min) testes resulted in selective and reversible damage to the seminiferous epithelium due to increased germ cell apoptosis (94, 98). Therefore a mild testicular heating has been established as a safe and reversible approach for suppression of spermatogenesis. Evidence has shown that the damaging effect of heat at 43 degrees centigrade for 30 minutes once daily for 2 consecutive days on spermatogenesis in cynomolgus monkey is completely reversible (94), as shown in Figure 1. Similar result with different time treatment of the testes at 43 degrees centigrade was also observed in rat (96). Notably, the spermatocytes and spermatids are the most sensitive cell types affected by heat stress (117). A redistribution of Bax from the cytoplasmic to nuclear localization in some germ cells was observed while its total expression levels remained unchanged. On the other hand, Bcl-2 (an important anti-apoptosis mitochondria protein) levels increased significantly in response to heat stress. The subcellular redistribution of Bax (an important pro-apoptosis protein) and the increase in Bcl-2 expression in the heat-treated testis suggest an involvement of Bcl-2 family members in the heat-induced germ-cell apoptosis (117). Our *in vivo* experiments also demonstrated that the heat-induced changes in the expression of Caspase 2 (118), nitric oxide synthase (119) and various heat short proteins (120) in the treated adult monkeys are all reversible and the signaling pathways of heat-induced germ cell apotosis are complected (121). Our experiment showed that Hsp70-2 expression was almost unchanged at early stage of germ cell apoptosis in the cryptorchid testis on day 5, and dropped dramatically along with loss of apoptotic germ cells on day10 after the operation. It is therefore suggested that Hsp70-2 might not take part in inhibiting germ cell apoptosis at the early stage in the experimental cryptorchid testis. It is therefore suggested that Hsp70-2 does not belong to the immediate early related gene responsible for germ cell apoptosis induced by the heat stress (122). On the other hand, Hsp60 signals were obviously elevated in the cryptorchid testis after the heat shock. The increased Hsp60 expression in the cryporchid testis was correlated with an increase in Bcl-2 expression. It is thus presumed that increased Hsp60 expression in the cryptorchid seminiferous epithelium might release free Bcl-2 by forming complex with Bax, which may be capable of promoting cell apoptosis via mitochondria pathway.

5.2. Heat induced changes in gene expression patterns in testis

To investigate the molecular mechanism of germ cell apoptosis induced by heat stress, the changes in the

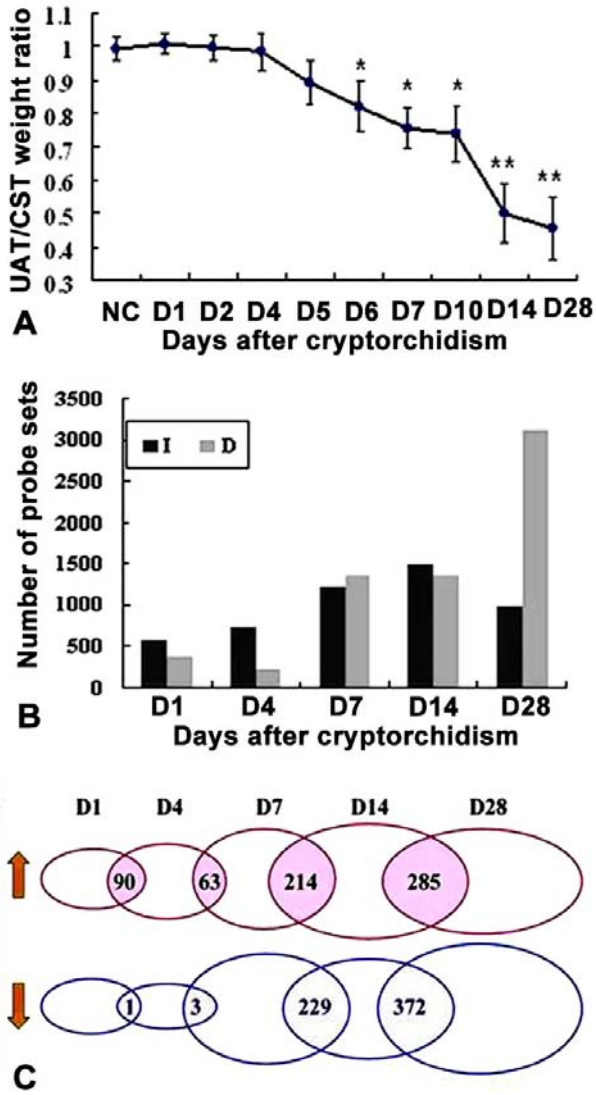

Figure 1. Weight changes in mouse cryptorchid testis and its genome expression pattern. Weight ratio changes in unilateral abdomen testis (UAT) to contralateral scrotum. testis (CST) on day 1, 2, 4, 5, 6, 7, 10, 14, 28 after EC operation in CD-1 mice. NC (normal control). Data are shown as the mean±SD. Values significant different compared with the normal control were labeled with asterisk (*, $p<0.05$ □ **, $p<0.01$) (one-way ANOVA). (B) Number of genes significantly up-regulated (signal log ratio (SLR) ≥1) and down-regulated (SLR ≤-1) on day 1, 4, 7, 14, 28 after EC. (C) Numbers in the space between overlapping elliptical circles represent the number of probe sets that were all significantly changed in the adjacent treatments. Number of probe sets in each treatment was represented by the size of respective circle. Up arrow denoted up-regulated probe sets, down arrow denoted down-regulated probe sets. IC, increase; DC, decrease. Reproduced with permission from reference 104.

gene expression patterns after experimental cryptorchidism (EC) in adult mouse testis were analyzed using Affymetrix MOE430A microarray on day 1, 4, 7, 14, and 28 as compared with the untreated control (104). As shown in Figure 2, the data showed that EC led to dramatical number of genes in the cryptorchid testis significantly up- or down-regulated in the first 28 days. Notably, both the oxidative stress and the related gene expression fluctuation were highly coincident in timing. The cryptorchid testis showed more effective antioxidative capability in the first 4 days, and suddenly lowered the capability from day 5 on, then gradually restored the antioxidation from day 10 to 14, and turned to worse on day 28 again. The extensive high gene expression on day 4 after EC, the up-rising of oxidative stress level on day 5 and the abrupt down-regulation of the gene expression on day 7 were closely related. From the chip data, the evidence showed that the high level of reactive oxidative species (ROS) was not only related to the dysfunction or abnormality of the direct origin of ROS generation, but also related to the abnormality of the more upstream physiological events in energy, lipid metabolism, and cell cycle regulation. The selective regulation of metabolic substrate transporter in different cell populations implied the existence of various regulations of the selective

