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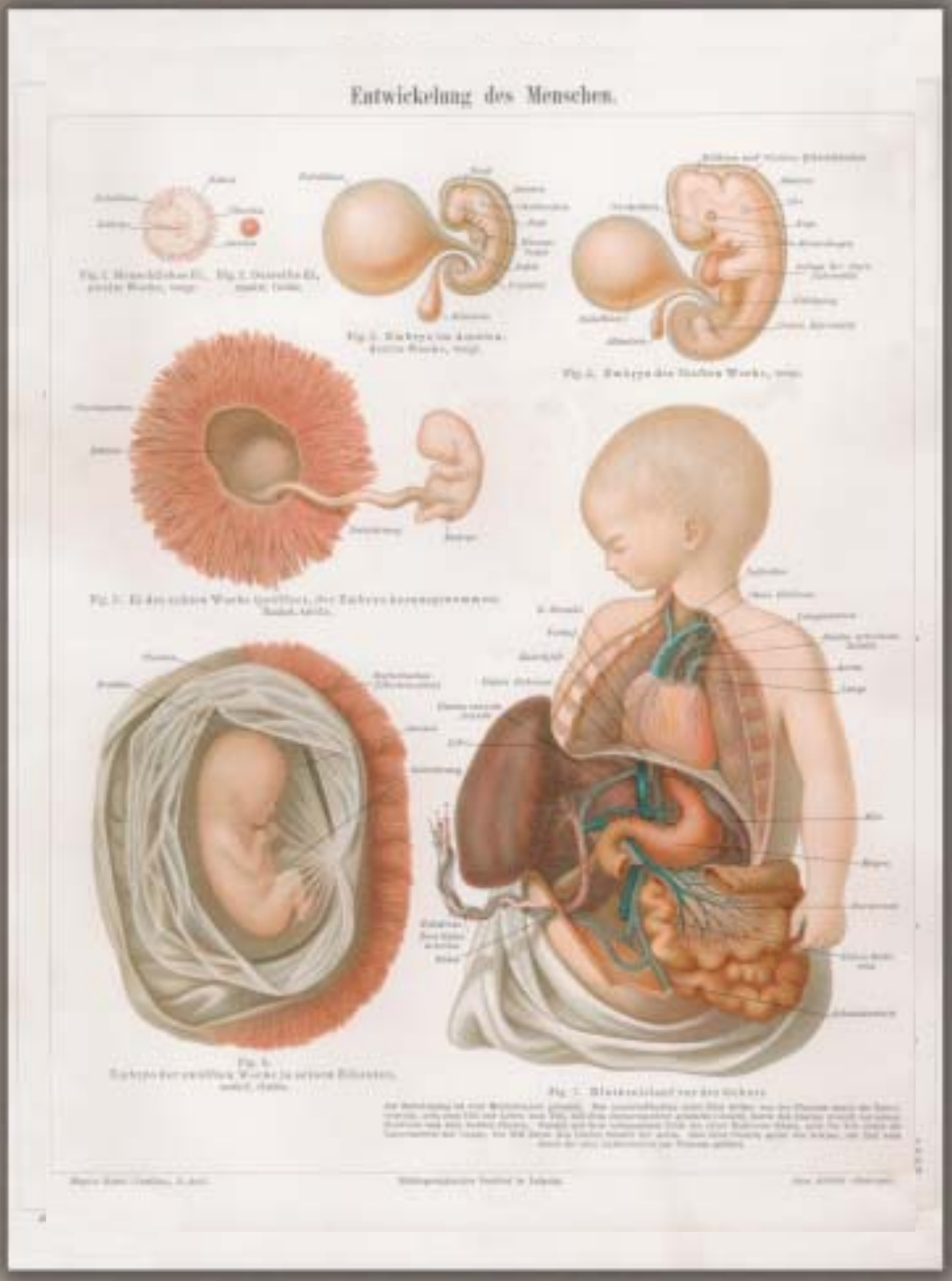

Main Feature Articles

CONTENTS

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EDITORIAL

This edition of A.R.T. & Science continues to cover a wide range of important issues that should be of interest to anyone working in the field of assisted reproduction. We are grateful to all the contributors that have done a great job in sharing their expertise in a manner that can be read by all.

The virtues of cryopreservation are well known, whether it be gametes or embryos that are stored at low temperatures for future use. However, our first author, John Morris, looks at the possible risks of contamination of stored material and ways in which this might be minimized. This aspect of our work will become of increasing concern as cryopreservation becomes routine and we begin to see possible adverse effects in addition to the benefits. Hopefully, measures can be introduced to prevent the transmission of infectious disease, such as that which happened with the case of hepatitis B transmission in the UK during the storage of frozen blood.

The enforcement of minimum standards is becoming the role of legislation rather than reliance upon the good will of clinicians and scientists, and it can be argued that this is a good thing for the protection of both ourselves and society. Stephen Junk summarises the new federal legislation that is coming into force in Australia that will prohibit cloning and lay down clear rules for research involving human embryos. This is an interesting development as the Federal legislation sits alongside laws already in place in several Australian States and will ultimately prevail should there be a conflict between the State and Federal laws.

Samer Ghunaim then takes a look at his experience with the performance of ICSI using round spermatids in cases of azoospermia. Gamete micromanipulation has enabled a large number of men around the world to achieve pregnancies that would otherwise not have occurred. Nevertheless, Samer's article serves to remind us that such techniques are not suitable to all kinds of problems and that the use of immature sperm cells is likely to yield very few pregnancies, particularly for those men with some form of maturational arrest present in the testes.

Whilst many of our efforts are directed towards the achievement of pregnancies for infertile or subfertile individuals, there are many more people in the world that are trying to prevent unwanted pregnancies. This becomes a natural extension of family planning, and John Amory tells of the options available to men. This is an increasingly important development that will remove some of the burden of contraception from women, and help us make serious efforts to stop the huge increase in world-wide population.

Our final author, Tracey Palmer, revisits some old texts and has a look at life in the early twentieth century. The profile of disease seen then and the attitudes of the day are real eye-openers, and it makes the huge contributions of people like Dr Max H_hner all the more remarkable.

Cover illustration:

"Original chromolithograph taken from the publications of the Bibliographisches Institut, Leipzig. These were published in Leipzig in 1884."

Cryopreservation and Contamination

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Storage at low temperatures, cryopreservation, plays a central role in IVF. Gametes, embryos and gonadal tissue are cryopreserved on the basis that cells will remain viable and unchanged during long term storage at the temperature of liquid nitrogen (-196°C). This has been substantially verified over many years, for example freezing to and thawing from liquid nitrogen is known not to be mutagenic (Ashwood-Smith and Grant, 1977). Further, although there is the potential of radiation damage to cells which could accumulate during storage and be expressed on thawing, it has been shown that with the current levels of background radiation significant effects would occur only after extremely long periods. Exposure to the equivalent of 2,000 years of background irradiation did not induce any obvious mutation in mouse embryos (Glenister *et al.,* 1984). The viability of cells, stored at liquid nitrogen temperatures, is now well known to be independent of the period of storage. Cryopreservation would appear to be ideal for the long term storage of cells. However, it has become apparent that contamination of samples may occur during the storage of cells in liquid nitrogen (Teddar *et al.,* 1995, Fountain et al., 1997) which is obviously undesirable. The two main potential sources of contamination are firstly other cryopreserved samples stored within the same container, and secondly the liquid nitrogen itself. This article briefly reviews the issues relating to these two aspects of potential contamination.

