J Assist Reprod Genet (2011) 28:711–724
DOI 10.1007/s10815-011-9582-0

TECHNICAL INNOVATIONS

Biological pH buffers in IVF: help or hindrance to success

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Received: 24 March 2011 / Accepted: 13 May 2011 / Published online: 26 May 2011

Abstract
Purpose Minimizing environmental stress helps maintain cellular homeostasis and is a crucial component in optimizing embryo development in vitro and resulting ART success. One stressor of particular interest is pH. Biologic buffers, such as HEPES and MOPS, are valuable tools for stabilizing pH. The objective of this manuscript is to summarize efficacy and impact of various pH buffers used during IVF lab procedures
Methods Keyword searches were performed using Pubmed and Medline and relevant literature reviewed.
Results Various pH buffers have been used with varying degrees of success for gamete and embryo processing in a variety of animal species, as well as in human.
Conclusion Though biologic buffers off a means to improve pH stability, not all buffers may be appropriate for use with gametes and embryos. Specific buffers may have undesired effects, and these may be buffer, species, cell type or concentration dependent. Continued research is needed to further refine and improve the use of biologic buffers for use in human ART.

Keywords pH · Zwitterion · Good's buffer · Gamete · Embryo

Capsule Various biologic pH buffers and their efficacy for use with gametes and embryos are reviewed and potential areas for improvement are discussed.

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Introduction

Since the first reports of culturing embryos in media formulated for somatic cells [1, 2], several improvements have occurred to vastly improve embryo culture conditions in vitro. Detailed studies on embryo metabolism, examination of tubal fluid composition and other physiologic, molecular and biochemical insights have led to improved modifications of media formulations. This has resulted in several excellent culture media, both mono and sequential systems, giving laboratories multiple choices in terms of selection of ingredients such as glucose concentration or amino acid complement [3–18]. However, despite the number of options in regard to substrate selection for embryo culture media for use within the laboratory incubator, the ability to select from a variety of pH buffers in handling media for use outside the incubator is extremely limited.

To be clear, pH is an important variable in the culture environment. External pH of culture media (pHe) influences sperm binding and motility [7–9], oocyte maturation [10, 11] and embryo development [12–16], though confounding factors such as bicarbonate and CO_2 levels exist. Improper intracellular pH (pHi) can impede sperm function [19–22]. Improper pHi is also detrimental to embryo development [23–27], impairs embryo metabolism [28, 29], alters organelle localization [30], and even retards resulting fetal growth [31]. Furthermore, periodic fluctuations in environmental conditions, such as the pHe of media, can be harmful as these can be transduced into deleterious intracellular perturbations [32]. This is more apparent in cell types like the denuded, mature oocyte [26, 33–35], or cryopreserved/thawed embryos [28], which lack robust pHi regulatory mechanisms. In particular, these cells are especially susceptible to deviations in pHe. Therefore, it is readily apparent that buffers used to stabilize pHe are extremely important

factors to consider in optimizing embryo culture systems and further examination and refinement of their use is warranted.

Importantly, independent of pH, buffers may impact cell development and function. Different cell types display different growth rates dependent on the type of buffer used as well as concentration [36–39]. Different buffers can also differentially impact various cellular processes or laboratory assays, including electron transport, photophosphorylation, and mitochondrial oxidation [36, 39]. Similarly, several reports examining impact of various pH buffers used in sperm storage or cryopreservation media demonstrate that some buffers are better than others in maintaining post-thaw motility and membrane integrity [40–44]. However, very few comparative studies have been performed to assess the impact of various biological buffers on oocyte and embryo development [45, 46]. At least one study shows that different pH buffers used in media to collect oocytes and embryos can result in differing levels of gene expression in bovine embryos [47], and this may have consequences on resulting embryo quality. Additionally, special concern may lie with procedures such as ICSI or embryo transfer, where some labs use buffered media. In these cases, buffer can be directly introduced into the oocyte or into the endometrium, where the impact of buffer is largely unknown. Finally, cryopreservation media contain buffers. Thus, in most laboratories, gametes and embryos are exposed to these buffers at some point during the IVF process. Therefore, optimization of the buffering system used for ART procedures may allow for further improvement of embryo quality and resulting success rates.

pH buffers

A pH buffer is a substance that acts as a weak acid and/or a weak base so that the pH of the solution to which it is added will be resistant to a change in pH in response to various insults. This occurs through accepting or donating hydrogen ions, which are ultimately responsible for establishing pH.

In cell culture, including IVF, the most common buffer used in media is sodium bicarbonate. The pH of sodium bicarbonate-containing media is sensitive to the amount of carbonic acid formed from the relative amounts of CO_2 in the atmospheric conditions immediately surrounding the culture dish. The pH of the media can be maintained as long as levels of CO_2 remain constant in the incubators; however, this can be problematic with repeated incubator openings/closings for cell observation as well as for manipulations performed at room atmosphere. Though some laboratories use isolettes, or portable working incubators to maintain elevated CO_2 and pHe for various procedures, these devices can be expensive and cumbersome. For procedures performed in room atmosphere, like gamete collection, ICSI, cryopreservation, and embryo transfer, many labs choose to utilize handling media with reduced bicarbonate levels and inclusion of another pH buffer to maintain pHe outside the incubator.

Buffers are generally selected based on their optimal pH buffering capacity or pKa value (Table 1). This is the log of the acid dissociation constant (Ka), or the point where equilibration is reached and equal portions of acid and conjugate base exist in solution, thus providing the highest

Table 1 Various pH buffers that have been used with gametes and embryos from various species, or whose buffering properties may warrant future examiniation. Buffer pKa values are listed at both 20° or 37°C to demonstrate impact of temperature of buffers (zwitterion values obtained from references 26, 28, 29). For maximal pH buffering, the pKa value should be close to the desired working pH of the solution/media

Common Name	pK_a at 20°C	pK_a at 37°C	Temp Effect dpH/dT in (I/K)	Full Compound Name
Tris	8.3	7.82	−0.028	tris(hydroxyrnethyl)rnethylarnine
Tricine	8.15	7.79	−0.02 1	N-tris(hydroxyrnethyl)rnethylglycine
TAPSO[b]	7.7	7.39	−0.018	3-[Ntris9Hydroxyrnehtyl)rnethylarnino]-2- hydroxypropanesulfonic acid
DIPSO	7.6	7.35	−0.015	2, 3[N-bis(hydroxyethyl)arnino]-2 hydroxypropanesulfonic acid
HEPES	7.55	7.31	−0.014	4-2-hydroxyethyl-1 -piperazineethanesulfonic acid
TES	7.5	7.16	−0.020	2{[tris(hydroxyrnethyl)rnethyl]arnino}ethanesulfonic acid
PhosphoricAcid[a]	7.21	7.19	−0.001	
MOPS	7.15	6.93	−0.013	3-(N-rnorpholino)propanesulfonic acid
BES	7.15	6.88	−.0.016	N.N-bis(2-hydroxyethyl)-2-arninoethanesulfonic acid)
PIPES	6.8	6.66	−0.008	Piperazine-N.N'-bis(2-ethanesulfonic acid)
Carbonic Acid[a]	6.38	6.30	−0.005	
MES	6.15	5.96	−0.011	2-(N-morpholino)ethanesulfonic acid

[a] Non zwitterionic buffers-Polyprotic acids with multiple pKa's

[b] Not tested with gametes or embryos, but may be appropriate based on pKa

buffering capacity (ability to resist pH change). Because many biological processes or laboratory assays are only functional over a small range of pHe, the ideal buffer utilized should have a pKa value close to the working pHe of the solution to efficiently maintain the pHe within this working window. When this occurs, a lower buffer concentration can be utilized, which is often safer than using higher concentrations. An additional consideration to selecting an optimal buffer is the general rule that amine or zwitterionic buffers are less likely to be inhibitory or reactive in their protonated form in comparison to their non-protonated form [38]. Thus, in these cases, it is usually safer to select a buffer with a pKa value slightly above the pHe.

