

MALE INFERTILITY

SPERM DIAGNOSIS, MANAGEMENT AND DELIVERY

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Foreword by Gedis Grudzinskas

Contents

Foreword v

Preface vii

Acknowledgements ix

Contributors xiii

Chapter 1
Basic anatomy and physiology 1
Fidelis T. Akagbosu

Chapter 2
Basic semen analysis 7
Bryan Woodward

Chapter 3
Sperm morphology reference values 21
Thinus F. Kruger, Danie Franken

Chapter 4
Culture media constituents 25
Jayant G. Mehta

Chapter 5
Sperm preparation: swim-up and density gradient centrifugation 37
Jaffar Ali

Chapter 6
Sperm function tests 45
Amjad Hossain, Ashok Agarwal

Chapter 7
Antisperm antibodies and immunological infertility 51
Jennifer E. Reifsnyder, Ranjith Ramasamy

Chapter 8
Causes and effects of poor semen quality 57
Jonathan Ramsay

Chapter 9
Treatment options arising from different semen analyses 65
Natarajan Pandiyan, D. Ramesh Raja, Radha Pandiyan

Chapter 10
Other factors to consider with sperm preparation for treatment 71
Tim M. Dineen, Bryan Woodward

Chapter 11
Preparing sperm from surgical retrieval 81
Sue Montgomery

Chapter 12
Retrograde ejaculation 89
Thomas R. Aust, Iwan Lewis-Jones

Chapter 13
Sperm cryobanking and oncology 93
Greta Verheyen

Chapter 14
Nurse-led insemination programs 103
Wendy Norton

Chapter 15
New developments 111
Markus H. M. Montag

Chapter 16
Sperm and legal aspects 119
Julius Hreinsson

Chapter 17
Sex selection of sperm 125
Andrew D. Dorfmann

Appendix **131**

Index **133**

Chapter 4 Culture media constituents

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INTRODUCTION

The turn of the 20th century witnessed an escalation in the development of culture media: aqueous solutions containing chemical and biological constituents that were suitable for the survival of cells and tissues. These developments were based on the pioneering studies of Arnold, Harrison, Ringer, Locke and Tyrode (for a review, see Waymouth 1972).

The potential importance of chemically defined media was first recognized by Lewis and Lewis (1912) while working on embryonic chick tissues. Their hope was that an artificial medium would be found that was as satisfactory as the plasma. The advantages of working with such a 'known' medium are obvious.

Although there has been an increase in our understanding of how physiology, biochemistry, genetics, and epigenetics influence the development of the preimplantation mammalian embryo, the development of culture media for the human embryo has been relatively slow, mainly due to the inability to perform empirical studies. As a consequence, the majority of what is known about the preimplantation human embryos comes from research conducted on animal models (Biggers 2002, Quinn 2004, Biggers & Summers 2008).

Furthermore, the development and design of specific media for sperm has also been neglected, possibly due to the fact that sperm are more resilient than oocytes. As a result, media for processing and manipulation of sperm have similarly defined chemical constituents as those for embryos.

Although 20 types of chemically defined culture media, each with a unique composition, are currently available commercially for human assisted reproductive techniques (ARTs), none are 'optimal' for embryo culture or sperm manipulation. Furthermore, some commercial media have been reported to cause imbalances and stress to the embryo (for a systematic review, see Mantikou et al. 2013).

In developing and designing media, two fundamentally different philosophies – 'back-to-nature' (sequential media) or 'let-the-embryo-choose' (global, monoculture media) – appear to have influenced all current commercial media (Biggers & Summers 2008).

'Back-to-nature' addresses changes in the physiological needs of the developing zygote and embryo as it transits from the oviduct to the uterine cavity as a morulae and a blastocyst (Biggers 2003, Lane & Gardner 2003) (**Table 4.1**). In essence, this acknowledges the existence of different physiological environment in the oviduct and the uterus (**Table 4.2**).

On the other hand, the monoculture 'let-the-embryo-choose' principle capitalizes on the knowledge that embryos may simultaneous use a cocktail of defined chemicals, because the effects of each constituent in the medium may depend on concentrations of the others (Leese 1997, Biggers & Summers 2008).

It is worth noting that *in vivo*, although millions of sperm may be deposited into the vaginal vault at ejaculation, normally only one spermatozoon will fertilize the oocyte (Williams et al. 1993). During the selection process, successful sperm are exposed to the same female reproductive tract fluids as the oocyte, confirming its physiological viability (**Figure 4.1**).