Screening of Samples

The first line of defence in reducing potential contamination is the effective screening of samples. In the UK semen donors are screened for infective agents, their semen is stored for 180 days and then may be used for treatment only after a further HIV test. Unfortunately, in many instances frozen semen from several donors may share the same liquid nitrogen Dewar during the "quarantine" period and samples released for use at intervals during this period. This means that semen from an uninfected donor could be stored with infected semen, and subsequently used for treatment before the infected samples are identified. With respect to donor embryos, the embryo must have been stored for 180 days before it can be donated and both people whose gametes were used to produce it must be shown to be HIV negative. However, again, the embryo may have been stored with embryos from unscreened donors.

There is however no requirement for clinics to screen semen, embryos or ovarian and testicular biopsies for their own use in fertility treatment, before oncology therapy or for other reasons, eg pre-vasectomy. Some clinics screen fertility patients as part of the process of assessing the welfare of any child born and to protect staff. Other clinics do not, and argue that routine screening is too costly, when only the minority of patients eventually store semen or embryos.

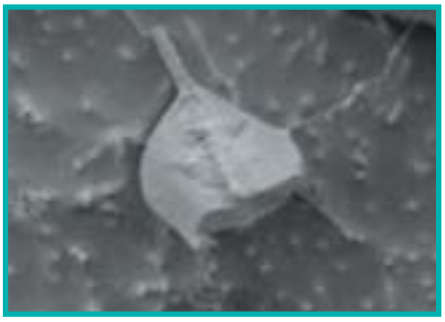
Cross Contamination of Samples

There has been a clear demonstration of the transmission of Hepatitis B between frozen bone marrow samples in a liquid nitrogen storage tank (Teddar *et al.,* 1995; Fountain *et al.,* 1997), which resulted in clinical infection on transplantation into patients. This incident caused major concern for medical cryobiology, especially in the use of materials which are transfused (blood, bone marrow) or transplanted (cornea). In the UK strict guidelines for cryopreservation of bone marrow and stem cells are now in place (Department of Health, 1997). There has been much discussion in the field of IVF about implementation of similar guidelines. These concerns have been highlighted by the experimental demonstration that when liquid nitrogen is contaminated with a virus then embryos stored in unsealed containers (cryovials, standard IMV straws, open pulled straws) in this nitrogen can become contaminated with these viruses (Bielanski, *et al.,* 2000).

Cross contamination of IVF samples may arise at various steps during cryopreservation (Wood, 1999) as discussed below:

1. Straws.

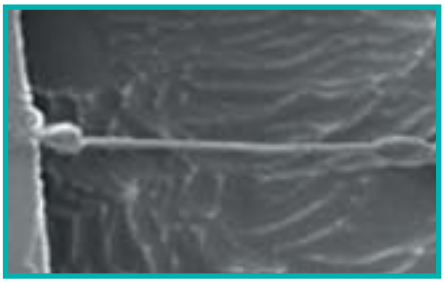
In an early study in the UK (Royal College of Pathologists, 1995) straws were described as being "microbiologically hazardous". Straws may be contaminated on the outside during filling by aspiration allowing particulates to transfer via the liquid nitrogen within the storage vessel. It



is advisable for asceptic filling procedures to be implemented for all applications using straws. The traditional way of sealing straws using PVA powder or straw plugs does not effectively seal straws. It has been suggested that effective containment of straws would be achieved by "double bagging", in which the filled straw would be placed in a clear plastic bag which can be effectively sealed, isolating the straw. Whilst this approach may isolate the straw, it makes controlled ice nucleation problematic and has not been taken up widely in IVF. IMV have developed a system, which claims to seal straws effectively and not allow ingress of liquid nitrogen. Importantly for the cryopreservation of embryos and oocytes the ice nucleation is not compromised.

2. Ampoules.

Because of the problems associated with straws it has been suggested that plastic ampoules might be used more widely in IVF. Whilst it is relatively common to cryopreserve semen in cryovials it has been reported that the viability of embryos cryopreserved in ampoules is reduced (reviewed in Wood, 1999). However this observation is complicated by the fact that the same cooling protocols were used in the comparison whereas it would be expected that the thermal histories experienced by the cells in the straws and ampoules would then be different causing differences in cell recovery on thawing. It is of course possible to redefine the cooling protocol to achieve the desired thermal history within the plastic ampoule.



3. Controlled Rate Freezing Equipment.

Vapour phase controlled rate freezers spray non-sterile, liquid nitrogen directly onto the samples (see below). Any direct contamination may be further compounded by liquid condensate accumulating within ducting between freezing runs, becoming contaminated by microbes and then blown onto samples. Ideally, freezing equipment should have the capability of being sterilised between freezing runs.

4. Vitrification.

As currently employed vitrification of samples requires rapid rates of cooling to be effective. Such rapid rates are achieved by the direct exposure of samples into liquid nitrogen for example loop techniques, microdrops, open pulled straws. In all these procedures it is difficult to see how contamination between samples may be contained. In addition, the samples are in direct contact with liquid nitrogen which itself carries risk (see below).

5. Vapour phase storage.

Immersion of samples in liquid nitrogen guarantees a stable storage temperature but provides a direct pathway for cross contamination. Storage of samples in the vapour phase above liquid nitrogen may reduce the risk although it is known that contaminants may circulate in the vapour (Fountain *et al.,* 1977); gas phase storage is microbiologically safer, but it is not microbiologically safe. However a major disadvantage of vapour phase storage systems is that vertical temperature gradients of 100ºC have been reported (Rowley and Byrne, 1992). The relatively high temperatures encountered by samples stored in the vapour phase together with temperature cycling would be expected to result in a reduction in viability with time of storage. Some simple mechanisms for reducing the size of the temperature gradient have been suggested (Pegg & Hunt 1996). An alternative solution would be to avoid liquid nitrogen and store cells in mechanical refrigerators. Conventional ultra low temperature refrigerators operate at temperatures down to –130ºC; various claims are made about the long-term stability of cells in such systems based on the fact that they operate below the "glass transition temperature of water". However recrystallisation of ice may occur at these temperatures leading to a reduction in viability. New mechanical systems capable of operating at temperatures close to liquid nitrogen are now becoming available.

Contamination Arising from Liquid Nitrogen

It is commonly assumed that liquid nitrogen is sterile and indeed when manufactured it usually has a very low microbial count. The microbial quality of the delivered liquid nitrogen varies widely with geographical region and some of the more extreme reports of microbial contamination reflect local industrial practices. However any contaminant (virus, bacteria, fungal spore etc) entering the liquid nitrogen will effectively be cryopreserved.

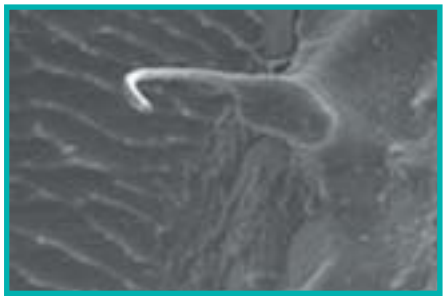
Sources of contamination.