Goods buffers

In an attempt to improve the selection of pH buffers for use with biological systems, Normann Good and colleagues developed a series of new buffers [36–39]. These new compounds were largely zwitterionic aliphatic amines, or altered amino acids, the majority consisting of N-substituted taurine and glycine. Zwitterions have the ability to act as either an acid or a base, and are excellent buffers of pH. In development of these new compounds, various criteria were set to ensure their usefulness for biologic research. These included:

1. **pK_a**. Because most biological reactions take place at or near neutral pH between 6 and 8, ideal buffers should have pK_a values in this region to provide maximum buffering capacity.
2. **Solubility**. For ease in handling and because biological systems are in aqueous systems, high solubility in water is required. Low solubility in nonpolar solvents, like fats and oils, is also considered beneficial, as this will tend to prevent the buffer compound from accumulating in nonpolar compartments in biological systems, such as cell membranes.
3. **Membrane impermeability**. Ideally, a buffer will not readily pass through cell membranes, as this will also reduce the accumulation of buffer compound within cells.
4. **Minimal salt effects**. Highly ionic buffers may cause problems or complications in some biological systems.
5. **Dissociation**. Buffer dissociation should be minimally affected by buffer concentration, temperature of the system and ionic composition of the medium.
6. **Well-behaved cation interactions**. If the buffers form complexes with cationic ligands, the complexes formed should remain soluble. Ideally, at least some of the buffering compounds will not form complexes.
7. **Stability**. The buffers should be chemically stable, resistant to enzymatic and non-enzymatic degradation.
8. **Optical absorbance**. Buffers should not absorb visible or ultraviolet light at wavelengths longer than 230 nm to avoid interference with commonly-used spectrophotometric assays.
9. **Ease of preparation**. Buffers should be readily prepared from inexpensive materials and easily purified.

Importantly, no one buffer meets every one of these ideals. Some buffers perform better than others, and careful comparisons are needed to determine which buffer is best suited for a particular purpose.

Buffers and temperature

Though often overlooked, temperature plays a significant role in pH and pH buffering. In general, as temperature increases, pH and pKa values decrease [36, 39] (Table 1). This has been measured for buffers and handling media commonly used in IVF [46] (Fig. 1a). This is important because many laboratories warm their handling media to around 37°C. Furthermore, cryopreservation procedures commonly performed in IVF also entail significant changes to temperature, and likely to pHe/pKa (Fig. 1a, c). Thus, a buffer that may provide optimal buffering for some procedures, may not be appropriate for other procedures performed at different temperature. Another consideration is how much pKa and pH change in response to temperature, as some buffers are more resistant to change than others (Table 1). Regardless of the buffer chosen, it is crucial to maintain an appropriate and constant temperature to avoid changes in pH. Due to this relationship, many studies detailing the effects of temperature on cellular structure and function, such as oocyte meiotic spindle organization, cannot rule out a role for pH in regulation of these processes. Therefore, in addition to refinement of buffer systems, continued research into the role of pH on control of gamete and embryo function is likely prudent.

At least one study has attempted to address this issue of temperature and change in pHe of buffered media by formulating a temperature independent pH (TIP) buffer [48]. By utilizing spectroscopic assessment of color change of a pH indicator included the media, researchers combined two buffers, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) and a phosphate-buffered solution. While the apparent pH of HEPES-buffered media rose when cooled and phosphate decreased, combining the two buffers resulted in a color stabilization indicating a stable apparent pH. This approach proved to be beneficial in storage of the pH sensitive compound, oxacillin, and improved stability compared to HEPES or phosphate buffered media. A similar approach could prove valuable for cryopreservation of gametes and embryos, especially for pH sensitive cells, like the oocyte (Fig. 1b, c).

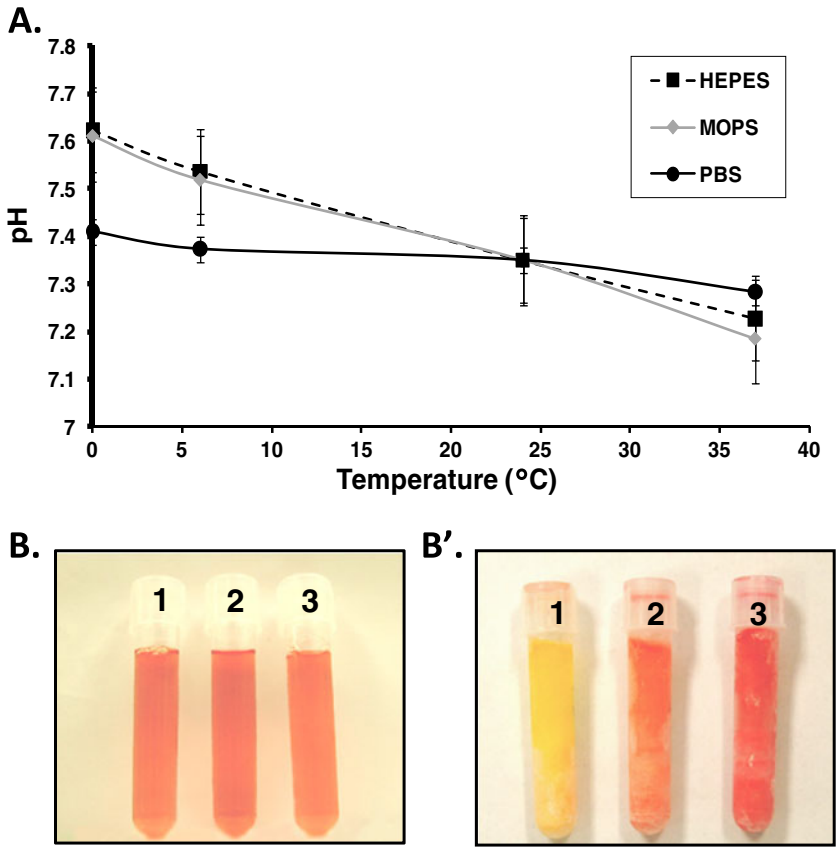

Fig. 1 Temperature can impact pKa as well as actual pHe of buffered solutions. **A** Change in pHe with change in temperature of various buffered media, as measured with a pH meter. While temperature can influence media pHe, direct measurement of pHe during freezing is difficult. The apparent change in pHe of media can be assessed by colormetric change of a pH indicator (phenol red). **B** Media at room temperature with buffer 1) phosphate, 2) 1:1 ratio of phosphate: HEPES 3) HEPES. pH of all media was ~7.3. **B'** Media following direct plunge into liquid nitrogen (~−196°C) 1) phosphate, 2) 1:1 ratio of phosphate:HEPES 3) HEPES. Phosphate buffered media appears to become acidic, HEPES media becomes alkaline, and a combination of the media is in between, as an apparent pH closer to that of media at room temperature. This approach of combining various buffers may allow for formulation of a temperature independent pH buffering system that may prove useful for cryopreservation of gametes and embryos