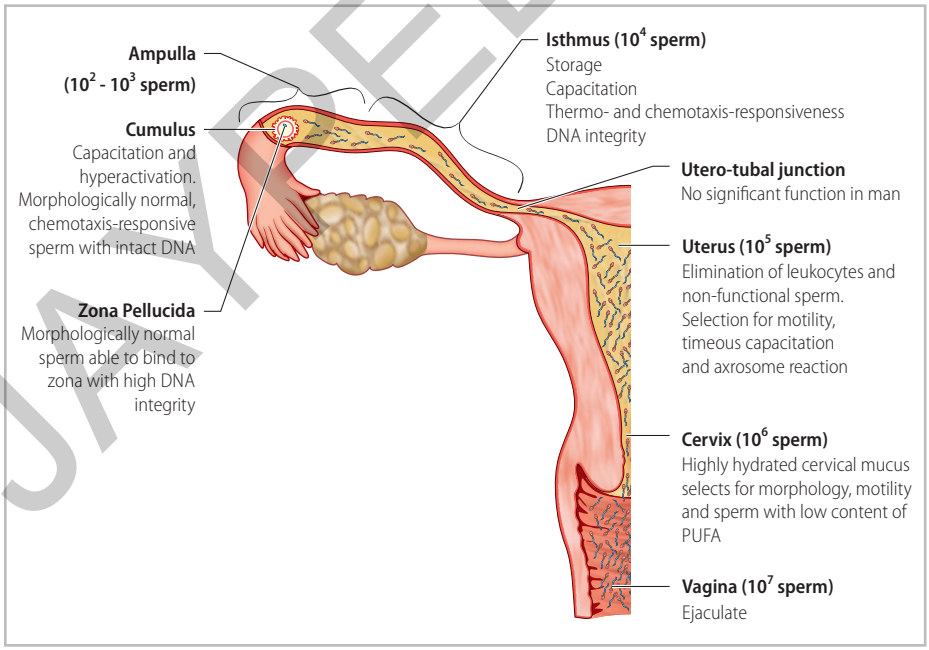

Figure 4.1 Diagrammatic representation of sperm selection within the female reproductive tract. Redrawn with permission from Nature Publishing Group, from Henkel R. Sperm preparation: state-of-the-art physiological aspects and application of advanced sperm preparation methods. Asian J Androl 2011;14:260–269.

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Table 4.1 Changes in the physiological needs of preimplantation mammalian embryo at different stages of development

Precompacting	Postcompacting
Low biosynthetic activity	High biosynthetic activity
Quiescent (low QO_2)	High active (high QO_2)
Ovoid mitochondria	Elongated mitochondria
Pyruvate-based metabolism	Glucose-based metabolism
Maternal genome	Embryonic genome
Individual cells	Transporting epithelium
Low ability to maintain cellular homeostasis	Complex systems for maintenance of cellular homeostasis
Identical cells	Cell differentiation into inner cell mass and trophectoderm

Table 4.2 The differences in constituents present in oviduct and uterus in the mammalian embryos

Constituents	Oviduct	Uterus
Glucose concentration	0.50 mM	3.15 mM
Pyruvate concentration	0.32 mM	0.10 mM
Lactate concentration	10.5 mM	5.2 mM
Oxygen concentration	8%	1.5%
Carbon dioxide concentration	12%	10%
pH	7.5	7.1
Glycine concentration	2.77 mM	19.33 mM
Alanine concentration	0.5 mM	1.24 mM
Serine concentration	0.32 mM	0.80 mM

With the introduction of ART, it became necessary to develop techniques to separate sperm from the seminal plasma, to mimic the *in vivo* selection process. These techniques were introduced to achieve a highly motile population of sperm (see Chapter 5). Because the ionic constituents present in commercial media are similar to those found in the female genital tract, sperm are able to undergo the necessary physiological events of capacitation prior to fertilization (**Figure 4.2**).

This chapter attempts to give an overview of the development of chemically defined media, focusing on the influences of various constituents imparted on to the preimplantation embryo and the sperm (for more extensive reviews, see Quinn 2000, Biggers 2003, Gardner & Lane 2003).

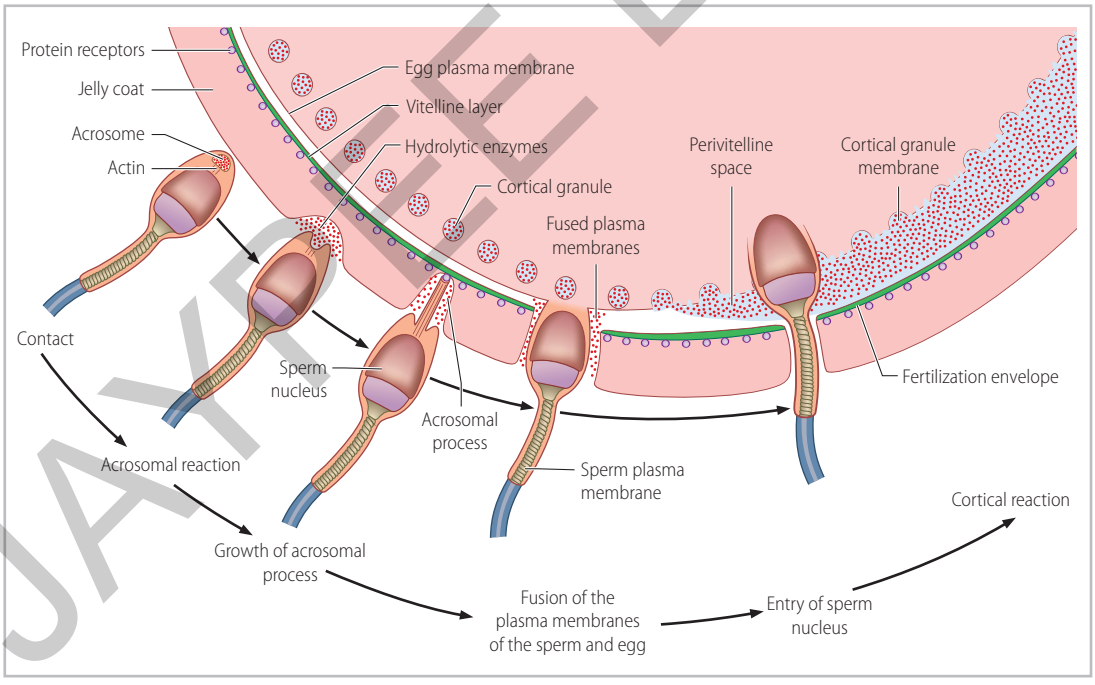

Figure 4.2 Diagrammatic representation of various steps leading to fertilization, including acrosomal reaction in step 2.