In Dewars open to the air the flow of gas is from the liquid nitrogen to the air i.e. at a slight positive pressure and it would not be expected that contaminants would directly enter the nitrogen. However, the formation of ice crystals in the air above the Dewar – visible as white cloud - may entrap air borne microbes and these ice crystals may fall into the open vessel. Removal of storage systems into the air either for sample removal or for routine checking of inventory may also lead to deposition of microbes from the air. If containers or inventory systems become iced during these operations this indicates that water vapour from the air is

condensing onto cold surfaces, inevitably bringing air borne microbes. Contamination is also introduced on the outside of straws, ampoules etc, carried on the outside of forceps used to remove samples from storage, and indeed may be present on the inside of new Dewars. In addition, serious problems may occur with any portion of the cold chain which periodically warms up. In particular "transfer" Dewars, which are allowed to warm up may accumulate pools of condensate which become heavily contaminated. In the most extreme of cases insects and rodents have been known to fall into empty Dewars. When re-filled with liquid nitrogen this microbial "soup" is effectively cryopreseved and then deposited onto samples. The addition of contaminated liquid nitrogen to top up the vessel or the introduction of contamination from other sources as indicated above will lead to increased contamination in the vessel with time because when the liquid nitrogen evaporates only gas is lost. This can reach unacceptable levels even though the microbes are cryopreserved and are not growing. A typical nitrogen Dewar topped up regularly with contaminated liquid nitrogen would be expected to increase its microbial load by a factor of 100 within 10 years. Plating out of nitrogen from storage Dewars has revealed very large numbers of bacteria: the micro-organisms isolated were the same as those isolated from contaminated cryopreserved samples (Fountain *et al.,* 1997). A related problem is that storage vessels which have been maintained for several years would be expected to contain a high level of contamination which may be a risk to operators. This aspect has generally been overlooked and should be included in the development of safety policy for laboratories.

Reducing contamination.

To reduce the microbial contamination carried within liquid nitrogen, filtration has been suggested. For some specialist pharmaceutical applications, liquid



nitrogen is filtered using special filters and housings capable of operating at temperatures of -196ºC. The use of general laboratory filters to filter liquid nitrogen will not be effective and will certainly be dangerous. In those storage systems which are automatically filled it would be possible to limit the contamination from the liquid nitrogen by placing a specialist filter into the pressurised filling line. In those systems which are manually filled the use of similarly filtered nitrogen would also be effective. Sterilisation of nitrogen Dewars has been proposed but this is difficult. Many of the available disinfectants cause severe corrosion problems with aluminium Dewars, which may result in a catastrophic loss of vacuum. Also it is not appropriate to autoclave Dewars: it is not the metal or the metal welds which are the problem but rather any joints in the neck region of the Dewars which are constructed from materials such as fibre glass. Autoclaving a vessel may also negate any warranty on the vessel as currently constructed. The contamination levels within contaminated storage Dewars and transfer Dewars may however be reduced by allowing the Dewars to warm, carefully disposing of any accumulated liquid, then washing with alcohol and drying.

Conclusions

The potential for contamination can occur at several stages in the cryopreservation process. In many applications for example the cryopreservation of cells (bone marrow, blood) and tissues (cornea) for transfusion and the preparation of drugs for injection it is essential that contamination does not occur. In these applications extensive steps are taken to ensure that contamination is minimised. Should this apply to all IVF applications?

Known infectious agents are best dealt with by screening and effective quarantine. General non-specific contamination is more problematical, for example some IVF samples are not bacteria free; it has been reported that it is possible to isolate bacteria in 87% of sperm ejaculates and that there is no negative influence of bacteria on spermatozoa during ART (reviewed in http://WWW.ferti.net/fertimagazine/hotopic/2001_05_01.asp). The situation with gonadal tissue for transplantation would be different; in this case microbial contamination must be avoided completely.

Much could be done to reduce contamination by simple improvement in procedures and many such changes should be implemented in IVF laboratories as a matter of "best practice". It would be appropriate to take more rigorous precautions with material for transplantation than for sperm for ART. ❑

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New Federal Laws in Australia Regarding Human Cloning And The Use Of Excess Human Embryos

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In response to community and ethical concerns about scientific developments in assisted reproductive technology (ART) and the use of embryos, the Council of Australian Governments (COAG) agreed to create nationally consistent legislation to ban human cloning and to regulate research and the use of excess ART embryos. Concerns have also been raised by the scientific community regarding human cloning and the creation of human embryos for commercial research purposes. As a result, two Acts relating to human embryos and ART were passed in December 2002. These are the *Prohibition of Human Cloning Act 2002* and the *Research Involving Human Embryos Act 2002*. The legislation bans certain unacceptable practices including human cloning and regulates the use of excess human embryos generated from ART. Only institutions operating under a license may undertake research, training or quality assurance schemes using excess human embryos.

Australian coat of arms.

Prohibition of Human Cloning Act 2002

There are a number of practices that are prohibited under the Act. In particular, the creation, implantation in a woman, or import or export of:

- a human embryo clone;
- a human embryo that has been created other than by the fertilisation of a human egg by human sperm;
- a human embryo created outside the body of a woman, unless the intention of the person who created the embryo was to attempt to achieve a pregnancy in a particular woman;
- a human embryo that contains genetic material provided by more than two persons;
- a human embryo that has been maintained outside the body of a woman for a period of more than 14 days, excluding any period when development is suspended;
- a human embryo that contains a human cell whose genome has been altered in such a way that the alteration is heritable by descendants of the human whose cell was altered;
- a human embryo removed from the body of a woman by a person intending to collect a viable human embryo; and
- a chimeric embryo or a hybrid embryo.

Parliament House, Canberra.

Additionally, it is an offence for payment of any kind to be made to persons supplying human gametes or embryos (ie: donors), apart from that considered reasonable reimbursement for expenses made in connection with the donation of the human gametes or embryos.

The *Prohibition of Human Cloning Act 2002* is likely to be well received by both the lay community and the scientific community. However, the area of research that will be most adversely affected is that involving *Cytoplasmic Transfer*. Though this technique is still in its research and development phase, it potentially offers benefits in cases where there is a mitochondrial DNA problem in the

oocyte, such as Leigh's disease and cases where it is thought that there are metabolic defects in the mitochondrial DNA. This research will be effectively banned as the technique would result in the embryo having genetic material from more than two parents.

Research Involving Human Embryos Act 2002

Under the Act, an excess ART embryo is defined as "a human embryo created *in vitro* for the purposes of attempting to achieve a pregnancy in a particular woman, that has been determined, in writing, by that woman (and her spouse) as being excess to their needs" [Section 9, (1) and (2)]. It is now an offence to use an excess ART embryo unless the use is authorised by a licence issued by the National Health and Medical Research Council (NHMRC), or the use is specifically exempt. These uses are:

- storage and removal from storage;
- transport;
- observation, which may include photography or video recording;
- allowing to succumb;
- diagnostic by an ART clinic where the embryo is biologically unfit for implantation, in order to directly benefit the woman for whom the embryo was created; and
- use in the ART treatment of a woman other than the woman for whom the embryo was created (embryo donation).