Concerns with buffers

As mentioned, buffers can have effects other than pH buffering [36–39]. This has been well demonstrated in somatic cells or in laboratory assays; however, thorough testing of buffer effects in human IVF is scarce (Table 2) [49]. Additionally, though continued research on buffers is warranted, some concerns with their use in human ART are unfounded, as poor experimental design and misinterpretation of the literature have propagated invalid conclusions [50]. For example, any concern with buffers and use for ICSI due to potential impact on pHi within the oocyte is unwarranted. It has been shown that use of media buffered with Good's buffer (TES, MOPS, HEPES) has no effect on mouse oocyte pHi compared to a bicarbonate-only buffered following microinjection [29]. In fact, many concerns raised in regard to zwitterionic buffers and IVF are confounded by simultaneous alterations in other media components that impact embryo development, such as CO_2 and bicarbonate levels [51–56]. Importantly, though procedures in ART using handling media with buffers generally entail only brief exposures, even brief exposure to inappropriate handling media can be detrimental to embryo development [47, 57, 58]. Therefore, attempting to isolate the potential impact of the specific buffer from other aspects of these media is warranted and may help improve the culture system. Keeping in mind the benefits and limitations of biological pH buffers, the efficacy of various buffer systems utilized in IVF can now be discussed.

Buffers in IVF

Phosphate Phosphate is a triprotic acid and the buffering base for phosphate buffered solutions (PBS). Because one

Table 2 Biologic pH buffers that have pKa values most appropriate for use with human gamete and embryo processing media, as well as the procedures in which they are commonly used

Buffer	Procedure				
	Sperm Washing	Ooocyte Retrieval	ICSI	Embryo Transfer	Cryopreservation
DIPSO	Unknown	Unknown	Unknown	Unknown	Unknown
HEPES	Used successfully[a]	Used successfully[a]	Used successfully[a]	Used successfully[a]	Used successfully[a] gamete/embryo slow rate & vitrification
MOPS	Used successfully[a]	Used successfully[a]	Used successfully[a]	Used successfully[a]	Used successfully[a] sperm slow rate & oocyte/embryo vitrification
Phosphate	Unknown	Used historically[a] not recommended	Unknown	Unknown	Used successfully[a] blastocyst vitrification
TEST	Unknown	Unknown	Unknown	Unknown	Used successfully[a] sperm slow rate
HEPES:MOPS mixture	Used successfully	Unknown	Used successfully	Unknown	Unknown

[a] commercially available

of the pKa values of PBS is 7.2, it offers efficient buffering for biologic procedures occurring around this pH. In the past, handling media in IVF have included phosphate buffered saline solutions, and some laboratories continue using this media for procedures like oocyte retrieval. However, while PBS possesses an appropriate pKa, its use may have damaging effects to cellular function. Use of phosphate buffer tends to precipitate most polyvalent cations, while also acting as a metabolite or inhibitor in various systems. Indeed, elevated levels of phosphate may compromise gamete and embryo metabolic activity, disrupt organelle distribution, and interfere with intracellular ionic homeostasis, including pHi [59–61]. Decreased motility of boar sperm was observed when stored in phosphate buffered diluents, compared to various zwitterion buffered media [41]. Authors proposed this may be due to metabolic disruption, possibly via mediation of the Crabtree effect [62]. Use of media buffered with phosphate yielded very low rates of fertilization in hamster oocytes compared to other buffers tested [63]. While these studies utilized longer period of exposure, even brief exposure to phosphate buffer as a handling media has been shown to compromise hamster and rabbit embryo development [57, 58] and results in aberrant gene expression in bovine embryos when compared to other buffers [47]. However, in these studies, results could not be attributed to phosphate alone, as the basal media and energy substrate composition also differed between treatments. When examined in a side-by-side fashion in media with the same substrate composition, use of phosphate buffer was inferior to media buffered with HEPES. When used for embryo culture, phosphate resulted in significantly lower blastocyst formation and live birth rates in mice following transfer in comparison to a HEPES buffered medium [56].

While evidence seems to support the notion that phosphate buffered solutions are a poor choice for use in IVF procedures, the media has been utilized successfully for cryopreservation procedures in various species. Phosphate buffered solutions can apparently provide excellent results when utilized with human blastocysts [65]. However, comparative studies examining efficacy of the buffer to other compounds is scarce. Though numbers of embryos tested were very low and precluded statistical analysis, early experiences with slow rate cryopreservation procedures with DMSO demonstrated phosphate buffered medium was able to successfully cryopreserve human embryos seemingly better than a HEPES buffered medium [66]. In contrast, when used with DMSO for vitrification of 2-cell mouse embryos, phosphate buffered media resulted in reduced embryo development and lower inner cell mass numbers compared to HEPES media [64].

One concern with use of phosphate buffer and cryopreservation remains the impact of temperature on pH. Using spectrophotometric methods, it has been observed that the apparent pH of phosphate buffered solutions decreases significantly upon slow rate freezing [48]. Similarly, Quinn noted that in examination of the color of a phosphate buffered medium with phenol red used during slow rate freezing, at −80°C, the "color is yellow", indicating a severe acidification [66] (Fig. 1b, c). Whether this is a true pH change, or simply an effect of the concentration of solutes in the remaining liquid phase as ice crystals form is unclear (Fig. 1a). What is known is that buffering capacity of phosphate compared to Good's buffers in response to an acid/base challenge is greatly reduced. Furthermore, it is plausible that with the cooler temperatures, cellular metabolism is slowed, thereby decreasing any potential detriment of phosphate on these functions. If the dramatic acidifica-

tion of pHe does occur, then a greater concern lies with slow rate freezing protocols, which extends the exposure time of cells to these altered conditions, in comparison to vitrification. In this respect, other buffering systems may be more appropriate.

Tris Tris(hydroxymethyl)aminomethane (Tris) buffer is used extensively as a buffer component for solutions used in various biochemical and molecular assays. However, as a primary aliphatic amine, Tris is highly reactive and often inhibitory to various cellular processes. Therefore, its use for cell culture is often limited. Further adding to the concerns of using Tris is that it provides poor buffering below pH 7.5 (27). Lastly, the pH of media buffered with Tris can change dramatically as temperature changes when compared to other buffers (29). As a result of these characteristics, reports of using Tris for oocyte or embryo manipulation are lacking.