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HISTORICAL DEVELOPMENT OF CULTURE MEDIA FOR PREIMPLANTATION MAMMALIAN EMBRYOS

A major breakthrough in the development of culture media was witnessed when Whitten (1956) reported successful *in vitro* development of eight-cell mouse embryos to the blastocyst stage in a simple chemically defined medium (**Table 4.3**). This medium contained only nine constituents yet resulted in birth of live pups after the first embryo transfer of *in vitro* grown blastocysts (Whitten 1956, McLaren & Biggers 1958).

Further supplementation of this medium with calcium lactate, as an additional metabolic substrate (in presence of glucose), allowed the development of late two-cell embryos to blastocysts (Whitten 1957). Similarly, if pyruvate, oxaloacetate, or phosphoenolpyruvate were supplemented as the energy source in the presence of lactate, an increase in blastocyst yield was observed (Brinster 1965). Because the mouse embryos studied were cultured in media designed for somatic cells, these observations suggested that the early embryo was unlike a somatic cell in that it was not able to utilize glucose for development and, instead, required a simpler energy source in the form of lactate and pyruvate (Whittingham 1969) (**Table 4.3**).

In vitro arrest at specific points in embryonic development has been shown to be species specific (**Table 4.4**). One possible reason for developmental arrest can be attributed to the depletion of the cellular reserves of inorganic phosphate (P_i), as this drives the metabolic machinery toward glycolysis. Without P_i, mitochondrial oxidative phosphorylation would be inhibited (Seshagiri & Bavister 1989a, 1989b). These observations led to speculation that the combination of glucose and phosphates together may be toxic for cleavage-stage embryos (Quinn 1995, Barnett & Bavister 1996, Barnett et al. 1997).

Furthermore, additional studies have shown that embryos produced from oocytes of F1 hybrid female mice did not arrest in pyruvate-based medium (Biggers 1998), suggesting that the possible cause of the arrest may be imbalances or deficiencies in the medium that induce embryonic stress, to which certain genotypes may be more resistant than others. Hadi et al. (2005) very elegantly demonstrated that 'non-blocking' F1 hybrid-derived embryos can be induced to arrest at the two-cell stage by exposing them to the same and somewhat more severe stressors that cause arrest at the two-cell stage in 'blocking' embryos.

Among the stressors, one possible candidate could be the osmotic pressure imparted on to the cell. It is well established that animal cells control their volumes by actively adjusting the cytoplasmic concentrations of osmotically active solutes, thus altering intracellular osmotic pressure (Hallows & Knauf 1994). Furthermore, cells regulate their volumes by exchanging osmolytes leading to temporary changes to the intracellular pressure. It is therefore likely that even small changes in external osmolality *in vitro* could influence the embryos to either shrink or expand and indirectly control the developmental cycle, such as a 'two-cell block.'

Similarly, sperm have high water permeability compared with other mammalian cell types (Noiles et al. 1993), which allows them to shrink or swell rapidly when exposed to extreme ranges of osmolality (70–500 mOsmol/kg) (Du et al. 1993). This knowledge enabled the hypo-osmotic swelling test to be developed as a simple clinical test for sperm membrane integrity and hence vitality (Jeyendran et al. 1992, see Chapter 6).

Potassium simplex optimized medium (KSOM) has been the most widely used chemically defined mouse embryo culture medium. KSOM also serves as the basis for the formulation of several chemically defined culture media that support successful development of preimplantation embryos in many other mammals, including humans.

The first successful fertilization of the human oocytes *in vitro* was accomplished in simple chemically defined media, such as Earle's balanced salt solution and modified Ham's F10 or T6 (Edwards 1981). These media were supplemented with maternal serum, thus converting them into biological media. Although Menezo et al. (1984) went on to formulate a successful complex medium with amino acids and without the need of a serum supplement, it was the human tubal fluid (HTF) that was recognized as the first medium specifically developed for human ART procedures and also used for sperm preparation (Quinn et al. 1985, Quinn 2003, Quinn & Pool 2004).

Table 4.3 The constituents present in Whitten's (1956), ground breaking culture medium used for *in vitro* culture of eight-cell preimplantation mammalian embryo to blastocyst stage. (values in mM)

Constituents	Whitten (1956)
NaCl	118
KCl	4.7
KH_2PO_4	1.2
$CaCl_2$	2.5
$MgSO_4$	1.2
$NaHCO_3$	25
Glucose	5.6
Bovine serum albumin	1 mg/mL
Eight-cell embryo cultured in a CO_2 bicarbonate buffering system.	

CONSTITUENTS OF HUMAN ART MEDIA

Preimplantation embryo development

During the initial stages of preimplantation development, the embryo resides in the protective environment of the oviduct and is relatively quiescent (**Table 4.1**). Because the early embryo does not preferentially metabolize glucose, it is dependent on its mitochondria to produce energy for development, via oxidative phosphorylation of pyruvate

Table 4.4 Characteristic cleavage stage blocks, which are species specific

Species	Cleavage stage arrest	First reported by
Mouse	2 cells	Whitten 1956
Bovine	8 cells	Camous et al. 1984
Hamster	2–4 cells	Schini and Bavister 1988
Humans	4–8 cells	Bolton et al. 1989
Rat	2 cells	Kishi et al. 1991

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or lactate (Leese 1988). Furthermore, the early stage embryo is more susceptible to stress exposure, as it has a limited ability to regulate against alterations in osmotic stress, pH, and possibly reactive oxygen species due to the absence of many key homeostatic pathways (Baltz et al. 1991, Lane 2001).