All other uses of excess ART embryos require a NHMRC licence. Though the Act was designed to regulate research, it also regulates the usage of excess human embryos for all other purposes. These uses would include training of personnel in embryo handling techniques and quality assurance programmes. To obtain a licence for all of these purposes, the proposal must initially be submitted and approved by the Institutional Human Research Ethics Committee. It is then submitted to the NHMRC licensing committee for consideration. The committee consists of nine members appointed by the Minister and have expertise relating to ethics, ART, law, consumer needs and embryology.

In deciding whether to issue a licence, the NHMRC licensing committee must consider the following:

- minimising the number of excess ART embryos that will be required to achieve the goals of the project;
- whether there would be a significant advancement in knowledge or improvement in technologies for treatment as a result of the use of excess ART embryos. This would include those embryos required for training purposes;
- if the use of an excess ART embryo involves the damage or destruction of an embryo, then only embryos created before 5 April 2002 may be used.

A uniform Australian law governing the use of human embryos is to be welcomed. The precise nature that it is likely to impact ART institutions depends largely on the state of Australia where the institution resides. South Australia, Victoria and Western Australia already have strict laws regarding the use of excess human embryos. Therefore, the new federal laws, that are intended to over-ride the existing state laws, are likely to allow research and usage of human embryos for purposes that were previously banned. The other states and territories have until now only ethical guidelines to follow. The new federal laws are therefore likely to place regulations and bans on the use of human embryos that were previously carried out in these institutions.

Sunset clause

Licence holders may only use excess human embryos that were created prior to 5 April 2002. However, this regulation for usage only applies until 5 April 2005 or if the COAG declares an earlier date.

Penalties

Offences involving the Research Involving Human Embryos Act 2002 carry a maximum of five years imprisonment, while the Prohibition of Human Cloning Act 2002 carries a maximum penalty of 15 years relating to human cloning and 10 years for other offences. ❑

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ICSI with round spermatids to by-pass azoospermia has major limitations

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The micro-injection of spermatids

Since the introduction of intracytoplasmic sperm injection (ICSI) in the treatment of male infertility, the ICSI technique has been developed to give high rates of fertilization, pregnancies and deliveries. Moreover, as a result of combining intracytoplasmic injection (ICSI) with surgical epididmal sperm aspiration (MESA) or testicular sperm extraction (TESE), men with obstructive azoospermia (Schoysman et al., 1993) and non-obstructive azoospermia had the chance to father their own children. However, in some cases no spermatozoa could be found in different testicular samples at the time of TESE-ICSI attempt due to a range of reasons, eg defective spermatogenesis as a result of spermatogenic arrest, undesended testicles, Kallman's syndrome, sertoli cell only syndrome, post pubertal tubular atrophy, Kleinfelter's syndrome, and damage caused by chemotherapy. To overcome such a situation, Edwards (1994) recommended the injection of spermatids into oocytes as an alternative and more direct approach to unite the two gametes containing haploid genetic material.

Spermatids are immature haploid germ cells that have not yet undergone complete biochemical and morphological changes that accompany spermiogenesis through the formation of spermatozoa. Encouraging results were obtained using spermatids in rodents, with sygnamy and embryo development and the formation of healthy fertile young after embryo transfer. This perspective encouraged some in-vitro fertilization (IVF) centers to inject spermatids into mature oocytes and viable embryos were obtained. These trials, termed ROSI (round spermatid injection) or ELSI (elongated spermatid injection), were reported to have produced a limited number of pregnancies in humans. Applying ICSI of spermatids as a method of treating male infertility needs to be evaluated for factors affecting the outcome of fertilization and conception, such as the severity of pathology, the type of injected spermatids, the successful identification of spermatids, viability and deficiency in the activation process of oocytes.

Morphological Classification

Spermatids are classified into 3 groups depending upon the stage of maturity, namely being either (i) round, (ii) elongating, or (iii) elongated. Round spermatids are distinguished from other round cells (lymphocytes, monocytes, spermatocytes, spermatogonia, polymorphonuclear leukocytes) by their round shape with smooth outline, size of 7.8 mm diameter, central rounded thickening of nucleus and by the developing acrosomal structure .

Our clinical experience

Fine needle biopsy was undertaken in 164 men and they were categorized on a previous biopsy or on the fine needle aspirate as:

- Focal sperm found in tissue (19 men)
- No sperm found, suggesting maturational arrest (74 men)
- Sertoli cells only (39 men)
- No germ cells of Sertoli cells (7 men)
- Non-obstructive azoospermia with no previous biopsy (25 men).

An open testicular biopsy was performed under general anaesthesia on the day of oocyte retrieval, tissue being taken from 9 different sites of each testis. The tissue samples for each man were crushed and minced between two sterile glass slides and the resulting suspension centrifuged through a discontinuous Percoll gradient. After washing, the pellet was resuspended in medium, the droplet spread out on a culture dish and then overlaid with mineral oil. After a 30 minute incubation, a meticulous search was made for sperm and spermatids using an inverted microscope (x200 and x400 magnification) with Hoffman optics. No sperm or elongating spermatids were found in this series of men, and only round spermatids could be isolated. ICSI was done with these round spermatids. The overall experience is summarized in Table 1.

Table 1. Clinical experience

Parameter	*Number of*
Patients	164
Injected oocytes with 2pn	198/662 (30%)
Injected oocytes with 1pn	96/662 (15%)
Embryos dividing	232
Good quality embryos	153
Embryo transfers	122
Pregnancies	0

There was no difference in the fertilization rate of any of the groups of men, and no pregnancies were recorded among the 164 couples included in this study.

The clinical value of ROSI

The aim of this article was to explore the effectiveness of ROSI in men with non-obstructive azoospermia, where no elongating/elongated spermatids were detected in the testicular tissue at the time of egg recovery. Our experience included 164 patients where no pregnancies had previously been observed. Our data, which represents one of the largest series reported to date, casts considerable doubt on the value of ROSI in the achievement of viable pregnancies. These results should stimulate both clinician and scientists to think more critically before adopting ROSI in its current format, and encourage the identification of methods to improve the chances of success.

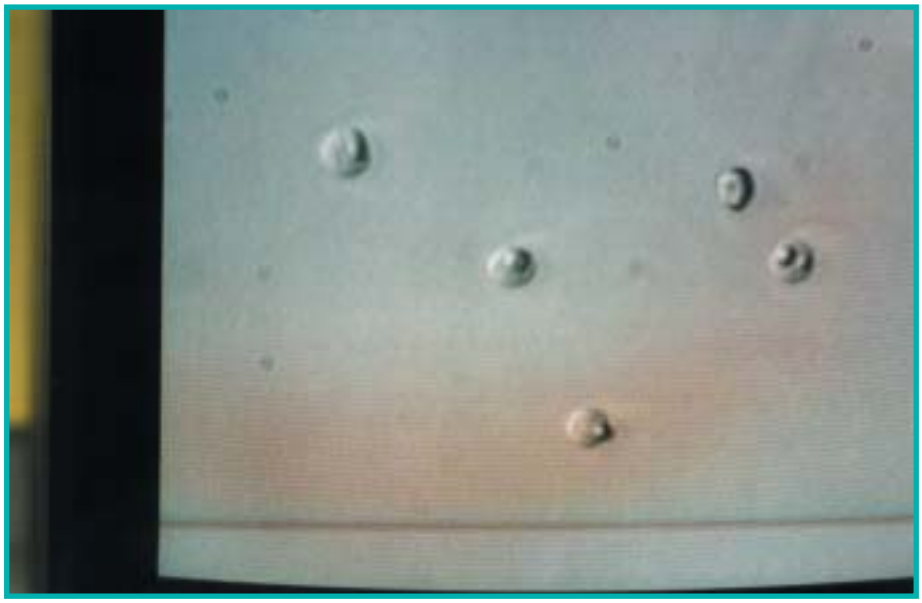

Fig. 1. Round spermatids can be distinguished by their round shape and smaller size than other round cells, as well as a central thickening around the nucleus. A developing acrosomal structure can also be seen as a bright spot on one side of the cell.