Despite these limitations, the combination of Tris with other buffers has been used extensively in diluents and cryopreservation solutions for mammalian sperm [40]. This is discussed later. When used alone, Tris-HCl buffer in boar sperm diluents yielded significantly lower sperm motility following storage at 37°C or 5°C compared to seven Goods buffers studied (MES, PIPES, BES, MOPS, TES, HEPES, Tricine). Furthermore, Tris-HCL also resulted in the greatest amount of sperm membrane damage, as indicated by the increased release of glutamine oxaloacetate tranaminase (GOT) from sperm in comparison to the other buffers studied [41]. However, when used to titrate pHe of media with various zwitterionic buffers, Tris resulted in higher bovine sperm motility after freezing than titration with other bases like NaOH [41]. Titrating various buffer solutions with Tris has also been shown to not adversely affect post-thaw motility of bull sperm [43], but significantly impaired motility of turkey sperm [42], suggesting perhaps some species-specific sensitivity.

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) is a zwitterionic organic buffer, also known as a Good's buffer, that is a modified taurine molecule. HEPES can be utilized as a free acid, or conjugated to various salts (Fig. 2). During their initial formulation, HEPES was one of the more superior buffers for supporting growth of various cell lines and in regard to performance of various biologic assays [36–39]. Similarly, HEPES has routinely been shown to be a safe and effective buffer when compared to other buffers for storage of sperm from a variety of species at various temperatures [41, 44, 67]. In one study, HEPES allowed for maintenance of boar sperm membrane integrity following storage at 5°C and resulted in the lowest amount of GOT release from sperm. This effect was not significantly different from BES, TES and PIPES, but was significantly better than MOPS, MES and Tricine [41].

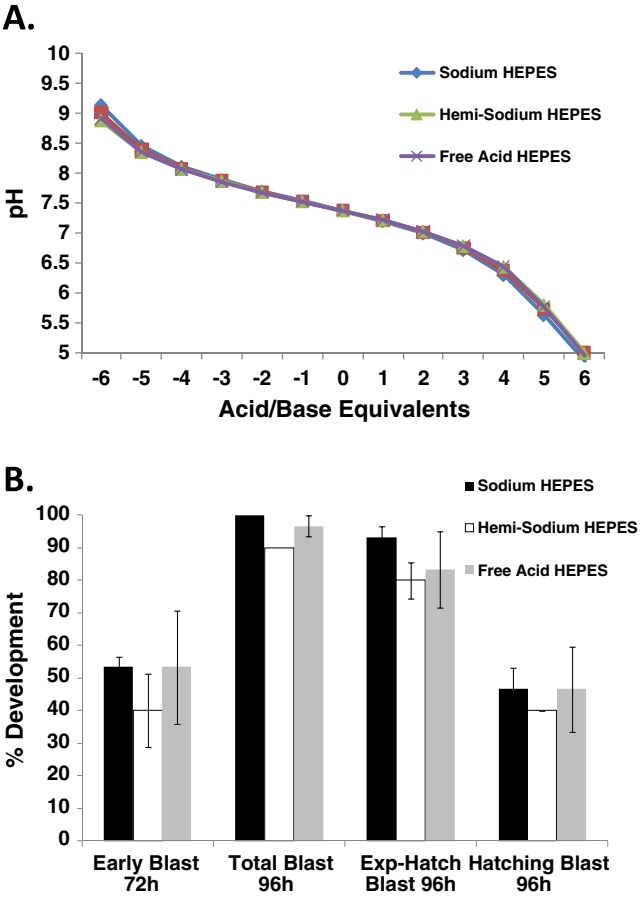

Fig. 2 Buffers, like HEPES, come in various forms. **a** Dose titration of HEPES forms indicate no difference in buffering capacity. **b** Frozen/thawed 1-cell mouse embryos cultured in media containing various forms HEPES showed no significant differences in development

While efficacy of HEPES with sperm is fairly widely accepted, its safety with oocytes and embryos is debated. This is largely due to the fact that HEPES is arguably the most-widely studied buffer. In regard to oocytes, it has been reported that HEPES-containing media used for ICSI of human oocytes compromised resulting embryo quality [50]. Additionally, it was reported that 10% HEPES used during maturation caused mouse oocyte degeneration [68]. However, careful examination of these studies point out key deficiencies in the experimental designs and conclusions. As previously mentioned, various confounders such as concentration, pH, and ionic composition are often not accounted for. Numerous studies actually indicate HEPES is able to support oocyte maturation [45, 69], fertilization [70–72] and embryo development [56, 72–74] at room atmosphere efficiently. Some studies have indicated inhibition of glucose uptake by mouse oocytes [75], lower fertilization rates [76], or compromised blastocyst formation in presence of HEPES [77], but are likely due, at least

in part, to the simultaneous reductions in CO_2 levels and bicarbonate concentrations. Embryo development is supported in the presence of HEPES when bicarbonate is present, but not when bicarbonate is absent [56]. Bicarbonate levels influence blastocyst development, possibility through activity of various HCO_3 dependent transporters. Furthermore, when embryos are cultured at room atmosphere and compared to controls cultured in 5% CO_2, differences in development cannot be attributed to HEPES alone. Elevated CO_2 of the laboratory incubator is utilized by embryos for various biochemical processes as a carbon source [51–53], and is likely beneficial over culture at room atmosphere, highlighting the importance of the surrounding environment despite which buffer media is chosen. Indeed, when CO_2 and bicarbonate levels are accounted for as variables, media with up to 50 mM HEPES yielded similar rates of blastocyst development and cell number compared to media without HEPES [46].

Despite numerous studies citing the safety and efficacy of HEPES with gametes and embryos, data exists to suggest buffer concentration may be an important consideration. Historically, HEPES at 21 mM has been a standard for IVF handling media utilized for handling of gametes and embryos. Inclusion of HEPES at various concentrations from 2.5–25 mM in media used in elevated CO_2 concentrations has been used to mature oocytes [78], fertilize eggs [76, 78] or culture embryos successfully [78–80], yielding rates similar to media with no HEPES present. Only when HEPES exceeded 35 mM in porcine embryo culture was any increased embryo fragmentation observed [79]. When used at 42 mM, HEPES supported hamster fertilization, but an increased incidence of abnormalities was observed. Again, these observations could also be due to reduced bicarbonate [63]. Reduction in HEPES concentration from 120 mOsm to 50 mM resulted in improved sperm survival following cryopreservation [81]. Also, when the concentration of HEPES was raised from 20 to 25 mM, there was a marked augmentation of meiotic arrest induced by chemical inhibitors [45]. Interestingly, it has been demonstrated that 25 mM HEPES has no adverse effect on mouse preimplantion embryo development, and that there are no adverse affects of up to 50 mM HEPES when cultured with 25 mM NaHCO3 in ~5% CO_2 [46]. Though there may be species specific sensitivities to HEPES, these data indicate that when adequately controlling for other factors such as osmolality, ionic composition, gas levels and pH, HEPES is able to successfully support mammalian embryo development.

Finally, it is also important to differentiate between the impact of the buffer itself, and interaction with other components in the media. Increasing HEPES concentration from 20 to 25 mM did not affect spontaneous oocyte maturation, but did suppress ability to induce FSH-stimulated meiosis in pharmacologically inhibited oocytes with dcAMP, but not hypoxanthine [45]. Importantly, these studies were conducted in MEM medium, which contains riboflavin. Early studies in somatic cells citing HEPES toxicity demonstrated that detrimental effects stemmed from light exposure and interactions with riboflavin [82, 83]. Also, this effect is reduced or blocked by pyruvate, though in concentrations typically higher than that used in culture media. Thus, it is important to examine use of these buffers in the context of specific base media. This evidence could explain the observations of oocyte degeneration in the presence of HEPES [68], which were also performed in the presence of MEM medium.