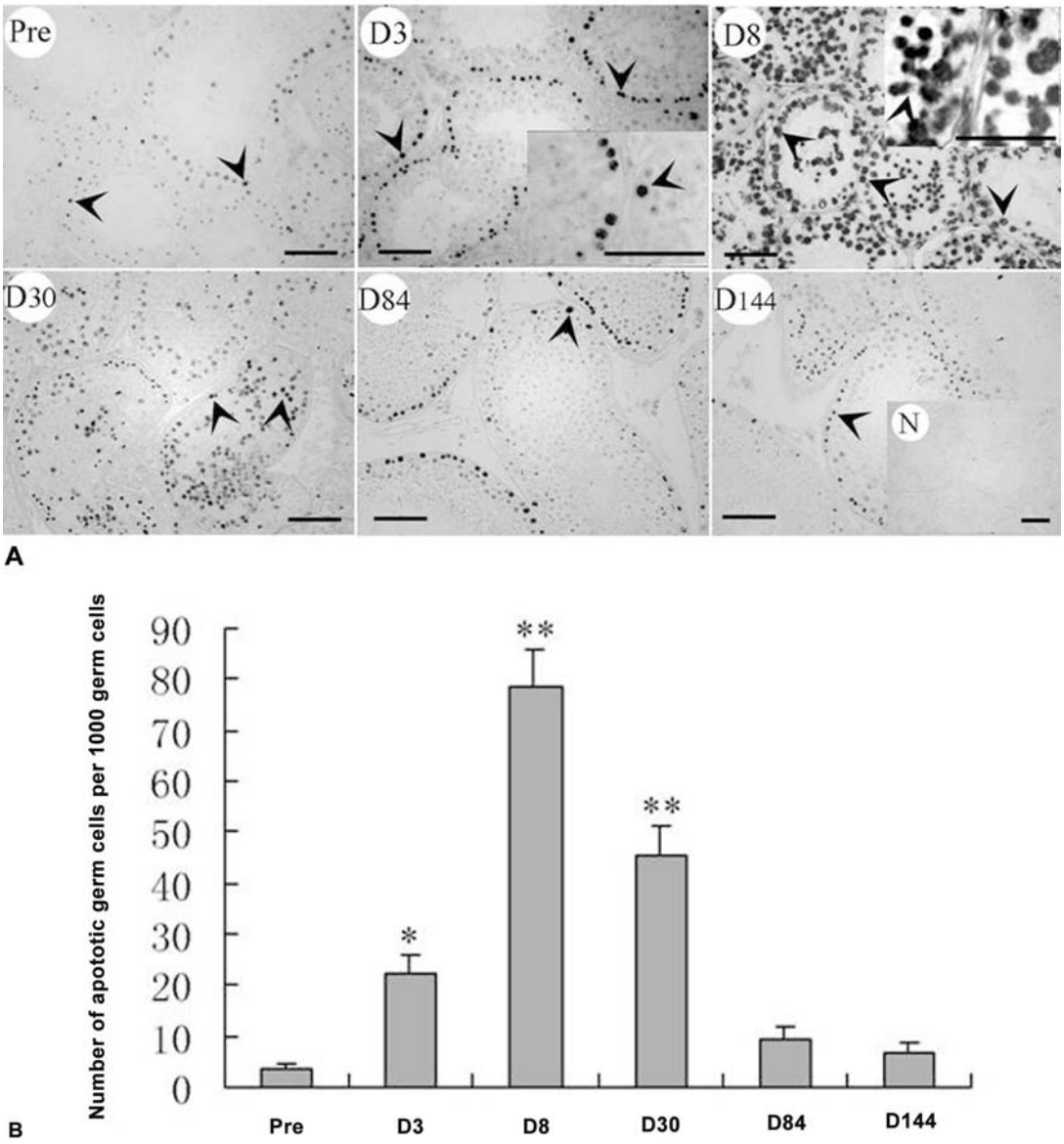

Figure 2. TUNEL detection of apoptotic germ cells before (Pre) and after heat stress on D3, D8, D30, D84, D144. (A) Apoptotic nuclei were stained dark (arrow head). The insets in D3 and D8 pictures were of higher magnification. N: a negative control with TdT left out. Bar= 100μm. (B) Statistical analysis of apoptotic index. AI, the number of apoptotic germ cells per 1000 testicular germ cells. Data are presented as mean±SEM (n=3). Number with * is significantly different ($P<0.05$) or ** ($P<0.01$). Reproduced with permission from reference 94.

signal pathways among cell populations induced by EC. In the studies, several physiological significant spermatogenesis-specific related novel genes have been cloned, such as a novel membrane-related protein, acrosome formation associated factor (Afaf) (123, 124), testis-specific sodium-hydrogen exchanger (tsNHE) (125, 126), temperature related sequence1 & 4 (Trs1, Trs4) and T6-441 (127-130).

Afaf was expressed abundantly in round spermatids, and localized exclusively at the site of the pre-forming and forming acrosomes during acrosome formation in these cells. Afaf expression obviously declined in the maturing acrosomes, its protein in transfected Hela cells was localized in the plasma membrane and EEA1-positive early endosome, occasionally was also observed in the nucleus. Therefore, we propose that the early endosome and the plasma membrane might be also involved in the acrosome biogenesis (123). We then further investigate the effect of Afaf antibodies on fertilization, acrosome exocytosis (AE) with streptolysin O-(SLO)- permeabili - zation and *in vitro* fertilizationas shown in Figure 3. The data showed that Afaf was involved in the calcium triggered AE, treatment of sperms with the Afaf antibodies was capable of significantly inhibiting the sperm penetration of the eggs, therefore reduced the rate of *in*

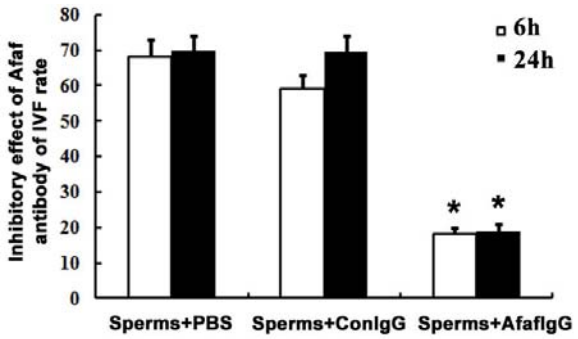

Figure 3. Inhibitory effect of anti-Afaf IgG on *in vitro* fertilization (IVF). IVF was performed according to the standard protocol. The mean and SD of three independent experiments was plotted against the IVF fertilization rate. In each experiment, at least 100-150 oocytes were used and the results were analyzed by two researchers independently. * denotes significant different ($p<0.01$) as compared with the control at 6 and 42 hours. Reproduced with permission from reference 124.

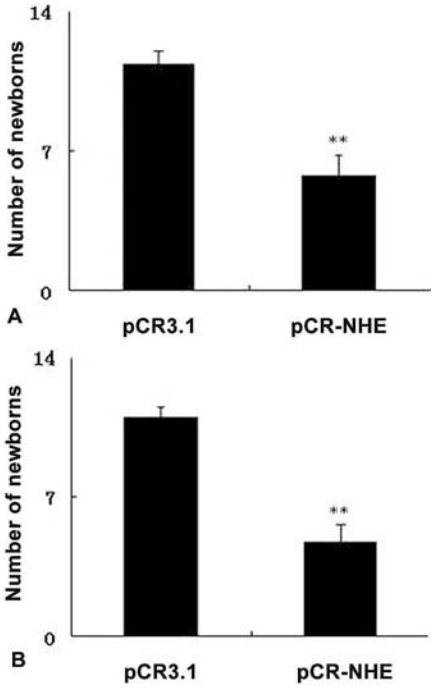

Figure 4. Effect of pCR-NHE DNA vaccination on number of newborns. Female mice were inoculated with pCR3.1 or pCR-NHE respectively via oral or nasal vaccination. To evaluate efficacy and safety of the DNA vaccine, the immunized mice were mated with normal adult males at one week after immunization. Number of newborns was scored. A. Number of newborns (mean ± SD) of the vaccinated female mice with pCR-NHE via oral feeding was significantly decreased from 11.3 ± 0.7 (pCR3.1) to 5.7 ± 1.1(pCR-NHE). B. Number of newborns (mean ± SD) of vaccinated female mice with pCR-NHE via nasal instillation was significantly reduced from 11.0 ± 0.5 (control) to 4.7 ± 0.9 (pCR-NHE). ** indicates significant differences ($P < 0.01$). Reproduced with permission from reference 125.

vitro fertilization (124). We suggested that Afaf might play an important role in AE and participate in fertilization.

Sodium-hydrogen exchanger as a channel for regulation of intracellular pH might be a crucial modulator of sperm capacitation and motility. The mtsNHE localizing on principle piece of sperm flagellum contained 12 predicted transmembrane regions without cytoplasmic fragment at carboxyl terminus. Polyclonal antibodies to trans-membrane region significantly reduced sperm motility, acrosome reaction and ratio of *in vitro* fertilization. By in-pouring the antibodies in sperm solution, intracellular pH and calcium concentration were decreased (125). To investigate the effect of the mtsNHE antibodies on fertility, the female mice were immunized muscally, orally or nasally with a *tsNHE* cDNA vaccine in 2-weeks interval. The number of newborns and fertility rate of the vaccinated female mice were significantly stepped down as compared to the controls (Figure 4). The antiserum or vaginal fluid from the *tsNHE* cDNA vaccinated female mice could specifically recognize the principle piece of sperm tail and triggered sperm agglutination. Evidence with the antibodies also showed a significant inhibitory effect on *in vitro* sperm motility and fertilization (126). The sodium-hydrogen exchanger might be an excellent target molecule for developing a new male contraceptive.