After the eight-cell stage, compaction causes blastomeres to flatten into one another to maximize cell–cell contact and result in clear cell polarization (Gallicano 2001). Cavitation then signals blastocyst formation (Watson et al. 2004), with formation of two distinct cell types: the inner cell mass and the trophectoderm.

Journey of ejaculated sperm

Similarly, the *in vivo* journey of ejaculated sperm from the vagina to the top of the fallopian tube involves a programmed physiological cascade of events and is highly dependent on the pH, osmolality, and availability of nutrients within the oviduct and uterus.

Prior to the acrosome reaction, a sperm undergo a series of biochemical transformations, collectively referred to as capacitation (Breitbart 2003). *In vitro*, these events need to be supported by the medium in which the sperm are prepared. The biochemical events include elevation of intracellular calcium and bicarbonate to activate adenylyl cyclase, production of cyclic adenosine monophosphate, activation of protein kinase A, and indirect phosphorylation of certain proteins on tyrosine. Calcium channels in the outer acrosomal membrane are activated with an increase in cytosolic calcium, resulting in F-actin dispersion aiding the fusion of the outer acrosomal and the plasma membrane and completing the acrosome reaction (Abou-haila & Tulsiant 2009, Esteves & Verza 2011) (**Figure 4.2**).

Table 4.5 lists the key constituents of sequential and monoculture media.

Nutrients

Energy source – carbohydrates

Pyruvate, lactate, and glucose are the triad of energy substrates (Quinn 2000, Pool 2002). Concentrations of these nutrients have been established via direct sampling (Gardner et al. 1996) and via vascular perfusion preparations of the fallopian tubes of various mammals (Leese & Barton 1984, Gardner & Leese 1990).

On the basis of these findings, Gardner et al. (2001) were able to adjust the concentration of the energy triad in their G1 (precompaction) and G2 (postcompaction) media, which led to the development of a sequential media for embryo culture (**Table 4.5**).

Pyruvate

The early preimplantation embryo is highly reliant on pyruvate for ATP generation (Leese & Barton 1984). Pyruvate also has a role in regulating intracellular pH (pH_i), scavenging free radicals and disposing of ammonia from amino acid breakdown by means of the transamination reaction (Leese et al. 2001).

Lactate

Lactate is known to regulate the pyruvate uptake and supports two-cell embryo development in the presence of pyruvate. Lane and Gardner (2000a) have demonstrated that pyruvate acts in tandem with lactate to facilitate the conversion of lactate to pyruvate, and this is dependent on the stage of embryo development. Although the precompaction

Table 4.5 Key constituents of sequential culture media and single/monoculture medium

Constituents	Sequential culture system phase 1	Sequential culture system phase 2	Monoculture system
Salts	Sodium chloride	Sodium chloride	Sodium chloride
	Potassium chloride	Potassium chloride	Potassium chloride
	Calcium chloride	Calcium chloride	Calcium chloride
	Sodium citrate	Sodium citrate	Monopotassium phosphate
	Magnesium sulfate	Magnesium sulfate	Magnesium sulfate
	Sodium dihydrogen phosphate	Sodium dihydrogen phosphate	
Energy substrates	Glucose	Glucose	Glucose
	Sodium lactate	Sodium lactate	Sodium lactate
	Sodium pyruvate	Sodium pyruvate	Sodium pyruvate
Non-essential amino acids (NEAA)	8 NEAA'	9 NEAA	NEAA
Glutamine dipeptide	Optional	Optional	Alanyl-glutamine
Essential AAs (EAAs)	2 EAAs	11 EAAs	EAAs
Chelator	EDTA		EDTA
Fatty acid	Lipoic acid	None	None
Vitamins	None	4 vitamins	None
pH Indicator	Optional	Optional	Optional
Antibiotics	Gentamicin	Gentamicin	Gentamicin
Buffer	Sodium bicarbonate	Sodium bicarbonate	Sodium bicarbonate
Macromolecules	Hyaluronan, HAS	Hyaluronan, HAS	Optional
Water	Yes	Yes	Yes

EDTA, ethylenediaminetetraacetic acid; HAS, human serum albumin.

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embryo has a significantly higher pyruvate uptake, Leese and Barton (1984) demonstrated that at compaction, glucose uptake increases while pyruvate uptake decreases. In culture media, lactate normally is included as a sodium salt of D- and L-isomers. The L-isomer is biologically active and can be metabolized, whereas D-lactate affects pH_i, which influences the cellular homeostasis (Lane & Gardner 2001).

Glucose

In humans, the presence of high levels of glucose in culture medium can cause retardation or developmental arrest of cleavage-stage embryos (Quinn 1995). Human embryos can develop through early cleavage and up to the blastocyst stage in the absence of glucose (Conaghan et al. 1993, Quinn et al. 1995, Coates et al. 1999). This apparent toxicity of glucose only manifests in medium lacking amino acid and containing phosphate. Furthermore, Seshagiri and Bavister (1991) demonstrated a reduction in the respiratory capacity due to reduced mitochondrial function. A reduction in the metabolic control is therefore likely to lead to a loss in ATP production and developmental arrest.

The switch of substrate from pyruvate to glucose at around the eight-cell stage is confirmed by expression of the protein GLUT2 at this time (Hogan et al. 1991), when the human embryonic genome is activated (Braude et al. 1988). Furthermore, GLUT3 is expressed at the morula and blastocyst stages, suggesting that the glucose utilization by the embryo increases as it develops and maximum utilization is observed at the blastocyst stage.