MOPS 3-(N-morpholino)propanesulfonic acid (MOPS), was one of the original Good's buffers developed. With a pKa~of 7.15 at 20°C, it is very useful for buffering of biologic systems and assays that utilize a more neutral pH. MOPS is supplied as both a free acid or sodium salt conjugate. MOPS was one of several buffers tested in diluents for cryopreservation of boar, ram, turkey and bull sperm [41–43, 84 40]. MOPS yielded similar motility after storage at 37°C compared to other buffers, but MOPS was one of the top three buffers in regard to maintaining motility after storage at lower temperatures [41–43]. However, MOPS was one of the worst three buffers in maintaining membrane integrity, as measured by GOT release [41].

Regarding use of female gametes, MOPS has been evaluated for use with oocyte maturation. MOPS buffer at 20 mM successfully enabled development of mature mouse oocytes [45]. Additionally, MOPS was used at 25 and 50 mM to culture mouse zygotes to blastocysts, with no significant difference in rates of development or cell number compared to media without a zwitterionic buffer present.[46]. Gene expression profiling of environmentally sensitive genes in bovine embryos indicate that expression levels of embryos handled in MOPS or HEPES buffered media were most similar to embryos derived in vivo [47]. The buffer is now included in commercial handling media and used successfully for human ART procedures, ranging from sperm washing to oocyte and embryo handling and vitrification (Table 2). Interestingly, MOPS has been reported to be superior to HEPES for vitrification, though the exact reason for this remains unclear, and the comparison was not made in a simultaneous side-by-side fashion controlling for changes in technique and other variables [85], thus making it impossible to attribute increased success to MOPS alone.

Importantly, use of MOPS is not without concern for unexpected cellular actions. MOPS has been noted to interfere with taurine uptake in tumour cell lines [86], interact with DNA in cellular preparations [87], and interfere with chloride conductance in neurons [88].

Whether these interactions occur in mammalian gametes or embryos is unknown.

DIPSO 3-(*N*,*N*-bis[2-hydroxyethyl]amino)-2-hydroxypropanesulphonic acid (DIPSO) is another zwitterionic Good's buffer that has potential applications for ART because at 37°C, the pKa for DIPSO is 7.35. DIPSO is commercially available as both a free acid or sodium conjugate. While information regarding DIPSO and sperm motility or function is lacking, it has been used for culturing oocytes and embryos. DIPSO was used in one study examining mouse oocyte maturation and showed no adverse effects on spontaneous maturation to metaphase II at 20 mM concentration [45]. Additionally, there were no adverse effects on mouse embryo development when exposed to 25 or 50 mM of DIPSO in the presence of 25 mM NaHCO3 in ~5% CO_2, [46].

TES N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid (TES) was another of the original Good's buffers. With a pKa value of 7.16 at 37ºC, it may be the best single buffer in regard to optimal buffering capacity for use in ART, as this is the closest to the pHi of oocytes and embryos at working temperature. TES can also be obtained as either a free acid or sodium conjugate. However, while TES is used extensively for molecular assays, it has received little use in IVF. As discussed later, the greatest use of TES has been for sperm storage, as a component of a combination buffer in conjunction with Tris (TEST). By itself, TES was successfully used for cryopreservation of ram spermatozoa, yielding higher post-thaw motility and acrosome integrity than controls frozen in Tris-citrate, and results were not different compared to 5 other zwitterions buffered media (HEPES, HEPES:Tris, MES:Tris, Pipes, Pipes:Tris) [44]. TES was judged as one of the two best buffers used in diluents for storage or freezing boar sperm. Sperm exposed to TES yielded some of the highest rates of sperm motility following storage at 5°C and lowest levels of GOT release compared to other buffers studied [41].

In an attempt to avoid use of CO_2, TES was used as the buffer in medium used to fertilize hamster oocytes. At 42 mM, TES permitted 55.8% fertilization, similar to that obtained with HEPES, though both were inferior to media buffer with bicarbonate [63]. Additionally, it was noted that anomalies were observed in pronucleii formed in TES buffered medium. However, this may be due to insufficient bicarbonate in the medium, which is required for efficient fertilization, unrelated to buffer action. Importantly, TES yielded significantly different expression of environmentally sensitive genes in bovine embryos compared to MOPS or HEPES [47]. Therefore, further examination of its efficacy is warranted.

Interestingly, the structure of the TES molecule is similar to known cryoprotective agents, containing a central amide group, with three side hydroxyl groups and a double oxygen bond. Thus, it is conjectured that in addition to its pH buffering capability, TES perhaps also offers additional protection as a cryoprotectant [89]. If this is the case, use of zwitterionic buffers in slow rate cooling or vitrification media need to be evaluated in a comparative fashion, as not all buffers may perform similarly.

Other buffers Additional buffers have been explored on a very limited basis, largely for use in media used for sperm storage. Efficacy for oocyte or embryo handling remains largely unknown.

PIPES—Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) is an ethanesulfonic acid buffer developed by Good et al. in the 1960s. With a pKa value of 6.66 at 37º, PIPES may not be an ideal buffer for IVF when used alone. That being said, the buffer has been used in media for cryopreservation of ram spermatozoa. This buffer at ~39.6 mM after dilution yielded higher post-thaw motility and acrosome integrity than the controls in Tris-Citrate buffer (120 mM:38 mM after dilution), with comparable rates of pregnancy following insemination of ewes [67]. PIPES was also used as a buffer in diluents for cryopreservation of boar sperm where it yielded similar sperm motility after storage at 37°C compared to six other zwitterionic buffers, but yielded significantly lower sperm motility when used at 5°C in comparison to BES, MOPS, TES, HEPES and Tricine [41]. PIPES has been shown to support mouse oocyte maturation in vitro to MII at 20 mM; however, there appeared to be interaction with a chemical inhibitor, hypoxanthine, as inhibition of germinal vesicle breakdown (GVBD) was reduced in presence of the buffer compared to MOPS or HEPES or DIPSO [45]. No reports of PIPES and embryo development exist.

MES—2-(N-Morpholino)ethanesulfonic acid hydrate (MES) is a Good's buffer with a pKa of 5.97 at 37ºC. Thus, the buffering capacity of MES is less ideal for use in IVF than other Good's buffers. MES was used as a buffer in diluents for cryopreservation of boar sperm where it yielded similar sperm motility after storage at 37°C compared to six other zwitterionic buffers, but yielded significantly lower sperm motility when used at 5°C in comparison to BES, MOPS, TES, HEPES and Tricine [41]. MES at 50 mM was the superior buffer of those tested and maintained bull sperm motility at 5 or 37°C [81].