Trs4 was identified as a testes-specific gene whose expression was sensitive to abdominal temperature changes induced by artificial cryptorchidism. In murine testes, Trs4 mRNA was detected in round spermatids, and its protein was localized mainly in the elongating spermatids as well as in the acrosomes and tails of mature spermatozoa. Using a yeast two-hybrid screening system, we identified Rshl-2, Gstmu1, and Ddc8 as putative binding partners of Trs4 in mouse testes. Their interactions were confirmed by *in vivo* and *in vitro* binding assays. Further studies demonstrated that Ddc8, a novel gene with unknown functions, displayed a similar expression pattern with Trs4 in mouse testes. In particular, Trs4, Ddc8 and Rshl-2 were co-localized to the tails of mature spermatozoa (130). These results suggested that Trs4 might be involved in diverse processes of spermiogenesis and/or fertilization through its interaction with its multiple binding partners.

T6441 is also a novel gene induced by artificial cryptorchidism. Its cDNA contains an open reading frame encoding a protein of 149 amino acids which showed no homology to any reported genes. T6441 gene has 4 transcripts in rat testis and was temporally regulated during testicular development. T6441 mRNA was specifically localized in spermatids, and its expression level varied in a stage-dependent manner during spermiogenesis, with the highest expression level observed at step 7-14. RT-PCR results showed that T6441 mRNA transcribed in most tissues, strongest in testis. T6441 expressed protein was mainly in the cytoplasm of spermatids, with the maximal levels at step 12-19. T6441 was mainly localized in the residual body at step 19 (127). It is suggested that T6441 may play an important role in cytoplasm movement and removal during spermiogenesis.

Because of all the cloned 4 genes, Afaf, mtsNHE, Trs4 and T6441 are specifically expressed in spermatids, and closely related to the sperm functions, such as acrome reaction, capacitation, sperm motility and fertilization. Disturbance of the gene expression locally does not affect human health, it is therefore suggested that they might be the excellent target molecules to further study for developing new safe and valid male contraceptives.

5.3. Hormone deprivation induced germ cell apoptosis

Spermatogenesis is dependent upon pituitary gonadotrophins, testicular androgen and estrogen as well as some other testicular endogenous factors. Testosterone produced by Leydig cells not only directly influences spermatogenesis in seminiferous tubules, but high concentration of testosterone is feedback to pituitary to inhibit the gonadotrophin secretion, leading to decrease in secretory activity of Leydig cells. Manipulation of spermatogenesis by deprivation of survival factors provides a basis for detailed study on the regulatory mechanisms of germ cell death. In immature rat, hypophysectomy or treatment with GnRH antagonist results in increased germ cell apoptosis, which can be inhibited by hCG or testosterone. Withdrawal of gonadotrophins and testosterone for up to 2 weeks by a potent GnRH-A treatment in adult rat results in acceleration of germ cell apoptosis at specific stages of seminiferous epithelial cycle (131). Ethane dimethanesulfonate (EDS) selectively and temporarily eliminates Leydig cells in testis resulting in a suppression of intratesticular and serum testosterone (132,133). Testosterone is needed for maintenance of spermatogenic process and for inhibition of germ cell apoptosis (134,135). A decline in androgen production by Leydig cells is a trigger for germ cell apoptosis, the seminiferous tubules were exposed to high concentrations of exogenous testosterone (25-100 folds higher than the circulating levels), a complete inhibition of intratesticular testosterone was observed and resulted in germ cell apoptosis at various stages (40,134). The predominant germ cell types undergoing apoptosis include pachytene spermatocytes and round spermatids at early (I-VI) and middle stages (VII-VIII) from the spermatogenic cycle. However, the molecular mechanisms by which gonadotropin and androgen regulate germ cell apoptosis are still unclear. Administration of exogenous testosterone can reduce secretion of pituitary LH, FSH and intratesticular testosterone levels and, as a consequence, inhibiting sperm output (40,94). Testosterone *in vivo* can be metabolized to either estrogen or DHT. Evidence has shown that both estrogen or DHT is capable of inhibiting germ cell death, and estradiol appears to be a more potent inhibitor of male germ cell death than testosterone or DHT (46). The localization of estrogen receptor beta and aromatase in pachytene spermatocytes was also demonstrated, indicating a role of estrogen in this step of spermatogenesis (47).

Evidence showed that inhibin B secreted by Sertoli cells is governed by a stimulatory and an inhibitory action of FSH and LH respectively (30). It is most likely that the interaction of pituitary FSH and inhibin B secreted by Sertoli cells is responsible for azoospermia or oligozoospermia induced by high exogenous T. Weinbauer *et al* demonstrated that treatment of cynomolgus monkey with testosterone buciclate (TB) significantly suppressed serum levels of FSH and inhibin–alfa in a dose-dependent manner, and pronounced inhibited spermatogenesis, whereas androgen receptor expression remained unchanged (30). Therefore, the authors suggested that the pronounced suppression of the primate spermatogenesis seemingly requires inhibition of FSH, rather than LH and testicular testosterone levels in the monkey. Estrogen is formed in Sertoli cells from testosterone by the enzyme P450 aromatase (136). Estrogen can cause alteration in circulating concentration of gonadotropins and testosterone and thus affect germ cell apoptosis indirectly (136). In the unilateral cryptochid cynomolgus monkey model, peripheral plasma testosterone level was significantly decreased after the unilateral cryptochid operation (137), but plasma estrogen level in spermatic vein was significantly increased (138,139).

In addtiton, expression of liver receptor homolog-1 (LRH-1) was obviously up-regulated in the cryptorchid monkey testis (137). LRH-1 could enhance P450 aromatase activity by converting testosterone to estrogen, implying that the increased LRH-1 expression in Sertoli cells might be responsible for the decrease in the circulating testosterone level. Estrogen was able to inhibit hypothalamus and pituitary function by a negative feedback loop in male animals, thus it was presumed that increase in estrogen production in cryptorchid testis might be responsible for the decrease in serum LH level (137)**.**

5.4. Sertoli cell impairment affected germ cell apoptosis

Sertoli cells foster development and maintain viability of germ cells by secreting hormonal and nutritive factors into a specialized compartment, formed by tight junctions between adjacent Sertoli cells. Germ cell apoptosis may be signaled by a decrease in Sertoli cell pro-survival factors, an increase in Sertoli cell pro-apoptotic factors, or both. Sertoli cells can also interact with Leydig cells and peritubular cells to regulate spermatogenic process (140-142). It has been reported that Sertoli cells undergo a radical change in their morphology and function, heralding the switch from an immature proliferative state to a mature non-proliferative state at the period around onset of puberty. Considerable evidences have demonstrated that elevated temperature could induce germ cell apoptosis via varours signal pathways and accompany with histological and hormonal changes in the seminiferous epithelium, leading to impairment of spermatogenesis (66, 74, 143-151).

Using cytokeratin-18 (CK-18) as a Sertoli cell immature molecule marker, we have demonstrated that only immature monkey Sertoli cells, but not the Sertoli cells from the adult could express CK-18 (146). Further studies showed that the adult monkey Sertoli cells from experimental cryptorchid testis or from 43 degrees centigrade local heating testis could induce re-expression of CK-18 (Figure 5) (74, 146, 147), indicating that the adult Sertoli cells have reverted to a dedifferentiated state in the heat treated testis, thus lost their supportive role leading to