There is considerable debate surrounding the clinical need for ROSI in the treatment of non-obstructive azoospermia. Some authors clearly see no need for treatment, arguing that elongating /elongated spermatids can be observed if a thorough and complete search is performed (Silber *et al.,* 2000). On the other hand, studies that have used ROSI failed to observe more mature cells and thus injected round spermatids (Vanderzwalmen *et al.,* 1997; Sousa et al., 1999). These are not necessarily mutually exclusive situations, and could represent different ends of a continuum as well as different underlying pathology. In our study, some of the cases had spermatozoa and spermatids seen in a previous biopsy or FNA, but no spermatozoa or elongating /elongated sperm were found on the day of oocyte retrieval despite extensive searching. During the same 12 month period, we treated a further 338 cases of non-obstructive azoospermia where testicular spermatozoa or elongated/elongating spermatids were found and injected. Thus, in our experience, men with complete spermiogenesis failure represent a significant proportion (32%) of men with non-obstructive azoospermia and, importantly, a subset of patients with currently no effective treatment using their own gametes if available.

The fertilization rate in our study of 35%, as shown in Table 1, is comparable to that of other workers using testicular round spermatids (22%: Ghazzawi *et al.* 1999; 48%: Sousa *et al.,* 1999). Interestingly, in contrast to the study of Ghazzawi *et al* (1999) where all embryos were classified as poor, over 60% of the embryos in our series were graded as good at the time of embryo transfer on Day 2. Based on this, we would have expected some pregnancies. However, particularly in cases of sever male factor infertility, the formation of good quality 2 days old embryos may not accurately reflect the functional competence of these embryos i.e. their ability to develop into blastocysts. In our study, we didn't culture embryos to blastocyst stage and so we don't know if these embryos were capable of developing further. Given the critical importance that the male genome plays in embryo development, and the likelihood that the round spermatids would have significant levels of DNA fragmentation, it is possible that many of these embryos wouldn't have developed to the blastocyst stage. This interesting hypothesis warrants further investigations. When life births have been reported in the literature following the use of round spermatids from men with complete spermiogenesis failure, it means that there were no spermatozoa or elongated/elongating spermatozoa found. In contrast, live births have been obtained when ROSI has been used in men were they have incomplete spermoigenesis failure. We can only speculate as to why no pregnancies were observed in our study; one explanation is that we did not correctly identify spermatids and thus were not injecting haploid cells. But this is very unlikely as we took great care to identify these cells, and we have considerable experience in examining a large number of testicular biopsies in both clinical and research environment (Dajani & Kilani, 1998). In addition to correct identification, we ensured that the cells injected were available by aspiration and releasing the cells and observing a return to the original shape. Our primary explanation of why no pregnancies developed is that the spermatids form these men are physiological defective.

Why don't round spermatids from men with complete spermiogenesis failure form functional pregnancies? Tesarik and colleagues showed that round spermatids form this group of men have very significant levels of apoptotic DNA damage (Tesarik *et al.,* 1998). Those high levels of DNA damage, at least in ejaculated cells, are strongly associated with poor fertilization success and subsequent embryo development. It is very likely, though not proven , that the spermatids would have a very poor developmental potential and implantation rates because of their high level of DNA damage. In addition to the damage in their DNA, it is possible that the round spermatids have many other physiological defects , which are not currently obvious , for example , they maybe unable to induce appropriate activations of the egg. Perhaps these cells have either absent or defective egg activation factor(s). This is a difficult question to address as the nature of the sperm is still under debate and no clear candidate has been identified.

Men with complete spermiogenesis failure represent a significant portion of patients in our clinic. Clearly the results in our study, and that from others, demonstrate that simply injecting round spermatids form these men is an ineffective treatment. On a practical level, it might be possible to improve success rates but only if we can identify and select the most competent gametes (possibly via in vitro culture of the cells). However, we are only likely to really improve the management of these patients when we understand why these men have complete failure of spermiogenesis. Once this is determined then effective rationale therapy can proceed. ❑

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Male Contraception

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Introduction

Despite currently available contraceptives, the world's population exceeds six billion and is increasing by 80 million yearly and much of this population growth is unintended. This high rate of unintended pregnancy is due to inadequate access and use of contraceptives. Inadequate contraception leads to undesired pregnancies and high rates of abortion and infanticide or results in unwanted children who suffer disproportionately from poverty and neglect. Therefore, there is a great need for better access to existing contraceptives, better contraceptive education, and more contraceptive options. In particular, male-directed contraceptive options are particularly limited.

Vasectomy and Condoms

The two contraceptive options currently available to men are condoms and vasectomy and they account for roughly one third of all current contraceptive use by couples, with 20% of couples using condoms and 11 % of couples using vasectomy. Vasectomy is a safe, simple, outpatient surgery performed under local anesthesia in which the ductus deferens is severed and the ends ligated through a small scrotal incision. There are approximately 500,000 vasectomies performed in the USA yearly and worldwide over 50 million men have undergone the procedure. Vasectomies are highly effective with a failure rate of less than 1% and a low incidence of complications. The relatively new "no scalpel technique", perfected in the Sichuan province in China (Li et al, 1991), in which a single puncture is made midline in the scrotal raphe with scissors, is probably superior to older techniques. Drawbacks to vasectomy include a delay in the onset of azoospermia of several months, pain and rare infections of the surgical site. While post-operative pain resolves quickly, upwards of one third of men will experience some

degree of chronic testicular discomfort. In one study of such men, the majority had relief of their symptoms with reversal of the vasectomy (Myers et al, 1997).

Vasectomies are most appropriate for men who no longer wish to father children. However, three to five percent of men with vasectomies do eventually request reversal, usually due to remarriage. Vasectomy reversal, a procedure termed vasovasostomy, has the potential to restore fertility although rates of pregnancy vary from 30-70% depending on the length of time between the vasectomy and the reversal procedure. In 20-30% of men vasovasostomy is unable to restore patency of the vas if more than 8 years have elapsed since the original vasectomy (Belker et al, 1991). In addition, 20-40% of men remain infertile despite restored patency of the vas (as documented by imaging techniques) possibly due to the presence of anti-sperm antibodies. For these reasons vasectomy cannot be considered to be a truly reversible method of male contraception.

The good news about vasectomy is that it appears to be safe in regards to overall male health. Earlier reports of associations between vasectomy and cardiovascular disease have proven incorrect, and more recent concerns about vasectomy and prostate cancer risk have not been substantiated (Peterson & Howards, 1998).