BES—N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)is a modified taurine molecule and

one of the original Goods buffers. With a pKa of 6.90 at 37ºC, the buffer was used as a buffer in diluents for cryopreservation of boar sperm where it yielded similar sperm motility after storage at 37°C, and was one of the top five buffers in regard to maintaining motility after storage at 5°C, similar to MOPS, TES, HEPES and Tricine [41]. Additionally, BES was one of the top three buffers used successfully for storage and freezing of turkey and bull sperm [42, 43].

Tricine—*N*-[Tris(hydroxymethyl)methyl]glycine (Tricine) is a modified glycine molecule. With a pKa of 7.80 at 37ºC, Tricine has been used as a buffer in diluents for cryopreservation of boar sperm. When compared with the other zwitterionic buffers, Tricine performed similarly to TES and HEPES in respect to preserving boar sperm motility, but did result in significantly higher release of GOT after plunging into liquid nitrogen for cold storage[41].

Combination buffers Combination of various pH buffers can be used to improve upon systems utilizing only individual buffers. As mentioned, some zwitterionic buffers like HEPES, MOPS, TES or DIPSO can be obtained as a free acid, or conjugated to various salts; like sodium and potassium. Combining free acid and salt forms of the same buffer in various ratios offers the ability to adjust the working pHe of the medium during formulation, without the need to adjust with an acid or base later (Fig. 3). This improves consistency of the media formulation. Additionally, use of these differing forms can be used to adjust levels/ratios of Na^+ or K^+ in the media, as well as osmolality, which can impact embryo development. However, it should be noted that solubility and purity of some buffer forms may be an important variable for consideration.

Furthermore, use of multiple different buffers allows for adjustment of pKa values to the desired range, while also permitting for a reduction in individual buffer concentration and potentially alleviating concerns for toxicity (Fig. 4). Varying ratios of buffer can be used to obtain the desired buffering. One of the first reports using combination of Goods buffers was by Eagle [37], who showed that different combinations of buffers can support growth of various cell lines. More recent attention has focused on use of combination buffers in handling media for IVF to further refine the current mono-buffered systems.

TES/TRIS (TEST)—While both Tris and TES have been tested individually and are not widely used in IVF, as discussed above, the combination of the two buffers is used extensively. The joint use of these two buffers is one of the first examples of a combination buffering system for ART, as TEST has long been included in media utilized for sperm processing [90]. TEST buffered media, with inclusion of egg yolk (TYB), is widely used for short storage of sperm around 4°C or for long term storage via slow rate cooling to approximately-196°C. One of the first reports of using TEST buffer with sperm was for storage at low temperature, where TEST gave some of the best results in regard to post-thaw motility and membrane integrity in comparison to seven other zwitterionic buffers titrated with four separate compounds [43]. TYB maintains human sperm motility for up to 96 h when stored at ~5°C [91, 92]. Subsequently, TYB was successfully used for cryopreservation of human sperm [93, 94]. The composition of the TEST used in current TYP medium is commonly 21 mM TES and 96 mM Tris, which is then usually diluted 1:1 with semen [90].

Use of TYB medium for sperm storage and shipment at 2–5°C maintained capability of sperm to bind for the

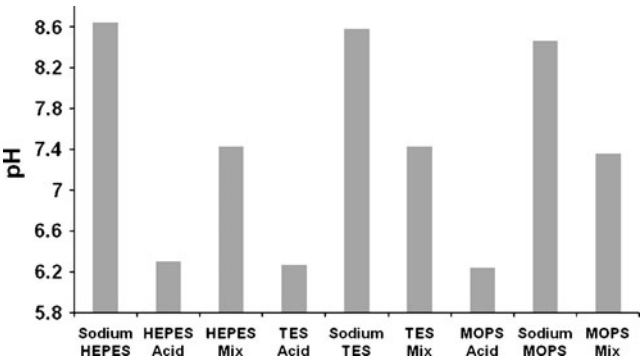

Fig. 3 Combining free acid and salt conjugated forms of various buffers offers the ability to adjust the working pHe of media during formulation, improving consistency by reducing need to adjust pHe with an external acid or base. Buffers were mixed in 1:1 ratios with a final concentration of 21 mM

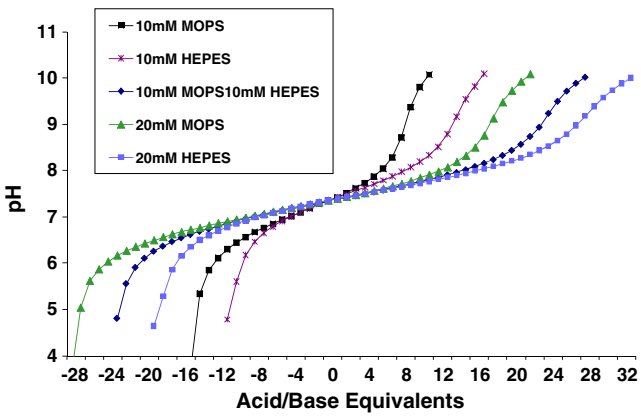

Fig. 4 Dose titration of various media using HCl and NaOH. Combination of two separate buffers, like HEPES and MOPS, allows for adjustment of pKa value to optimize buffering, while also allowing for use of lower buffer concentrations to reduce toxicity concerns (figure modified from reference 36)

sperm penetration assay. Interestingly, it was found that in some samples, the percent penetration was improved by exposure to the TEST-yolk buffer [95]. This improved penetration has been verified and 42 h was better than 18 h [96]. Preincubation in TYB at ~5°C improved results of the acrosome reaction test [97, 98], SPA [95–101], and binding in the hemizona assay [101]. Finally, and perhaps more importantly, incubation of human sperm in TYB for 2 or 24 h at 4°C prior to insemination has been reported to increase fertilization rates in couples with poor or prior failed fertilization in IVF cases [101, 104]. Though these impacts cannot be attributed to TEST buffer alone [105], the results do indicate TEST is an apparently safe buffering system and does not compromise sperm function.

While the vast majority of work involving TEST buffer involves storage of sperm, at least one study has examined the impact of the buffer system on storage of oocytes. Zona-intact hamster ova were stored in TYB at 4°C and were subsequently able to be used for the SPA, yielding 100% penetration similar to fresh ova [106]. Further studies are required to determine the true impact of TEST on oocyte and embryo function and development.

Tris:Citrate—This buffer combination has been used to freeze semen from a variety of domestic species including ram [44, 107], boar [41], bull [43] and turkey [42], though post-thaw motility and acrosome integrity was often lower than that obtained from other zwitterionic buffers studied, like HEPES, TES and PIPES [44, 107]. The buffer was also used to successfully freeze human spermatozoa [108, 109]. In early attempts to achieve fertilization in vitro in hamster, Bavister examined the use Tris-Citrate buffer (25 mM). Though an excellent pH buffer, and despite the fact that it stimulated sperm motility, no fertilization was obtained from 207 oocytes inseminated [63]. Because fertilization is obtained with TEST buffers, as mentioned above, a likely explanation for the lack of fertilization observed in Tris:Citrate is the chelation of calcium ions by citrate.