In light of these observations, Gardner et al. (2001) modified glucose concentrations of G1 and G2 media to 0.5- and 3.15-mM glucose, whereas Quinn's ART media contains glucose at concentrations of 0.1 and 2.8 mM in the cleavage and blastocyst media, respectively.

For decades, researchers have been debating whether sperm get ATP from mitochondrial oxidative phosphorylation or glycolysis. It was recognized that the absence of glucose in fertilization medium resulted in decreased sperm progression and lower fertilization, mainly due to poor capacitation (Quinn 1995, Mahadevan et al. 1997). To correct these observations, glucose levels were raised to 2.8 mM in Quinn's fertilization medium.

To satisfy the high rates of glucose utilization, by millions of sperm during swim-up method, Bertheussen et al. (1997) have suggested use of even higher concentrations of glucose in the culture medium. Dan-Goor et al. (1997) have reported that GLUT1 expression and glucose uptake in preimplantation embryos is triggered after sperm penetration, fertilization, and pronucleus formation. However, in a recent series of experiments, Amaral et al. (2013) demonstrated that apart from the ATP derived from sugars, sperm may also get their ATP from fatty acids metabolized in mitochondrial and peroxidase pathways. This suggests that sperm may use lipidic beta-oxidation to contribute to sperm motility and metabolism. The existence of peroxisomal proteins also suggests that sperm may be able to get energy from internal sources of substrates, such as the long-chain fatty acids, to guard against external energy source fluctuations.

IONIC COMPOSITION

The dynamic interplay that exists between pH_i, pyruvate, and lactate levels and the manner in which calcium (Ca^{2+}) and magnesium (Mg^{2+}) influence the cellular homeostasis and embryo development has been studied by Lane and Gardner (2000b). Embryo development may be impaired as a result of an increase in intracellular Ca^{2+} concentration (Lane et al. 1998), but this can be corrected simply by increasing the Mg^{2+} concentration in the culture medium. Mg^{2+} blocks the increase in Ca^{2+} influx through L-gated Ca^{2+} channels, leading to a reduction in stress.

Furthermore, it should also be noted that internal Ca^{2+} is released as part of the changes occurring in sperm during fusion with the oocyte membranes. Observations by Rogers and Yanagimachi (1976) show that when levels of Mg^{2+} are too high in relation to Ca^{2+}, the acrosome reaction in guinea pig sperm is inhibited. These observations are contrary to what happens in the cleaving embryo (Lane et al. 1998). It is therefore essential that when formulating media, the requirements of both the male and female gametes should be considered.

Phosphate (P_i)

In the absence of P_i, glucose does not appear to have a negative effect on early embryo development. However, glucose is needed to maintain the activity of pentose phosphate pathway (PPP) (Gardner & Lane 1996). Although in the presence of amino acids, a high level of phosphate (1.19 mM) has no negative effect (Gardner & Lane 1996), absence of phosphate in the medium is compensated by the internal P_i levels, which are sufficient to maintain numerous metabolic processes of the postcompaction embryo (Quinn & Wales 1971). Furthermore, it is important to consider the negative effects of high P_i that exists when phosphate buffered medium is used (Quinn 2000) and its subsequent effect on sperm.

Miraglia et al. (2010) have reported that swim-up capacitated sperm exhibited enhanced PPP activity and increased expression of glucose-6-phosphate dehydrogenase. However, PPP activation during capacitation is not a response to increased oxidative stress, but it exerts a role by supplying reducing equivalents to oxygen.

pH

Environmental conditions are known to be harmful stressors, as these can be easily transduced into deleterious intracellular perturbations. pH is one such parameter that requires strict attention as it is dynamic (Pool 2004). pH is highly dependent on the association and dissociation of compounds in the solution and is sensitive to factors, which influence this balance, such as temperature.

Since the extracellular (pH_e) of the culture medium is the result of a balance between concentration of CO_2 in the culture incubator and the amount of bicarbonate in the media, the only way to achieve the desired pH_e is to adjust the CO_2 concentration of the culture incubator (**Table 4.6**).

To calculate the CO_2 concentration, use the Henderson–Hasselbalch equation:

$$pH = pKa + \log_{10} ([HCO_3^-]/[CO_2])$$

Other components in the medium, such as amino acids, also contribute toward buffering capacity and will influence the final pH of an equilibrated medium.

Table 4.6 The concentration of CO_2 required to achieve a given pH value, for a solution containing 25-mM NaHCO3 at 37°C, at sea level

% CO_2	pH
4.6	7.5
5.8	7.4
7.3	7.3
9.3	7.2

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Because this is an inverse relationship, it is essential to appreciate the equilibration timing for the desired pH_e to be achieved also depends on the volume of media, surface area, use of oil overlay, and the respective oil volume. Use of premixed gas cylinders makes it difficult to adjust the required pH_e by altering the CO_2.

Intracellular pH

Intracellular homeostasis is regulated by intracellular pH_i. Although intracellular acidosis involves the sodium-dependent HCO_3^-/Cl^- exchanger (HCE) and the Na^+/H^+ antiporter, which adds and removes bicarbonate and hydrogen ions, respectively, from cells, the short-term regulation of pH_i is maintained by limited physiochemical buffering capacity of the cytoplasm and proteins (Lane & Gardner 2000b).