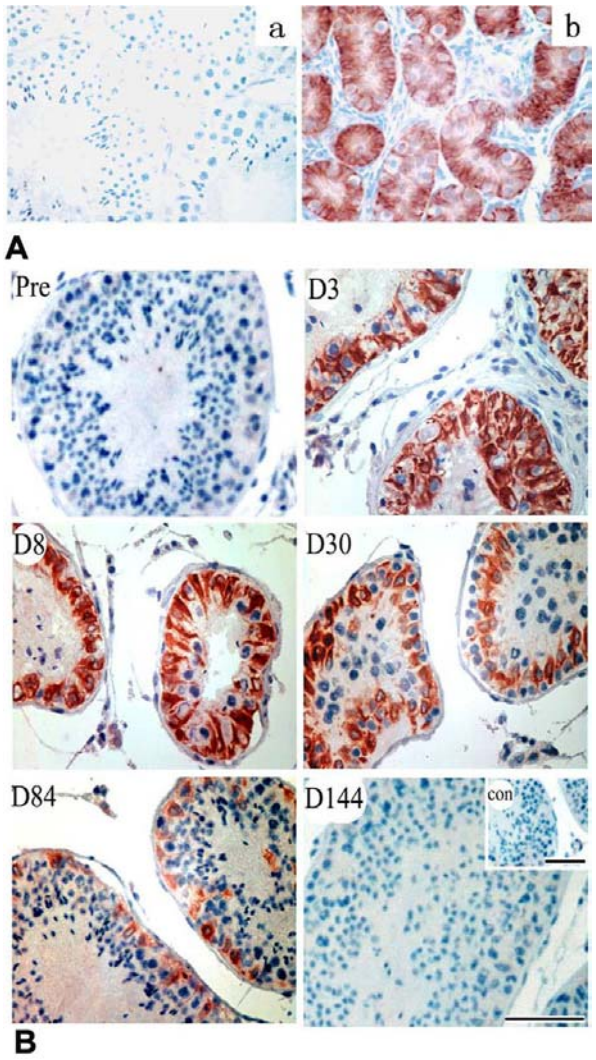

Figure 5. Immunohistochemical staining of cytokeratin-18 (CK-18) protein in monkey testes. A CK-18 expression in adult (a) and immature monkey testes (b). Only immature Sertoli cells expressed CK-18. B. Immunohistochemical staining in 43°C treated adult monkey testes. Before (Pre) and after 43°C heat stress on D3, D8, D30, D84 and D144. Red color shows the positive staining of CK-18 in Sertoli cells (arrow heads). Blue color is background counter staining, con: the negative control without primary antibody on D84. Bar=100μm. Reproduced with permission from reference 146.

a cessation of spermatogenic activity. Exposure of cultured mature monkey Sertoli cells to 43 degrees centigrade water bath could also induce CK-18 re-expression, and this transition has been demonstrated via activation of a PKA-dependent ERK MAPK pathway (74, 147). These findings suggest that not only germ cells, but also Sertoli cells may be affected by the heat-treatment, subsequently leading to germ cell apoptosis. Furthermore our evidence showed that after local warming (43°C) the adult monkey testis, LRH-1 was also induced in the Sertoli cells in coincidence with activation of cytokeratin 18 (CK-18), as shown in Figure 6, suggesting that the heat-induced dedifferentiation of the mature Sertoli cells might be related to LRH-1 regulation.

Close interactions in adjacent Sertoli cells and between Sertoli and germ cells base on various cell junctions present in seminiferous epithelium. It has been reported that compounds such as glycerol or cadmium chloride ($CdCl_2$) could perturb TJs in testis and underlying associated AJs (152). Evidence also showed that gossypol, lonidamine and its analog AF-2364 could perturb AJs between Sertoli and germ cells (153-156). All these compounds are known to be capable of inducing germ cell loss from the epithelium and inducing infertility without any apparent effect on serum FSH, LH and testosterone levels. They perturb cell junctions by changing the expression of junction-associated molecules or the interactions between these molecules. Our evidence has further demonstrated that local heat treatment of pubertal monkey testis could disrupt cell junctions in seminiferous epithelium, and affect expression of junction-associated molecules in the Sertoli cells (74). Furthermore, treatment of monkey Sertoli cells *in vitro* with 43°C warming could also affect expression of AJs-, TJs-associated molecules.

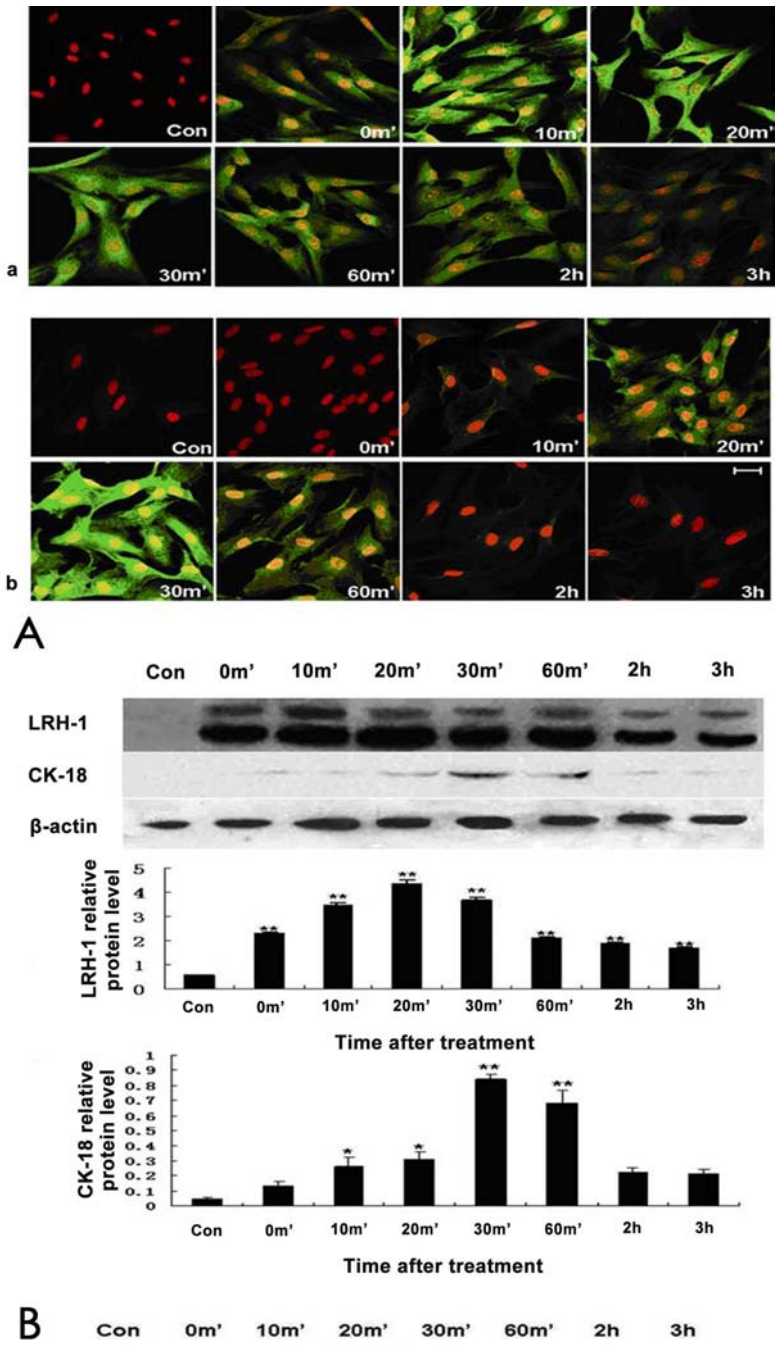

Figure 6. Confocal immunohistochemistry and Western blot analysis of LRH-1 and CK-18 expression induced by 43°C treatment in adult rat primary Sertoli cells. A: LRH-1: The mature Sertoli cells were isolated from rat testes at the age on day 60 after birth and cultured in DMEM plus F-12 medium with 10% FBS for 48 h. The cells were washed and further cultured in serum free medium for 12h, and then the cells were underwent at 43°C water bath for 15 min. 0 min was the time justly ending the 43°C water treatment. 10, 20, 30, 60 min, 2 and 3 h indicate the time points respectively after ending the heat treatment. After fixed in mixture of methanol and acetone (1:1), the cells were incubated with the primary antibody LRH-1 (1:300) at 4°C, and then with the flurescein isothiocyanate conjugated antirabbit IgG (1:200). Bar, 50 μm. B: CK-18: the mature Sertoli cells were incubated with the primary antibody CK-18 (1:100) at 4°C, and then with the flurescein isothiocyanate conjugated antirabbit IgG (1:200).The nuclei were stained in red using PI, and the green is the positive signal. Bar, 50 μm.C: Western blot analysis of LRH-1 and CK-18 expression. The Sertoli cells were isolated from adult rat testes and collected at 0, 10, 20, 30, 60 min, 2 and 3h after ending 43°C warming treatment. β-actin was used as an internal control. Data are presented as mean ± SEM (n=3). Bar with ** is significantly different (P<0.01). Reproduced with permission from reference 77.