HEPES/MOPS—The first report of combining HEPES and MOPS for embryo culture demonstrated that the use of both buffers at 10 mM provided buffering at a point between HEPES or MOPS alone (Fig. 4) and also yielded similar blastocyst formation and cell number compared to use of the individual buffers [46]. Furthermore, preliminary studies suggest that a combination buffer of HEPES/MOPS in a 1:1 may be useful for procedures such as ICSI, as it yielded similar rates of normal fertilization of human oocytes (71%) abnormal fertilization (5%) and blastocyst development (74%) as media buffered with HEPES alone (63%, 12%, 52%, respectively) [110].

HEPES/MOPS/DIPSO—In the first examples of a tri-buffered media utilized for mammalian preimplantion embryos, the combination of HEPES, MOPS and DIPSO at 6.7 mM each supported mouse blastocyst development, while allowing for the adjustment of pKa and lowering of individual buffer concentrations compared to mono-buffered media [46].

Others—Various zwitterionic buffered media have been titrated to a working pH using Tris as diluents for semen. These include BES:Tris (BEST), HEPES:Tris (HEPEST), MOPS:Tris (MOPST) and PIPES:Tris (PIPEST) [111]. These are technically combination buffers, though insight into their efficacy for cell culture is limited.

Conclusion and future directions

Buffers of pH are extremely important factors of an IVF culture system. Utilization of these compounds help stabilize media pHe for procedures used outside the laboratory incubator and minimize stresses imposed upon gametes and embryos. Zwitterionic buffers, such as HEPES and MOPS, appear to be superior to buffers such as phosphate and Tris. Additionally, closer examination of additional buffers like DIPSO or TES for human ART procedures may be useful, as their pKa values lend themselves to buffering in the range used for embryo culture.

Importantly, concern does exist with use of some buffers. For procedures such as sperm washing or oocyte retrieval, these effects could be directly on the gamete. Though Good's buffers are largely membrane impermeable, use for ICSI represents a condition where buffer can be directly introduced into the cell, and could conceivably convey some undesired effect, though this remains unproven. Furthermore, use of buffers for embryo transfer or cryopreservation represent examples where buffers may impact embryo quality, or even influence the endometrium, though again, this remains untested. What is known is that deviations in pHe can be detrimental, so exclusion of buffers in procedures performed outside the laboratory incubator in itself represents a potentially harmful environmental condition. Therefore, closer examination of buffers used is required to improve upon current approaches. To this end, further exploration of combination buffer systems with new buffers may lead to further improvements in the IVF culture system. Combination buffers have proven useful for sperm cryopreservation, and emerging data demonstrates their efficacy during embryo culture. These combinatorial systems can utilize different buffers to adjust and optimize pKa, or optimal buffering, while allowing use

of reduced buffer concentrations to alleviate toxicity concerns. In addition to use of separate buffers, combination buffer systems can also use different forms of the same buffer, including different salt conjugated forms or free acid preparations. These allow for the refinement of the final working pHe without the need for titrating with HCl or NaOH during preparation, which may improve media consistency. Additionally, combination buffer systems may provide a means to improve cryopreservation media, allowing for compensation of pH changes due to temperature. Important to the endeavor of optimizing the buffering system for gametes and embryos is determining the optimal pHe for culturing gametes and embryos. Once this value is established, a combination buffer system could be formulated with a pKa value slightly above this pHe for optimal buffering.

Another area of research regarding improvement of buffers in IVF entails examination of the total concentration used. Generally, 21 mM HEPES or MOPS are used in medium with ~4 mM sodium bicarbonate. However, the rationale for the concentration is not entirely clear. A plausible explanation would seem to be that most culture media in the past had a bicarbonate concentration of ~25 mM. When bicarbonate levels were reduced to 4 mM, keeping the Na-conjugated buffer at 21 mM helps maintain media osmolality. Data from our laboratory suggests that lower concentrations of buffer may be adequate to maintain stability of pHe, especially if pKa values can be optimized via combining buffers. This reduction in concentration again may help alleviate possible toxicity concerns.

Additional benefit of pH buffers may arise from their use within media used in the incubator. Though bicarbonate concentration and external CO_2 levels will primarily regulate pHe stability, addition of zwitterionic buffers can help further stabilize pHe. This may be useful for culture approaches using extremely small volumes, including emerging microfluidic technology.

It is clear that there exists potential to improve upon current IVF handling media. Further experiments examining biochemical, molecular and genetic endpoints will aid in this endeavor. Employing these approaches may lend itself to formulation of various specialized handling media, with different media used for specific procedures, constructed with specific buffer combinations and specific pHe/pKa for specific cells types.

Acknowledgements The authors would like to thank Rusty Pool for his insight and assistance with preparation of this manuscript.

References

1. Steptoe PC, Edwards RG, Purdy JM. Human blastocysts grown in culture. Nature. 1971;229(5280):132–3.
2. Steptoe PC, Edwards RG. Reimplantation of a human embryo with subsequent tubal pregnancy. Lancet. 1976;1(7965):880–2.
3. Brinster RL. Studies on the Development of Mouse Embryos in Vitro. I. The Effect of Osmolarity and Hydrogen Ion Concentration. J Exp Zool. 1965;158:49–57.
4. Hershlag A, Feng H. The effect of CO2 concentration and pH on the in vitro development of mouse embryos. Fertil Magazine. 2001;4:21–2.
5. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. Biol Reprod. 1987;36 (5):1155–63.
6. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. Fertil Steril. 2011;95(4):1291–4.
7. Emmens CW. The motility and viability of rabbit spermatozoa at different hydrogen-ion concentrations. J Physiol. 1947;106(4): 471–81.
8. Pholpramool C, Chaturapanich G. Effect of sodium and potassium concentrations and pH on the maintenance of motility of rabbit and rat epididymal spermatozoa. J Reprod Fertil. 1979;57(1):245–51.
9. Dale B, Menezo Y, Cohen J, DiMatteo L, Wilding M. Intracellular pH regulation in the human oocyte. Hum Reprod. 1998;13(4):964–70.
10. Bagger PV, Byskov AG, Christiansen MD. Maturation of mouse oocytes in vitro is influenced by alkalization during their isolation. J Reprod Fertil. 1987;80(1):251–55.
11. Downs SM, Mastropolo AM. Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. Mol Reprod Dev. 1997;46(4):551–66.
12. Leclerc C, Becker D, Buehr M, Warner A. Low intracellular pH is involved in the early embryonic death of DDK mouse eggs fertilized by alien sperm. Dev Dyn. 1994;200(3):257–67.
13. Zhao Y, Chauvet PJ, Alper SL, Baltz JM. Expression and function of bicarbonate/chloride exchangers in the preimplantation mouse embryo. J Biol Chem. 1995;270(41):24428–34.
14. Zhao Y, Baltz JM. Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. Am J Physiol. 1996;271(5 Pt 1):C1512–20.
15. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. Dev Biol. 1999;208(1):244–52.
16. Lane M, Bavister BD. Regulation of intracellular pH in bovine oocytes and cleavage stage embryos. Mol Reprod Dev. 1999;54 (4):396–401.
17. Berthelot F, Terqui M. Effects of oxygen, CO2/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos. Reprod Nutr Dev. 1996;36(3):241–51.
18. Kane MT. The effects of pH on culture of one-cell rabbit ova to blastocysts in bicarbonate buffered medium. J Reprod Fertil. 1974;38(2):477–80.
19. Hamamah S, Gatti JL. Role of the ionic environment and internal pH on sperm activity. Hum Reprod. 1998;13 Suppl 4:20–30.
20. Babcock DF, Rufo Jr GA, Lardy HA. Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. Proc Natl Acad Sci USA. 1983;80(5): 1327–31.
21. Babcock DF, Pfeiffer DR. Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. J Biol Chem. 1987;262(31): 15041–7.
22. Marquez B, Suarez SS. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca2+ influx. Biol Reprod. 2007;76(4):660–5.
23. Leclerc C, Becker D, Buehr M, Warner A. Low intracellular pH is involved in the early embryonic death of DDK mouse eggs fertilized by alien sperm. Dev Dyn. 1994;200(3):257–67.