Although the pH_i of the human embryo initially follows the pH_e, the HCO_3^-/Cl^- exchanger only gets activated when pH_i rises above 7.2–7.3 (Phillips et al. 2000). Furthermore, when pH_i drops below 6.8, the Na^+/H^+ antiporter is activated. The Na^+-dependent HCO_3^-/Cl^- exchange operates below pH 7.0. However, due to the formation of tight junctions between cells, it is possible that morula and blastocyst-stage embryos have more rigorous control over their pH_i (Edwards et al. 1998).

Although few studies have addressed the question of mechanism implicated in the regulation of sperm pH, Babcock (1983) proposed that the inhibition in motility of the sperm from the hamster caudal epididymis is due to an acidic pH, maintained by the presence of lactate. Although sperm are immobile in epididymal cauda fluid, their motility initiation is accompanied by an intracellular alkalization mediated possibly by a Na^+/H^+ exchange mechanism (Wong et al. 1981). It is also likely that Ca^{2+} may play a role in this mediation (Armstrong et al. 1994). Furthermore, it is worth noting that pH_i alkalization stimulates both metabolism and protein phosphorylation (Carr & Acott 1989).

In a study on ejaculated human sperm diluted in different ionic conditions (with or without Na^+ and K^+ ions), it was shown that the pH_i was highly dependent on the pH_e and that it increased linearly with pH_e (Hamamah et al. 1996). The optimum pH_e was found to be 7.2.

Zeng and colleagues (1996), in a study carried out on mouse epididymal sperm found two regulatory mechanisms; an NaCl/HCO_3^- exchange mechanism that appears to be specific to the sperm, an ion-dependent mechanism that allows the cell to recover after alkalinisation and acidification. The latter was not precisely defined.

Is there an optimal pH_e?

Many commercial companies recommend a pH range for their different media to ensure that pH_e is slightly higher than pH_i, as it will help offset the acidification that occurs as a result of intracellular metabolic processes (**Table 4.7**). Unfortunately, there is no optimum pH_e, as this varies from media to media based on its constituents. It is also known that certain amino acids, such as glycine, taurine, and glutamine, act as zwitterions and help in buffering pH_i (Edwards et al. 1998). Most laboratories culture their embryos in the range of pH 7.2–7.4 and have found that culturing cleavage stage embryos closer to pH_e 7.2 may give better embryo development (Swain & Pool 2009).

Table 4.7 pH_i of the oocyte and preimplantation embryo at different stages of its development (Phillips et al. 2000)

Different stages of development	pH_i
Germinal vesicles – intact oocyte	7.04 ± 0.07
Metaphase I oocyte	7.03 ± 0.04
Metaphase II oocyte	6.98 ± 0.02
2–8 cells embryos	7.12 ± 0.0

Human sperm – pH requirements for fertilization

With respect to fertilization, the human sperm requires a slightly higher pH than that which may be optimal for the oocyte. Dale et al. (1998) have demonstrated that binding of sperm to zona is optimum at pH 7.5. Whether this binding is less efficient over the pH range of 7.0–7.5 still remains unanswered. Miyamoto et al. (1974) have shown that the rates of fertilization of mouse, hamster, and rat oocytes decreased at pH < 7.2. As a consequence, companies offering sequential media have adjusted the pH of their fertilization, cleavage, and blastocyst media at 7.1, 7.2, and 7.3, respectively (**Table 4.8**).

Furthermore, periodic fluctuations in environmental conditions, such as the pH_e of media, can be harmful as these can be transduced into deleterious intracellular perturbations (Phillips et al. 2000). This is more apparent in cell types like the denuded, mature oocyte (Fitzharris & Baltz 2006, Fitzharris et al. 2007) or cryopreserved/thawed embryos (Lane et al. 2000), which lack robust pH_i regulatory mechanisms.

pH buffering – good buffers

Although the availability of more complex culture media formulation has drastically improved the quest to produce better embryos, formulation of oocyte and sperm handling media outside the laboratory incubator has lagged considerably.

It has been documented that different buffers impact various cellular processes or laboratory assays differentially, including electron transport, photophosphorylation, and mitochondrial oxidation (Good et al. 1966, Ferguson et al. 1980). Similarly, several reports examining the impact of various pH buffers used in sperm storage or cryopreservation media demonstrate that some buffers are better in maintaining post-thaw motility and membrane integrity (Graham et al. 1972, Garcia & Graham 1989, Molinia et al. 1994).

Currently, two Good's buffers are available as *in vitro* fertilization (IVF) handling media (Good et al. 1966). 4-(2-Hydroethyl)-1-piperazineethanesulphonic acid (HEPES) is a modified taurine molecule and at 25 mmol/L is a standard IVF handling media. HEPES has routinely been shown to be a safe and effective buffer for storage of sperm from a variety of species at various temperatures (Molinia et al. 1994). Although efficacy of HEPES with sperm is fairly widely accepted, its safety with oocytes and embryos is debated.

The HEPES buffer and human serum albumin have a protective effect against the action of free oxygen radicals. Concentrations of free oxygen radicals are increased in the seminal plasma of infertile men (De Lamirande & Gagnon 1995), leading to sperm membrane and DNA damage. Because HEPES medium is a potent DNA protector (Ermilov et al. 1999), it can be postulated that early incubation of the ejaculate in HEPES buffered medium may enhance the chances of successful fertilization.

The other Good's buffer that has been incorporated by some commercial companies in their IVF handling media is 3-(*N*-morpholino)-1-propanesulfonic acid (MOPS). MOPS, offers a pKa of 7.2, which happens to be closest of the 20 zwitterion buffers to pH_i of embryos of 7.12 (Phillips et al. 2000).