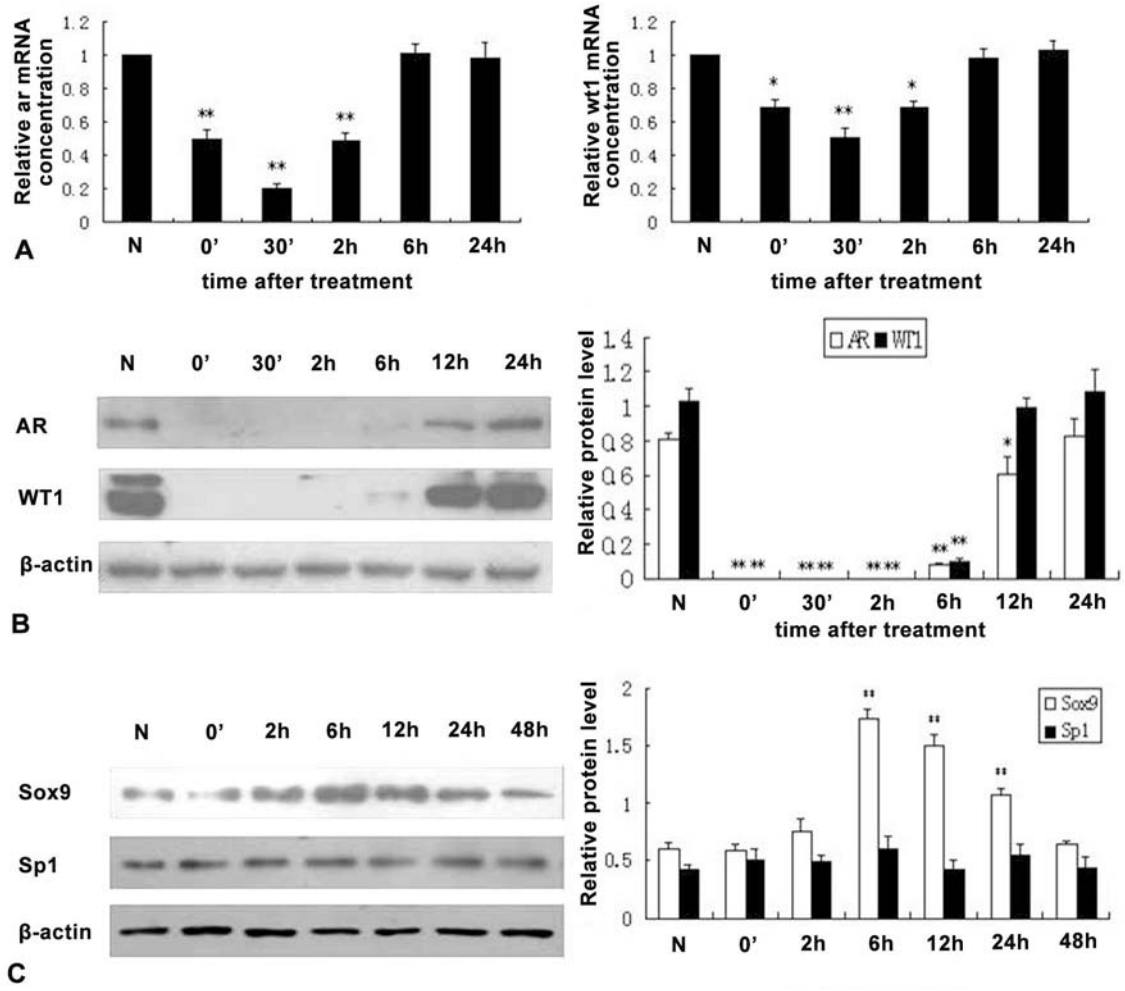

Figure 7. 43°C treatment induced dramatical decrease in AR and WT1 expression in monkey Sertoli cells. Sertoli cells were isolated and cultured as described in the text. After hypotonic shock and culture in serum-free medium for 24h, the Sertoli cells were incubated in 43°C for 30 min. N indicates the untreated control Sertoli cells. The time immediately after terminating the heat treatment was designated as 0', and 30'-48h represent the time point respectively after terminating the treatment;. A, Real-time PCR analysis of AR and WT1. The relative concentrations were calculated as $2^{-\Delta\Delta CT}$ and the relative concentration of N was 1; B, Western blot analysis of AR and WT1; C, Western blot analysis of Sox9 and Sp1. In B and C, β-actin was used as an internal control. The relative levels were determined by the ratio of target protein to β-actin as measured by densitometry. Data in A, B and C are presented as mean ± SEM (n=3). Bar with * is significantly different ($P<0.05$); **, $P<0.01$(Cited from reference 74).

N-cadherin, β-catenin and the TJs-associated molecule ZO-1 significantly decreased at 24h and 48h, while the expression of vimentin increased dramatically from 6h to 48h after the heat treatment, indicating the heat treatment affects the junction-associated molecules in Sertoli cells. It has been hypothesized that vimentin may have diverse functions as structural support, plasma membrane-nucleus communication and cell signaling (153,154). Further experimental evidence showed that the action of "heat stress" on various Sertoli cell junctions in seminiferous epithelium was also through ERK MAPK signal pathways (74, 146, 147).

WT1 is essential for both embryogenesis and spermatogenesis. 43°C warming monkey Sertoli cells induced a reversible loss of both AR and WT1 protein expression (74). As in Figure 7 both protein levels in the heat-treated Sertoli cells dramatically decreased at 15 min, and disappeared at 30 min. However, their mRNAs in the cells did not disappear, but significantly decreased in a time-dependant manner. Cycloheximide, PMSF and aprotinin could not block the ablation of AR and WT1 induced by the heat treatment (74). However, when the Sertoli cells were pre-incubated with MG-132, a significant appearance of the disappeared AR and WT1 expression by the heat treatment could be observed. We suppose that the disappearance of AR and WT1 proteins in the Sertoli cells in a short time after the heat treatment might be degraded quickly by some type (s) of unknown proteases, in addition to 26S proteasome, or by other unknown mechanism. The

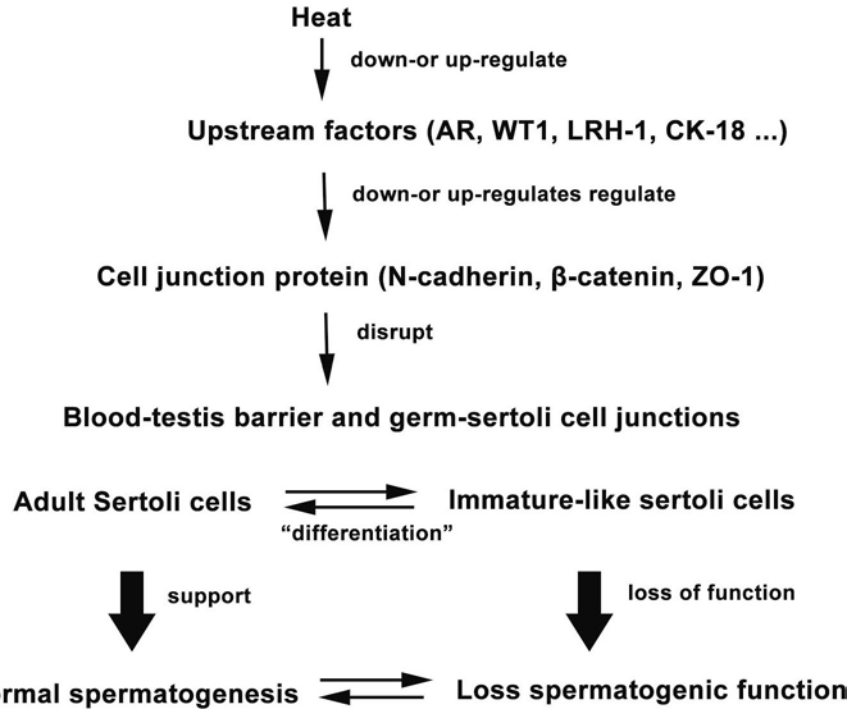

Figure 8. Systematic regulation of heat-induced a reversible change in Sertoli cells from an adult differentiated state to an immature-like dedifferentiated state.

disappearance of AR protein after heat treatment has also been observed in human prostate cancer cells (LnCaP). Pajonk *et al* (157) placed the LnCaP cells in 44°C for 1 hour and then returned to 37°C for 90 min, the AR expression in cells was completely abrogated and did not recover in 24 hours. However, the mechanism of the 44°C-induced LnCaP cell AR loss is not known. To further verify the disappearance of AR and WT1 expression in the cultured monkey Sertoli cells by 43°C treatment, we designed unilateral cryptorchid experiment using adult rats. A dramatical decrease in the AR and WT1 expression in the Sertoli cells was also observed (unpublished data).