Condoms made of animal intestine have been used as a means of male fertility control for several hundred years. Since 1920, most condoms have been made of latex rubber, which offers some protection against sexually transmitted diseases including HIV. Condoms are mostly free from adverse side effects; however, condoms have a marginal contraceptive efficacy. This is mainly due to improper or inconsistent usage, although condom breakage occurs in up to 2% of cases. Pregnancy rates for couples using condoms as their sole means of contraception approach 15% per year (Trussell & Vaughan, 1999). A drawback to condoms is their poor long-term compliance as they have to be used correctly during 100% of sexual encounters. In addition, some men dislike condoms because they feel condoms diminish sexual pleasure or are difficult to use. Latex condoms can lead some men and women to develop allergic reactions to the latex, which can cause skin irritation and even death (Turjanmaa, 1994).

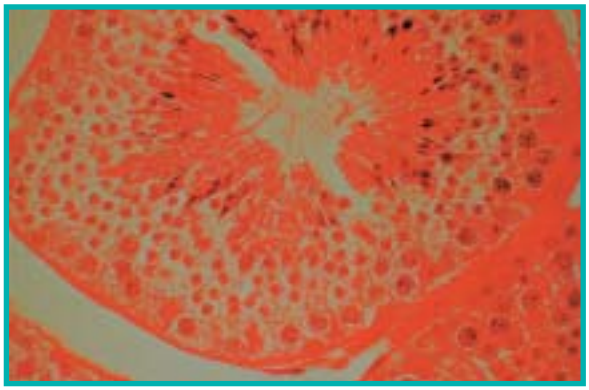
As a result of latex allergies, polyurethane condoms have been developed and tested; however, clinical studies with these condoms have demonstrated a slightly higher rate of condom breakage and slippage when compared to latex condoms. In addition, some subjects had greater difficulty with putting on the polyurethane condoms as compared to latex condoms. More importantly, polyurethane condoms appear to be as be as effective at latex condoms at pregnancy prevention, with one study reporting a roughly a 5% pregnancy rate after six months of usage (Frezieres et al, 1999).

Experimental Male Contraceptives

Because of the shortcomings of existing methods of male contraception, efforts have been made to develop a hormonally derived contraceptive for men analogous to the estrogen/progesterone pill used by women. Such a hormonal contraceptive has the potential to be safe, effective and easy to use. When surveyed, a majority of men indicate a willingness to use such a contraceptive if available (Martin et al, 2000). Important-ly, 98% of women surveyed were wil-ling to trust their male partner to use a hormonal method of contraception (2000)

When administered to a normal male, testosterone (T) functions as a contraceptive by suppressing secretion of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Low levels of LH and FSH deprive the testis of the signals required for normal spermatogenesis, leading to markedly decreased sperm counts and reversible infertility in most, but not all, men. Because of the failure of T alone to completely suppress sperm production in some men, compounds such as gonadotropin-releasing hormone analogues and progestins that further suppress pituitary gonadotropins are being studied in combination with T to optimize its contraceptive efficacy.

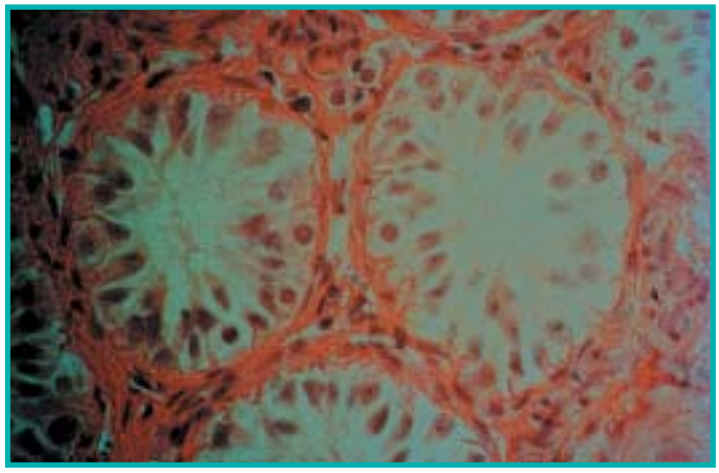
In normal men, sperm counts vary from 20-200 million sperm per ml. of ejaculate. The absence of spermatozoa in the ejaculate (azoospermia) renders fertilization impossible and is the ultimate goal of hormonal male contraceptives. Most studies to date, however, demonstrate that while most men's counts are suppressed to azoospermia some men sustain partial but incomplete reduction of their sperm counts (ie oligozoospermia). There is good evidence from efficacy trials of T alone as a contraceptive that sperm counts below 3 million sperm per ml. of ejaculate are associated with decreased rates of pregnancy (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1996). Severe oligozoo-spermia (ie < 1 million sperm per ml.) decreases the chances of conception even further and is therefore considered a reasonable short-term



Normal spermatogenesis (note sperm in center of seminiferous tubule).

goal for male contraceptive research.

Most hormonal contraceptives do not incapacitate existing sperm; they hinder sperm production. Since sperm take an average of 72 days to reach maturity, it is likely that any contraceptive based on manipulation of the hormonal axis will be associated with some delay in the onset of full contraceptive effect. In addition, it is important to consider ethnic differences in interpreting results of contraceptive trials. Study volunteers in Asia are more susceptible to T- induced suppression of spermatogenesis, with rates of azoospermia in the 90-100% range. Men studied in Europe, North America and Australia, however, have rates of azoospermia closer to 60% on the same T-only regimens (World Health Organisation Task Force on Methods for the regulation of Male Fertility, 1996). While the explanation for this difference is unclear, it is important in the interpretation of trial results and complicates extrapolation of data to different populations.



Testicular histology after six months of hormonal contraceptive treatment.

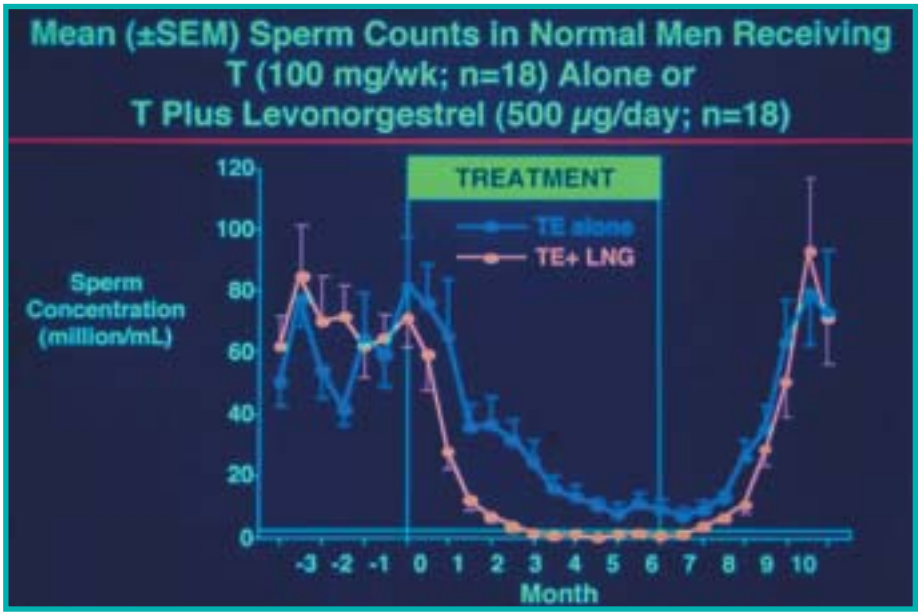
Future Directions

Given the encouraging results from recent combinations of T and progestins, many researchers now feel that this combination is the most likely to result in a viable contraceptive method for use in non-Asian populations, while long-acting injections of T (e.g. T undecanoate) alone may prove effective in Asian men. Current research is focused on both improving the method of androgen administration and finding combinations that optimize sperm count suppression in all populations while minimizing side effects.