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Table 4.8 Recommended pH-ranges of sequential and monoculture media for human IVF

Sequential culture media system		
Company	**Medium**	**pH-Range**
Cook Medical	Cleavage	7.3–7.5
	Blastocyst	7.3–7.5
FertiPro	FERTICULT IVF Medium	7.2–7.6
	FERTICULT G3 Medium	7.3–7.6
InVitroCare	IVC-TWO	7.25–7.45
	IVC-THREE	7.3–7.6
Irvine Scientific	ECM	7.2–7.25
	MultiBlast	7.3–7.4
Origio	EmbryoAssist	7.3–7.5
	BlastAssist	7.3–7.5
	ISMI	7.2–7.4
	ISM2	7.2–7.4
	Quinns Advantage Clevage	7.1–7.3
	Quinns Advantage Blastocyst	7.2–7.4
Vitrolife	G-1 PLUS	7.27 ± 0.07
	G-2 PLUS	7.27 ± 0.07
	IVF	7.35 ± 0.10
	CCM	7.35 ± 0.10
Monoculture one medium system		
Company	**Medium**	**pH-range**
LifeGlobal	Global	7.2–7.4
Gynemed	GM501	7.2–7.4
Irvine Scientific	SSM	7.28–7.32
CellCura	Protein Free Medium	7.2–7.4

IVF, *in vitro* fertilization.

Although both these buffers have been found to be efficient, there is still room for improvement when it comes to pH buffering.

Use of phenol red as a colored pH indicator

Quinn and Pool (2004) reviewed the advantages and disadvantages of using phenol red as a pH indicator. Although the pH range specified by most commercial suppliers is in the range of 0.2 units (**Table 4.8**), it is a good practice to measure pH of each new lot of culture medium that is being used, once equilibrate in the culture incubator. Frequency of opening of the culture chamber also affects pH making its measurement sometimes difficult to discern.

Since it is not practical to measure the pH of the culture medium during actual culture of gametes and embryos, an alternative to monitor pH is to supplement the media with a colored pH indicator. In the majority of the commercial media, the most widely used indicator is sodium salt of phenol red because of its greater solubility. The concentration normally used varies from 0.003 to 0.010 g/L. At a high pH, e.g. >7.45, the phenol red color is more purple; at a low pH, e.g. <7.15, the color becomes more yellow, and over the preferred range of 7.15–7.35, the color is pinker. Although Ernst et al. (1989) have demonstrated mild estrogenic effects of phenol red, it is accepted that the use of phenol red has been more helpful in providing greater pregnancy rates than hindrance. The concern of estrogenic effect was laid to rest by Moreno-Cuevas and Sirbaskus (2000), who demonstrated that the estrogenic effects of phenol red may be more of 'red herring' than real, as the concentration used in a typical culture media had no estrogenic effect when thoroughly tested in a number of estrogenic sensitive cell lines. However, embryologists, who remain concerned about the estrogenic effect of phenol red, can use culture medium that has no phenol added to it. The pH can still be monitored in a separate culture dish with a droplet of culture medium containing phenol red or alternatively along with culture droplets without phenol red in them. No deleterious effects have been reported on the sperm function during preparation and when cultured with the oocyte.

AMINO ACIDS

Gardner et al. (2001) and Pool (2002) have extensively reviewed the role of amino acids in the culture media. In brief, amino acids, a collective term, which includes all 20 common and naturally occurring amino acids, may be considered the most important constituents of all handling and culture media. They play vital roles: acting as chelators, osmolytes, pH_i buffers, antioxidants, regulators of energy metabolism, biosynthetic precursors, and energy substrates (Bavister 1995, Gardner

JAYPEE BROTHERS

1998, Ali 2006). Analysis of the oviduct and uterine fluids has revealed the presence of significant levels of free amino acids (both essential and non-essential) (Gardner & Leese 1990), which have been shown to enhance development to blastocyst stage and increased viability (Devreker et al. 2001).

VITAMINS

Lane and Gardner (1998) have demonstrated the beneficial effects of vitamins contained in Eagle's minimal essential medium (MEM). When added as antioxidants to the culture media containing glucose and phosphate, they helped prevent a loss in respiration and metabolic control. In the presence of amino acids, vitamins helped maintained a more normal metabolic activity in mouse and rat blastocysts. Although McLeod (1943) reported that oxygen radicals are toxic to the motility of human sperm, the significance of these radicals was not appreciated until recently.

It is established that vitamin E is one of the most important antioxidative molecule, residing mainly in the cell membranes. It may interrupt the chain reactions with lipid peroxidation and scavenge free radicals generated during the univalent reduction of molecular oxygen and during normal activity of oxidative enzymes (Palamanda & Kehrer 1993). These radicals, if left alone, will lead to peroxidation of phospholipids in the mitochondria of sperm and reduce motility accordingly (De Lamirande & Gagnon 1992a, 1992b).

Based on these studies and, since vitamins are essential nutrients for cultured mammalian cells, it is now a common practice for most commercially prepared media to have Eagle's MEM vitamins included in their media for the culture of postcompaction human embryos (McKiernan & Bavister 2000) and protection of the sperm from reactive oxygen radicals. Among the known vitamins used in different ART culture media, ascorbic acid, cyanocobalamin, folic acid, and tocopherol are the most commonly used.

MACROMOLECULES

It is recognized that serum provides beneficial factors including amino acids and vitamins, which are essential for development to the blastocyst stage (Bavister 1995).