Earlier studies showed that androgen antagonist, flutamide, could reduce occludin expression in rat testis (158-160), however no evidence has addressed its action on junction-associated molecules in Sertoli cells *in vitro*. Our evidence showed that flutamide as an AR antagonist, similar to heat treatment, could decrease junction-associated molecule expression, while increase vimentin production, implying that the decreased AR expression by heat treatment may be involved in regulation of changes in the junction-associated molecule expression in Sertoli cells (74). Based on the data available we proposed a systematic regulation of Sertoli cell dedifferentiation induced by heat stress (Figure 8)

6. TWO MAJOR PATHWAYS REGULATING GERM CELL APOTOSIS

Germ cell apoptosis is controlled in a cell-type specific fashion, but basic elements of the death machinery may be universal. Most of morphological changes in apoptotic cells are caused by specific proteases, caspases. Two major pathways, intrinsic and extrinsic, are involved in the process of caspase activation and apoptosis in mammalian cells (161-169). The intrinsic pathway for apoptosis involves release of cytochrome c from mitochondria into the cytosol, where it binds to apoptotic protease activating factor 1 (Apaf-1), resulting in activation of the initiator caspase 9 and the subsequent proteolytic activation of the executioner caspases 3, 6, and 7. This pathway is also called mitochondria-dependent apoptotic pathway. The active executioners are then involved in cleavage of a set of proteins, including poly-(ADP) ribose polymerase (PARP), lamin, actin, and gelsolin, and cause morphological changes in cell and nucleus typical apoptosis. Members of Bcl-2 family proteins play a major role in governing this pathway with protein such as Bax functioning as inducer of apoptosis, and protein such as Bcl-2 as suppressor of cell death (161, 162). The extrinsic pathway (death receptor pathway) for apoptosis involves ligation of death receptor (such as Fas) to its ligand (FasL). Binding of Fas L to Fas induces trimerization of Fas receptors, which recruit FADD (Fas-associated death domain) through shared death domains (DD). FADD also contains a death effector domain, or DED, in its N-terminal region. The Fas/FADD complex then binds to the initiator caspase 8 or 10 through interactions between the death effector domain of the FADD and these caspase molecules. Caspase 8 or 10 then activates the effector or executioner caspases 3, 6, and 7, resulting in cellular disassembly. Both pathways converge on caspase 3 and other executioner caspases and nucleases that drive the terminal events of

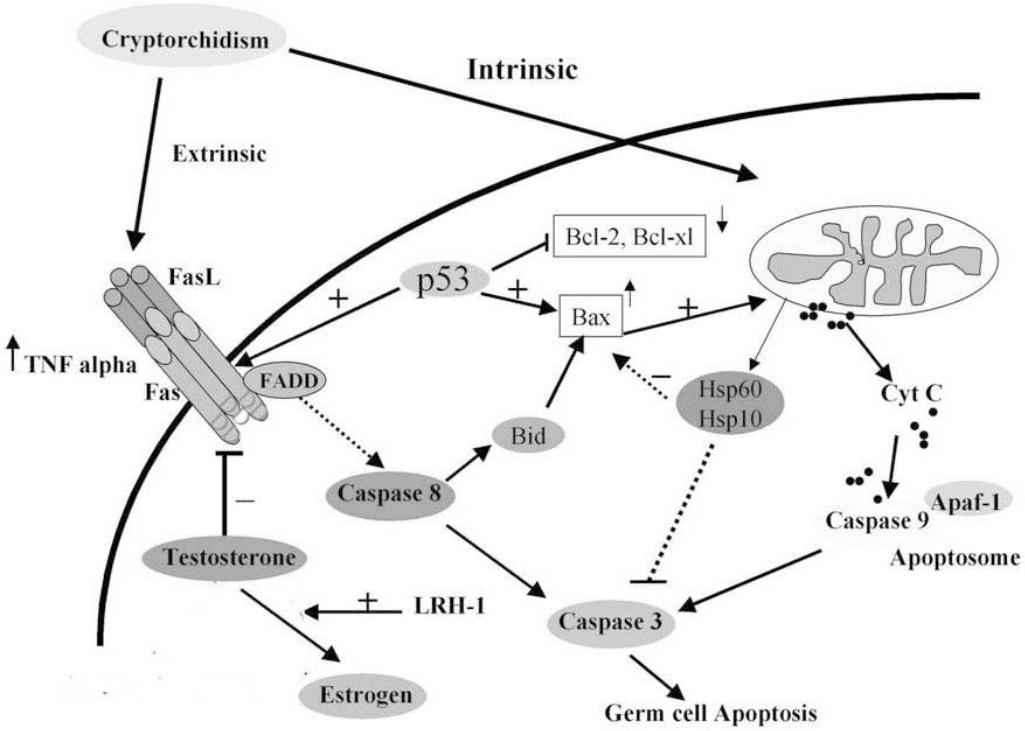

Figure 9. A schematic representation of germ cell apoptosis induced by cryptorchidism. Two of the major pathways for caspase activation in germ cells are presented, the extrinsic and intrinsic. The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as TNFR and Fas. Trimerization of the Fas receptor by Fas ligand results in activation of caspase 8 mediated by the adapter protein Fas-associated death domain [FADD), which then binds DED-containing pro-caspase-8. The intrinsic pathway can also be activated by cryptorchidism, resulting in release of cytochrome c from mitochondria. The release of cytochrome *c* from mitochondria binds and activates Apaf-1, allowing it to bind and activate pro-caspase-9, assembling of the high molecular weight caspase-activating complex called apoptosome. Both active caspase-9 and caspase-8 can directly cleave and activate the downstream effector protease, caspase-3, an executioner of apoptosis. p53 can result in increased Fas expression and hence induce killing by the Fas/Fas ligand pathway. It can also interfere with Bcl-2 family proteins to regulate the fate of germ cell, including elevations in the levels of pro-apoptotic members such as Bax or down-regulation of anti-apoptotic Bcl-2 expression level. On the other hand, up-regulation of mitochondrial Hsp60/Hsp10 expression by cryptorchidism helps the inhibition of activating caspase-3, thus inhibiting gem cell apoptosis. LRH-1 might accelerate transformation of testosterone into estrogen leading to the decrease in the serum testosterone concentration, subsequently enhancing germ cell apoptosis by activating testosterone-dependent Fas/FasL death receptor pathway. Reproduced with permission from reference 137.

programmed cell death (Figure 9) (164,170). Crosstalk among these pathways does occur at multiple levels. For example, in certain types of apoptosis, proper activation of effector caspases by Fas depends on caspase 8-mediated cleavage of the proapoptotic Bcl-2 member, Bid, and subsequent release of cytochrome c from mitochondria, in turn, results in caspase 9 activation via apoptosome formation (166, 167).