One mystery in the field of male contraceptive research is why some men fail to suppress their sperm counts to zero despite the extremely low-levels of serum gonadotropins. It is clear from recent contraceptive trials using testosterone/progestin combinations that the additional suppression of serum gonadotropins mediated by the addition of a progestin improves rates of azoospermia to 70- 90% compared with 50-60% with TE alone. In these studies, the levels of circulating gonadotropins in subjects receiving TE in combination with a progestin are significantly lower than control groups receiving TE alone. However, there are no apparent differences in the gonadotropin levels among men who suppress to azoospermia and those who do not. One must conclude, therefore, that while further lowering of serum gonadotropin levels improves the percentage of subjects who achieve azoospermia, it is also suggests that serum gonadotropin levels within a given hormonal regimen may not distinguish between men who will achieve azoospermia and those who will not.

Explanations for differences between subjects who attain azoospermia and those who do not on contraceptive regimens have been proposed. For example, it has been suggested that this difference may be due to greater 5a-reductase act-ivity in the testes resulting in higher intratesticular DHT levels in subjects who failed to suppress to azoospermia on 200 mg weekly TE. In support of this theory, it has recently been demonstrated that intratesticular DHT is relatively preserved in men on contraceptive dose steroids (McLachlan et al, 2002). However, recent studies have demonstrated that the combination of testosterone and a type II 5a-reductase inhibitor (finasteride) did not enhance suppression of spermatogenesis any more than T alone or T plus DSG.

Recent research in cynomolgus monkeys implies that FSH suppression may be more important than low levels of intratesticular T in the maintenance of spermatogenesis



on contraceptive dose T. Moreover, a recent highly sensitive gonadotropin assay has detected FSH immunoreactivity in men on hormonal contraceptive regimens (Robertson et al, 2001). Since it has recently been demonstrated that estrogen and not T is the major source of feedback inhibition of FSH in men at the pituitary, and the addition of estrogen has been shown to enhance T-induced suppression of spermatogenesis, enhancing estrogen feedback to limit FSH activity may be crucial in future male contraceptive trials. Of note, the number of CAG repeats in the androgen receptor does not influence responsiveness to hormonal suppression of spermatogenesis. Lastly, a recent study combining TE with CPA found that sperm suppression was actually less when 200 mg of weekly TE was administered compared with the group receiving 100 mg of weekly TE (Meriggiola et al, 2002). Clearly, further investigation will be needed to understand the innate differences in the intratesticular environment that allow some men to continue to produce sperm in the extremely low gonadotropin environment of a male contraceptive regimen. ❑

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Reflections on another time

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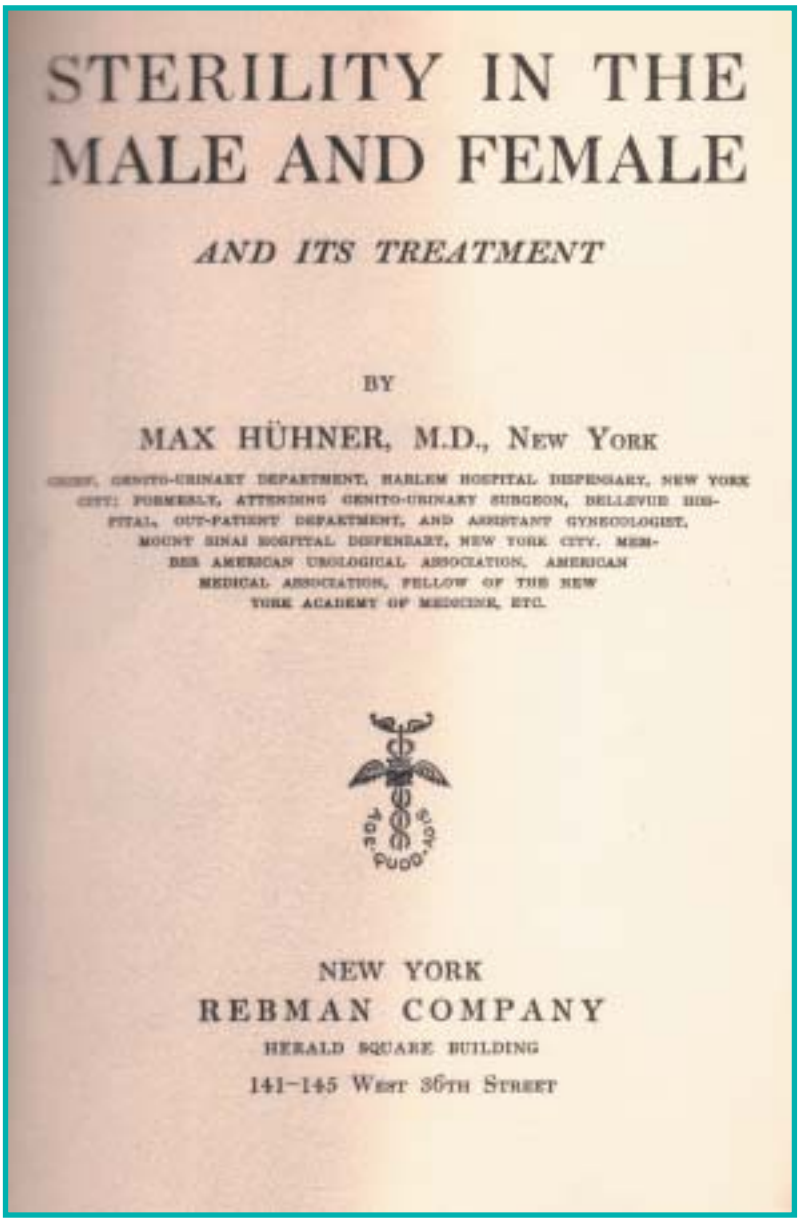
I was recently given some old books and amongst them were 2 books that have become linked in my mind for several reasons, as I am an avid reader of Charles Dickens one was an old book printed in 1904 that contained a photographic record of the places that were important to him and that figured in his books. Also in this book was biographical material on Dickens. I have always had a great deal of respect for his radical position on the values and politics of the contemporaneous society but as I read his history I was touched by the amount of physical suffering in his world. He suffered from "nervous disorders " which sound like depression and anxiety, a painful gouty foot, intermittent paralysis and painful facial neuralgia. Not to mention bouts of severe infectious illnesses and renal colic. His family were struck down by a variety of complaints, his wife from post-natal depression (10 times or more), a baby dying suddenly of some complaint, a brother from suffocation from a burst abscess of the lung and his father from complications of surgery without an aneasthetic to remove a bladder stone that was obstructing his urinary tract. There was some suggestion that his son-in-law was impotent. A litany of suffering and misery. I

was forced to wonder if this man's literary output would have been the same if contraception, antibiotics and other medications were available. His suffering and that of those he loved touched me.