However, Gardner et al. (1994) have observed an abnormal increase in lamb offspring weight when sheep embryos were cultured in medium supplemented with serum. Because human serum albumin is pooled, each batch of serum contains differing levels of metabolites, growth factors, hormones, and protein, making it impossible to standardize culture conditions within a laboratory (Maurer 1992). However, today, most of commercial media include synthetic serum, which eliminates any risk of potential contamination and infection, batch-to-batch variation and has a known composition, thus standardizing the culture media.

Proteins help maintain the stability of cell membranes by preventing membrane peroxidation and chelating trace amounts of toxic components that may be present in the media and culture dishes. Furthermore, albumin provides up to 80% of the total colloid osmotic pressure in the plasma and is involved in the transport of CO_2 and further acts as pH buffer. Capillary membrane permeability and osmoregulation is also attributed to albumin. By altering the surface tension, albumin facilitates manipulation of gametes and embryos by preventing them sticking to the culture vessels (Gardner 2008). In sperm, albumin also has a protective effect against the action of free oxygen radicals.

OSMOLALITY

The regulation of the cell volume is directly related to the osmolality of the fluid in which it is cultured. Animal cells control their cell volumes by actively adjusting the cytoplasmic concentrations of somatically active solutes, thus altering intracellular osmotic pressure (Hallows & Knauf 1994). As cell volume decreases, intracellular pressure increases, resulting is osmolytes being imported to restore the cell volume. Cell volume regulation is critical for embryo health and normal development.

Glutamine, glycine, betaine (*N*, *N*, and *N*-trimethylglycine), proline, and β-alanine are potential organic osmolytes, which are used by preimplantation embryos because they each ameliorate the detrimental effects of increased salt concentration in media (Biggers et al. 1993, Dawson & Baltz 1997). Organic osmolytes are a diverse array of small, neutral organic compounds that can provide intracellular osmotic support without perturbing cell function even at high intracellular concentrations, in contrast to ions (Garcia-Perez & Burg 1991).

Mammalian sperm, freshly produced in the testis and discharged into the epididymis, are exposed to fluids of increasing osmolality (**Table 4.9**). These changes in osmolality require the regulatory volume in sperm to be decreased to counteract the tendency of swelling (Yeung et al. 2006). It has been reported that human ejaculated sperm fail to penetrate cervical mucus if the volume regulation is inhibited, possibly due to reduced swimming velocity of the swollen sperm cells (Yeung & Cooper 2001). Furthermore, in infertile men, sperm volume regulation capacity is drastically reduced compared with that in fertile men (Fetic et al. 2006) and donors (Yeung & Cooper 2008).

In addition to various ion channels and transporters (Lang 2007), sperm also regulates water volume through organic osmolytes, such

Table 4.9 Different osmolality faced by sperm in mammalian male and female reproductive tracts

Site	Osmolality (mmol/kg)	Reference
Post testes (retetestis)	280–320	Cooper and Young 2003
Corpus – epididymis	480 (Hamster)	Cooper and Young 2003
Vas deference	340 (Human)	Hinton et al. 1981
Seminal fluid	294 (Human)	Cooper et al. 2005
Cervical mucus	287	Rossato et al. 2001
Uterine fluid	284	Casslen and Nilsson 1984
Oviduct fluid	268–280	Granot et al. 1998

JAYPEE BROTHERS

as myo-inositol, carnitine, sorbitol, taurine, and glutamate, all found at high concentrations in epididymal fluid (Yeung et al. 2006).

CONCLUSION

It has been shown that human embryos can develop *in vitro* in rather different types of media from basic systems to sequential complex culture media. Current ART culture media contain only a subset of the constituents, which are found under *in vivo* conditions. Hence, embryos cultured *in vitro* are exposed to constant stress possibly leading to lower pregnancy and higher abortion rates.

It is also evident that all necessary steps in ART treatment of infertility can influence the epigenetic programming during early development (Rivera et al. 2008). It therefore warrants that high level of quality controls should exist in the laboratory.

Although no specific media have been designed or developed for sperm, *in vivo*, sperm function optimally in the fluids of the female reproductive tract. Because most of the commercially prepared media have the same chemical constituents found in tubal fluid, it is reasonable to use these media for sperm preparation and manipulation, thus achieving successful outcome.

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JAYPEE BROTHERS

MALE INFERTILITY
SPERM DIAGNOSIS, MANAGEMENT AND DELIVERY

Male Infertility: Sperm Diagnosis, Management and Delivery provides a comprehensive review of male infertility and the clinical and laboratory tools currently available for its treatment. It highlights the significance of advances in assisted reproductive technology, including sperm preparation for insemination, the role of cryopreservation, techniques for obtaining sperm after retrograde ejaculation and the preparation of sperm from men who are infected with blood-borne viruses.

The book opens with an introduction to the anatomy and physiology of the male and spermatogenesis. Subsequent chapters discuss the causes and effects of poor semen quality, semen analysis, cryo-banking and oncology, ethical and regulatory requirements in the use of male gametes for fertility treatment, and future technologies.

Richly illustrated throughout, this is a key resource for clinicians and biomedical scientists who are training in reproductive medicine, gynaecology, oncology, infertility and embryology.

- Covers all aspects of sperm diagnosis, management and delivery
- Written and edited by leading experts in the field
- Up-to-date reference values are highlighted as well as the benefits of using the most advanced technology available
- Reviews of cutting-edge matters such as sperm DNA fragmentation tests and their role, and the relevance of immunological phenomena in modern infertility practices

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