6.1. Mitochondrial death pathway

The Bcl-2 family can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and pro-apoptotic members, such as Bax, Bak, Bid, and Bad (166,170). It is generally believed that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members is critical in determining whether occurrence of germ cell apoptosis (22, 163). During the early apoptotic wave, a high level of Bax expression is normally observed in germ cells of testes (163,164). Bax deficient mice are infertile by disrupted spermatogenesis due to an inappropriate accumulation of premeiotic germ cells, and the deficient mice display similar phenotypes to those overexpressing Bcl-2 or Bcl-xl in premeiotic germ cells. In addition, transgenic mice that over-express *Bcl*-2 or *Bcl*-xL are infertile and display a disorganization of the cells in seminiferous epithelium (170). While those expressing lower level of Bcl-xl show an increased germ cell death, similar to Bax overexpression. Thus, it appears in mice that the early apoptotic wave required for formation of mature sperm is dependent on proper balance of anti-apoptotic and pro-apoptotic genes (28). The testes of Bax knockout mice contain excessive numbers of spermatogonia and pre-leptotene spermatocytes, consistent with failed apoptosis during the first wave of spermatogenesis (170). Expression of Bcl-2 or Bcl-xL transgene in germ cells reduced the incidence of apoptosis and produced the pathology similar to that of Bax knockout animals (171,172). In addition, mice misexpressing bcl-2 in spermatogonia displayed an

accumulation of spermatogonia before puberty, but during adulthood, exhibited loss of germ cells in majority of the tubules (172). Interestingly, the preferential Bax expression in round spermatids and in a few spermatocytes is consistent with the numerous apoptotic cells found among round spermatids in normal human testis, indicating that the preferential expression of Bax in these cells may be related to induction of spontaneous apoptosis (173). Bcl-2 and Bak showed very similar and preferential expression in spermatocytes and spermatids, therefore, the co-localization of Bcl-2 and Bak and the ratio between them may also indicate an important function in regulation of human testicular apoptosis. One of the intriguing aspects of apoptosis regulation by members of this family is their subcellular localization and translocation. Some Bcl-2 family members, such as Bcl-2 and Bak, constitutively localize to the mitochondrial membrane, whereas others, such as Bax and Bid, translocate from cytosol to mitochondria early during apoptosis (174-176). Furthermore, insertion of Bax into mitochondrial membranes has been shown to play an essential role in releasing cytochrome c from the mitochondrial membrane space to the cytosol in various cell systems (177-179).

In heat-induced testicular germ cell apoptosis, the initiation of apoptosis was preceded by a redistribution of Bax from a cytoplasmic to paranuclear localization in heat-susceptible germ cells (180). The relocation of Bax is further accompanied by sequestration of ultracondensed mitochondria into paranuclear areas of apoptotic germ cells. One of the main roles of Bcl-2 proteins is to regulate release of cytochrome c, allowing it to complex with Apaf-1 and activate caspases. The release of cytochrome c from mitochondria into cytosol is further associated with activation of initiator caspase 9 and executioner caspases 3, 6, and 7, and cleavage of PARP. Recently, Smac (stands for second mitochondria-derived activator of caspase), also known as DIABLO, was identified as a mitochondrial protein just like cytochrome c, which is released from mitochondria into cytosol following apoptotic stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins (IAPs) (100). The IAPs and their counteraction by the mitochondrial protein DIABLO have also emerged as important regulators of caspase activation and apoptosis in germ cell system (162). Of particular importance, the expression of DIABLO mRNA as well as the protein has been found to be the most abundant in the adult testis. Immunocytochemistry of heat-treated testes revealed diffused staining of DIABLO in those heat-susceptible germ cells before their eventual apoptosis at later time intervals, whereas in the untreated cells, staining is mostly punctate, suggesting cytosolic translocation of mitochondrial DIABLO, as reported for various mitochondrial proteins, including DIABLO in various extragonadal cell systems (181-183). Furthermore, because, both cytochrome c and DIABLO were simultaneously released from mitochondria and accumulated in the cytosol after heat-stress, it is possible that the same mechanisms responsible for release of cytochrome c from the mitochondria are responsible for the translocation of DIABLO. Thus, one could assume that while cytochrome c initiates caspase activation through interaction with Apaf-1, DIABLO could bind and inhibit the cellular IAPs and, in turn, promote apoptosis. Meanwhile, some members of the Bcl-2 family are also involved in other induced germ cell apoptosis (184). And there also exists evidence suggesting that Bcl-2 family proteins may also serve to regulate germ cell apoptosis via an endoplasmic reticulum (ER) dependent pathway (163). Induction of apoptosis in rat testicular cells caused translocation of Bax to the ER shortly after induction (176). In control rats, only trace amounts of Bax were found in the ER, with most localized to the cytosol and mitochondria. This indicates a role for Bax and potentially other Bcl-2 proteins in regulating early apoptosis via the ER pathway.

6.2. Death receptor pathway

Apoptosis can be induced by ligation of a subset of plasma membrane tumor necrosis factor receptor (TNFR) family members, referred to as death receptors (185). The best characterized subset of the death receptors are Fas (CD95/Apo-1) and TNFR1 (p55/CD120a). The corresponding ligands of these two receptors, FasL (CD95L) and TNFα, are structurally related proteins that belong to the TNF subfamily (186). Though the Fas-system may play a role in maintaining the immune privilege status of the testis, it also plays a role in regulating germ cell apoptosis in the testis (187). FasL has been found in mouse, rat, and human Sertoli cells. Some reports have also shown that FasL is expressed in germ cells. In general, it is assumed to be constitutively expressed by Sertoli cells (184,188-190). Fas expression, in turn, has been demonstrated in germ cells of rat and human testes, and also in Sertoli cells (184, 187, 189). After exposure of rat testis to Sertoli cell toxicants, expressions of both Fas and FasL are upregulated concomitantly with increased germ cell apoptosis. Furthermore, when expression of FasL is blocked by FasL antisense oligonucleotide treatment a decrease in germ cell apoptosis in the co-cultures of Sertoli-germ cells *in vitro* was demonstrated (187). These imply that the Fas system mediates testicular germ cell apoptosis and Fas up-regulation takes place at the initiation of male germ cell death. In addition, a variety of other mediators associated with the cytoplasmic domain of Fas such as FADD have been recently identified in testis (189-192). Radiation induced injury or testicular hyperthermia, which both directly affect germ cells, results in an increased expression of Fas mRNA, but not FasL gene expression (99). The increase in germ cell apoptosis following androgen withdrawal by EDS has also been temporally correlated with changes in Fas protein expression in germ cells. Fas protein expression increases after androgen suppression, but before the onset of germ cell apoptosis. If EDS and testosterone are administered simultaneously, apoptosis is suppressed, and testicular Fas levels remain unchanged (193). Administration of mono-(2-ethylhexyl) phthalate and 2,5-hexanedione, two widely used Sertoli cell toxicants, resulted in up-regulated expression of both FasL and Fas. Thus, if Sertoli cells are injured, Sertoli cells may up-regulate FasL to eliminate Fas-positive germ cells, however, this hypothesis can not be supported adequately (99). Though only a few testicular toxicants are clearly assigned to a target cell (194,195), Lee *et al* have suggested that differential up-regulation kinetics

of the Fas system may be useful in predicting cell type-specific toxicity of toxicants (99). Up-regulation of FasL gene after testicular exposure may be a novel useful marker to identify Sertoli cell toxicant. If a toxicant induces up-regulation of FasL, followed by massive germ cell loss, it is likely that the toxicant alters function of the Sertoli cell (99). Testicular hyperthermia is one of these exceptions. Using the FasL-defective gld mice, it has been shown that germ cells of wild-type and gld mice are equally sensitive to testicular hyperthermia-induced germ cell apoptosis. These results suggest that the Fas signaling system may be dispensable for heat-induced germ cell apoptosis in the testis. It is possible that other members of the tumor necrosis factor (TNF) family, such as TNF or TNF-related apoptosis-inducing ligand, may bind to Fas receptor and induce apoptosis (155, 196-198). TNF is expressed by spermatocytes and spermatids, with its receptor found in Sertoli cells (199). Upon induction of cell apoptosis, the increased FasL expression could be decreased in the presence of TNF, indicating that TNF may regulate Fas-mediated germ cell apoptosis (28). While death receptors are able to activate apoptosis through many unique signaling components, they often converge on downstream caspases and other substrates similar to those activated by the mitochondrial and possibly ER pathways (28). Evidence suggests that the different pathways may also exhibit some degree of crosstalk, such as is the case with Fas-mediated caspase cleavage of Bid, which can then elicit cytochrome c release from the mitochondria (28,199).