In the same batch of old books was one by Max H?hner. In 1913 Max Hühner, an American surgeon specializing in the genitourinary tract, published a book titled Sterility Treatment and in this text he described the post-coital test. He described this as "an original method….for studying the progress of spermatazoa in the tubes in normal and pathological conditions" In this book the role of sperm seemed to be well established but the time of highest fertility in the menstrual cycle was still open to debate. I found the way in which the book was written and the way Max Hühner thought about his subject an interesting insight. He seems to talk to his audience and argues for and against his thoughts. He is also very aware of the idea of bias in his thinking and discusses the confounding factors that may influence his results to give false conclusions. Hühner then tries very valiantly to take this bias out.



STERILITY IN THE MALE AND FEMALE

AND ITS TREATMENT

BY

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Max Hühner states "all that is necessary is for the woman to come after coitus, the sooner the better". He also mentions "we need not care what the condition of his sexual apparatus is, for we know at once that as far as impregnation is concerned his organs are satisfactory". Another advantage as far as Max Hühner is concerned is that" the husband need know nothing about the fact that he and his wife are being investigated for the cause of sterility.

It appears that ethics and the English language and its usage have changed since 1913.

Hühner reports that several studies using statistics available to them reveal that the average period between marriage and the birth of the first child is between 12 and 26 months. He believed at that time that 1 in 10 marriages are sterile with the highest percentage being in France where it was 1 in 5. He also thought that 24% of marriages were sterile after the birth of the first child. He studied the characteristics of the sterile and made some interesting observations. He concluded that the sterile woman was much more likely to be stout. Some thought this was the tendency of the sterile to become fat whereas others felt the obesity was related to the cause of the sterility. I can only think the population with PCOS had already been identified. Hühner did not find age a significant feature of infertility, nor did he find that periods for a sterile woman were different to those of a fertile one.

Two conditions that struck me with a combination of amusement and horror were *receptaculum seminis* and *effluvium seminis*. The former appears to be the belief that a pool of semen in the upper vagina should form after coitus so that a very active cervix can dip into and drink from it by being able to open and close and suck up the sperm. The latter theory was debunked when Hühner interviewed 132 women who had had children and found that 80% of these women reported that semen leaked from the vagina after intercourse. Hühner found this surprising and " it occurred to me, however that the flowing out of semen after coitus might possibly not be a rare condition in married life". One of his conclusions from observing the behaviour of spermatazoa in the cervix is that "amputation of the cervix for sterility in the case of a long conical cervix is sensible, for then the semen can be more easily ejaculated on to the cervix than on either side of it".

Hühner thought the more " passionate a man is, or rather the more forcibly his semen is expelled, the greater the chances of impregnation, as more of it would hit the cervix and less drop into the

vagina". When you think of the anaesthetics available and the risk of infection the thought of operations such as this performed on the cervix or the formation of a pouch in the upper posterior vagina to allow the formation of the pool of semen previously alluded to one wonders how many women were rendered truly sterile or even paid the price of their sterility with their lives.

Hühner in general attempts to recognise potential sources of bias and does his best to describe accurately what he sees and draw conclusions from them. One incorrect assumption is that the sperm has both tail and independent head motility. He ascribes this conclusion to the fact that although the tail appears still the head may be seen to move from side to side. Hühner then goes on to report that various authorities believe that only a single sperm enters the ovum and that the body and the tail of the sperm then disappear when it enters the ovum. The head then goes toward the nucleus and fuses with it. Hühner makes the point that this could be due to the independent motion of the sperm head.

Not only does he describe the post-coital test in this book but also testicular aspiration. He aspirated the testicles 54 times, 28 in cases of sterility ie. No sperm found in a condom after coitus and 26 cases from the general genito-urinary clinic he attended. He makes the observation that the finding of immotile sperm in a condom that has not been allowed to become cold and which is examined shortly after coitus means that the sperm are dead whereas the immotile sperm found on a testicular aspirate may be normal as the sperm only becomes motile shortly before ejaculation. In his sterile patients he found sperm in 2 out of 5 cases and if he did not find sperm on one occasion he did not find any on subsequent occasions. Hühner went so far as to aspirate sperm and then place them within the cervix of the wife. None of his cases were successful "in as much as the wife had her periods at the regular time". This result is not surprising when Hühner goes on to explain that he tried the technique just after the menses, as early as day 4 or as late as 3 days before the expected period. This is despite earlier quoting research which suggested that mid-cycle was the most fertile time as the fertility of orthodox Jews who abstained from intercourse at other times was very high.

It is amazing to find out that 15 cases involving the operation of epididymo-vasostomy in men with azoospermia was reported by Martin in 1909. In 6 cases live spermatazoa were found in the condom after the operation and in 3 cases pregnancy followed.

I think however the vision that will stay with me apart from the thought and sight of the illustrations of the equipment for obtaining fundal specimens of uterine secretions to look for sperm or the deep prostatic massage with the instillation of deep dilute silver nitrate into the prostate and down the urethra is the thought of all the patients in the waiting room clutching their used condoms. "While I was experimenting with spermatazoa, it was a common occurrence in my office to have several women, each with a condom for investigation. I generally have on hand also patients with acute gonorrhoea, with pus oozing from the meatus. I was thus well supplied with live spermatazoa and live gonococci. I have very often made the following experiment". He then quite astutely goes on to say that the gonococci themselves are not lethal to the sperm but the exudate that goes with them. I can't help but have a vision of this waiting room in my head. All the women with their used condoms and all the men with pus dripping from the meatus of their penises.

When Hühner describes the cause of the male infertility it is a role call from a different era. There is acute gonorrhoea, scarring from previous gonorrhoea, syphilis and TB. He continues to attempt to get these women pregnant to the husbands even when one husband has the rash of active secondary syphilis. Hühner even described the birth of one child and then secondary infertility as occurring as a result of the gonococcal infection the wife caught from her husband during the first pregnancy. There is certainly no comment or concern about getting the couples to have intercourse when nearly all the men had or had had infections complete with what I know crudely as "drippy dicks". At no point was the issue of the health of the female partner raised. Or of the unborn children.

I admit to liking Hühner when he describes the prevailing attitudes of his fellow physicians who investigated and treated the women with no regard to the state of the men or their genitalia. He felt that women were being singled out for blame as the men would not tolerate being responsible for the sterility. Hühner thought this both unfair and unprofessional.

Old medical textbooks and biographies of those from long ago give us food for thought. They remind us of the wonderful advances in medical science, public health and treatments available enabling us to reduce suffering. It also makes me realize that what may be thought of as a medical fact at one time becomes a curiousity and an amusing one at that in another. The first rule of medicine is to do no harm therefore we should all look critically at what we do as Hühner tried to do. ❑

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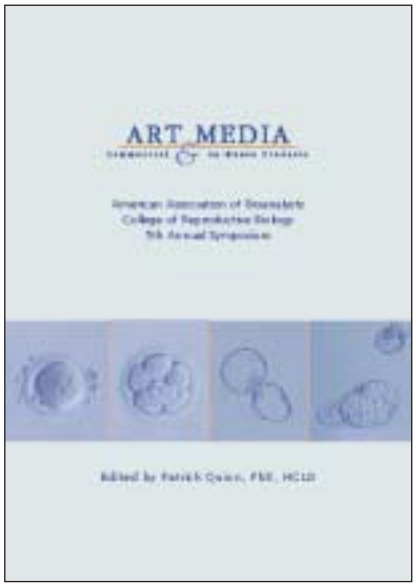

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