7. MALE CONTRACEPTION: STRATEGY AND PROSPECT

7.1. Gossypol: an anti-sperm agent

Gossypol had been showed to be an effective anti-fertile agent in men. Supported by grants from World Health Organization and Rockefeller Foundation, systematical studies by Chinese researchers from 14 provinces or cities were carried out and over 10,000 men as volunteers used the drug for clinical trial (200-202). The statistical analysis from 8806 cases showed that the men became infertile in 75 days if the pills of 20 mg gossypol were orally taken every day and the validity approached to 99.9%. However, with longer period of the drug administration (up to 6 years), following increase of the drug in the body, variation of individual reaction to the drug as an incidence of the donors appeared two kind of side-effects, hypokalemic paralysis (0.75%) and irreversible infertility (9.9%) (200, 201). However, further experiments by Ye *et al* showed that low dose gossypol in combination with testosterone did not damage the testicular and other viscera function (203). The validity of anti-fertility of such combination attained 100% (203, 204). Therefore, an international collaboration group further evaluated gossypol as a contraceptive pill for men at lower doses. Total of 151 men from 4 countries were received with 15 mg gossypol daily for 12 or 16 weeks to reach spermatogenesis suppression, the subjects were then randomized to either receiving 7.5 or 10 mg /day for the maintains. All the subjects attained spermatogenesis suppression, the potassium levels fluctuated within the normal range, no obvious side-effect on sexual desire and frequency of intercourse could be observed (205). A similar clinical study with a changed regimen on 77 male volunteers in China could induce infertility without developing hypokalemia and irreversibility. The serum levels of potassium, FSH, LH and T in the treated subjects were not significantly changed as compared with the control subjects (206). It is therefore suggested that a regimen with 10 to 12.5 mg of gossypol as the daily loading and 5-6.25 mg as daily maintenance dose could induce infertility in men without developing hypokalemia or irreversibility (207,208). Reports *in vivo* and *in vitro* also demonstrated that gossypol could dose-dependently inhibiting sperm motility by blocking sperm cAMP formation (209). These data suggest that gossypol may be also used by females as an effective antifertility agent for vaginal contraceptive. On the basis of the recent conclusive reports, the early reported hypokalewmia of gossypol has been not confirmed in the latest trials with the effective lower doses. Professor Xue has published a review paper (210) and suggested a low dose of gossypol in combination with the steroid may be hopeful as a prospective male contraceptive.

7.2. Testosterone: a male contraceptive agent

At the beginning of 1990s Chinese scientists with international cooperative groups supported by World Health Organization succeeded in making use of high dose of testosterone undecanoate (TU) as male contraceptive agent to block spermatogenesis, and has been applied clinically (211,212). High doses of exogenous T were thought to be capable of suppressing release of gonadotrophins from the pituitary with a resultant deprivation of intra-testicular androgen, and spermatogenesis would be finally blocked. Our further experiment on rhesus monkeys clearly showed that germ cells began to apoptosis on day 7 after TU injection and the apoptotic germ cells reached a peak level on day 30, the sperm account in the semen reduced to zero in 7-8 weeks. More importantly, we demonstrated that the treatment would be reversible, the sperm account and the changes in all the molecules induced by exogenous testosterone return to the normal levels 3 month after stopping TU injection.

7.3. Testis warming in combination with testosterone or gossypol for developing male contraception

Our experiments with local warming monkey testes at 43 degrees centigrade water for consecutive subsequent two days (30 minutes per day) showed that the sperm amount in the semen decreased 80% as compared with the untreated control at 28 days after the two day' warming treatment. Furthermore, the "heat" in combination with testosterone implant, the sperm account reduced to zero in 2 month time. Administration of exogenous testosterone can reduce secretion of intratesticular androgen level, as a consequence, inhibiting sperm output. Withdrawing the T implant, the density of semen sperms recovered to the normal levels after 2-3 months. All the changes in the regulated molecules in the testes completely restored to the normal expression levels. These findings provide an important theoretical basis for designing combined male contraceptives (Figure 10

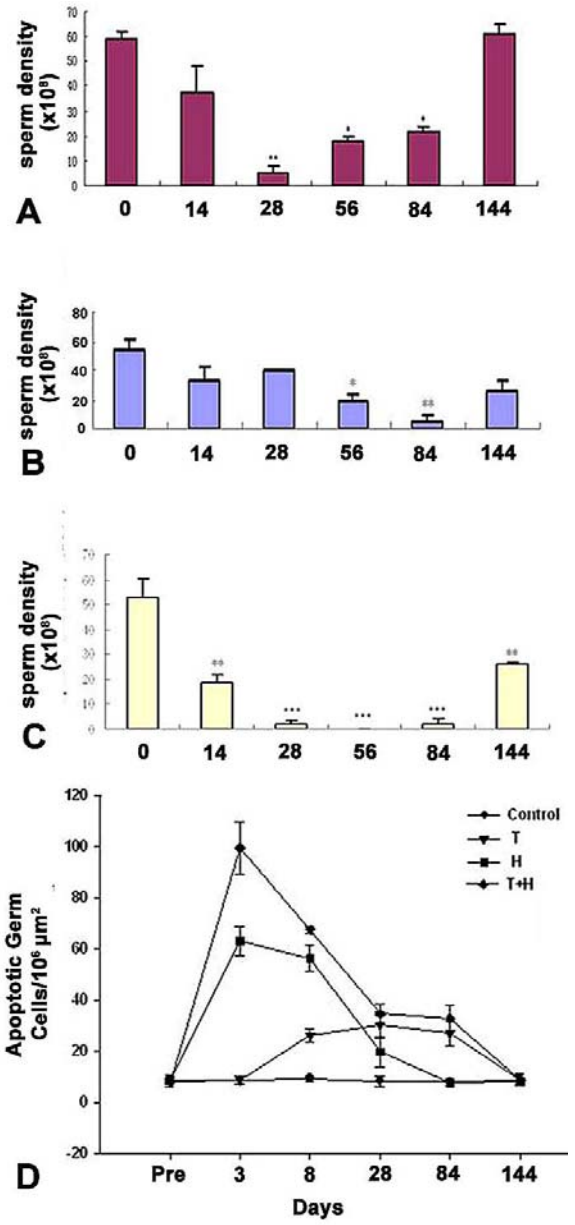

Figure 10. Effect of local 43°C warming testes (H), testosterone implant (T) and in combination(T+H) on monkey sperm density (A,B,C) and germ cell apoptosis (D) on the day before (0) and days 14 (D14), 28 (D28), 56 (D56), 84 (D84) and 144 (D144) after the heat treatment. Data are presented as mean±SEM (n=3). Value with * is significantly different ($P<0.05$) or ** ($P<0.01$). Germ cell apoptosis in heat-treated monkeys recovered to pretreated level on day 144 after T withdrawal on day 84. The apoptotic index is expressed as number of apoptotic germ cells per versibility was further substantiated by the histologic data. Reproduced with permission from references 3, 94 and 98.

A single testicular heat exposure could enhance the effect of testosterone-induced germ cell apoptosis. An additive and/or a synergistic effect of the two distinct stimuli on germ cell apoptosis at various stages could be observed.

Low dose of gossypol, in addition to acting on the testicular spermatids, mainly influences the sperm activity and the sperm structures in the epididymis. In combination with local testicular warming an additive effect on reducing germ cell function has been demonstrated (unpublished data). Based on the evidences available and acceptable, several combination regimens for male contraception would be strongly suggested for further clinical investigation: (1) Wearing specially designed 43 degree centigrade undershort in combination with testosterone implant; 2) Wearing the undershort in combination with low dose of gossypol (5-6 mg/day); T, gossypol and "heat stress" in combination act respectively on different sites and stages of spermatogenesis in testis or sperm activity in epiddymis. Apoptosis induced by testosterone occurs mainly at stages 7-8 and the "heat stress" induces germ cell apoptosis mostly at stages 1-4 and 10-12 (Figure 2). The combination actions of "heat", T or gossypol should meet the standard for developing new male contraceptives.

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Abbreviations AE: Acrosomal Exocytosis;AR: Acrosomal Reaction; BoNT/E LC: Botulinum Neurotoxin E, light chain;ZP: Zona pellucid; ABP: androgen-binding protein; AJ: adherens, junctions;AR:androgen receptor; BTB: blood-testis barrier; CK-18: cytokeratin-18; ERK:extracellular-signal regulated kinase; ES:ectoplasmic specialization; FSH:follicle stimulating hormone;FSHR: follicle stimulating hormone receptor;GJ:gap junction;HSP: heat shock protein; LRH-1:liver receptor homologue 1;MAPK:mitogen-activated protein kinase;NO:Nitric oxide;NOS:Nitricoxide synthase; PI3K: phosphatidylinositol 3-kinase;T: testosterone; TGF-β:transforming growth, factorβ;TJ: tight junction;WT1: Wilms' tumour gene 1; ZO-1: zonula occluden 1

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