24. Zhao Y, Chauvet PJ, Alper SL, Baltz JM. Expression and function of bicarbonate/chloride exchangers in the preimplantation mouse embryo. J Biol Chem. 1995;270(41):24428–34.
25. Zhao Y, Baltz JM. Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. Am J Physiol. 1996;271(5 Pt 1):1512–20.
26. Lane M, Baltz JM, Bavister BD. Na+/H+antiporter activity in hamster embryos is activated during fertilization. Dev Biol. 1999;208(1):244–52.
27. Lane M, Bavister BD. Regulation of intracellular pH in bovine oocytes and cleavage stage embryos. Mol Reprod Dev. 1999;54 (4):396–401.
28. Lane M, Lyons EA, Bavister BD. Cryopreservation reduces the ability of hamster 2-cell embryos to regulate intracellular pH. Hum Reprod. 2000;15(2):389–94.
29. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. Mol Reprod Dev. 1998;50(4):434–42.
30. Squirrell JM, Lane M, Bavister BD. Altering intracellular pH disrupts development and cellular organization in preimplantation hamster embryos. Biol Reprod. 2001;64(6):1845–54.
31. Zander-Fox D, Mitchell M, Thompson JG, Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. Reprod Biomed Online. 2010;21 (2):219–29.
32. Phillips KP, Leveille MC, Claman P, Baltz JM. Intracellular pH regulation in human preimplantation embryos. Hum Reprod. 2000;15(4):896–904.
33. Fitzharris G, Baltz JM. Granulosa cells regulate intracellular pH of the murine growing oocyte via gap junctions: development of independent homeostasis during oocyte growth. Development. 2006;133(4):591–9.
34. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. Development. 2007;134(23):4283–95.
35. Phillips KP, Baltz JM. Intracellular pH regulation by HCO3-/Cl-exchange is activated during early mouse zygote development. Dev Biol. 1999;208(2):392–405.
36. Ferguson WJ, Braunschweiger KI, Braunschweiger WR, Smith JR, McCormick JJ, Wasmann CC, et al. Hydrogen ion buffers for biological research. Anal Biochem. 1980;104(2):300–10.
37. Eagle H. Buffer combinations for mammalian cell culture. Science. 1971;174(8):500–3.
38. Good NE, Izawa S. Hydrogen ion buffers. Meth Enzymol. 1972;24:53–68.
39. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RM. Hydrogen ion buffers for biological research. Biochemistry. 1966;5(2):467–77.
40. Garcia MA, Graham EF. Development of a buffer system for dialysis of bovine spermatozoa before freezing. I. Effect of zwitterion buffers. Theriogenology. 1989;31(5):1021–8.
41. Crabo BG, Brown KI, Graham EF. Effect of some buffers on storage and freezing of boar spermatozoa. J Anim Sci. 1972;35 (2):377–82.
42. Brown KI, Graham EF, Crabo BG. Effect of some hydrogen ion buffers on storage and freezing of turkey spermatozoa. Poult Sci. 1972;51(3):840–9.
43. Graham EF, Crabo BG, Brown KI. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa. I. Bull J Dairy Sci. 1972;55(3):372–8.
44. Molinia FC, Evans G, Maxwell WM. In vitro evaluation of zwitterion buffers in diluents for freezing ram spermatozoa. Reprod Nutr Dev. 1994;34(5):491–500.
45. Downs SM, Mastropolo AM. Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. Mol Reprod Dev. 1997;46(4):551–66.
46. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. Reprod Biomed Online. 2009;18(6):799–810.
47. Palasz AT, Brena PB, De la Fuente J, Gutierrez-Adan A. The effect of different zwitterionic buffers and PBS used for out-of-incubator procedures during standard in vitro embryo production on development, morphology and gene expression of bovine embryos. Theriogenology. 2008;70(9):1461–70.
48. Sieracki NA, Hwang HJ, Lee MK, Garner DK, Lu Y. A temperature independent pH (TIP) buffer for biomedical biophysical applications at low temperatures. Chem Commun. 2008;7:823–5.
49. Swain JE. Optimizing the culture environment in the IVF laboratory: impact of pH and buffer capacity on gamete and embryo quality. Reprod Biomed Online. 2010;21(1):6–16.
50. Morgia F, Torti M, Montigiani M, Piscitelli C, Giallonardo A, Schimberni M, et al. Use of a medium buffered with N-hydroxyethylpiperazine-N-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization. Fertil Steril. 2006;85(5):1415–9.
51. Graves CN, Biggers JD. Carbon dioxide fixation by mouse embryos prior to implantation. Science. 1970;167(924):506–8.
52. Quinn P, Wales RG. Fixation of carbon dioxide by pre-implantation mouse embryos in vitro and the activities of enzymes involved in the process. Aust J Biol Sci. 1971;24(6):1277–90.
53. Quinn P, Wales RG. Fixation of carbon dioxide by preimplantation rabbit embryos in vitro. J Reprod Fertil. 1974;36(1):29–39.
54. Lawitts JA, Biggers JD. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol Reprod. 1991;45(2):245–51.
55. Kane MT. Bicarbonate requirements for culture of one-cell rabbit ova to blastocysts. Biol Reprod. 1975;12(5):5525.
56. Mahadevan MM, Fleetham J, Church RB, Taylor PJ. Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, hepes, or phosphate. J In Vitro Fert Embryo Transf. 1986;3(5):304–8.
57. Farrell PS, Bavister BD. Short-term exposure of two-cell hamster embryos to collection media is detrimental to viability. Biol Reprod. 1984;31(1):109–14.
58. Escriba MJ, Silvestre MA, Saeed AM, Garcia-Ximenez F. Comparison of the effect of two different handling media on rabbit zygote developmental ability. Reprod Nutr Dev. 2001;41 (2):181–6.
59. Barnett DK, Clayton MK, Kimura J, Bavister BD. Glucose and phosphate toxicity in hamster preimplantation embryos involves disruption of cellular organization, including distribution of active mitochondria. Mol Reprod Dev. 1997;48(2):227–37.
60. Barnett DK, Bavister BD. Inhibitory effect of glucose and phosphate on the second cleavage division of hamster embryos: is it linked to metabolism? Hum Reprod. 1996;11(1):177–83.
61. Lane M, Ludwig TE, Bavister BD. Phosphate induced developmental arrest of hamster two-cell embryos is associated with disrupted ionic homeostasis. Mol Reprod Dev. 1999;54(4):410–7.
62. Koobs DH. Phosphate mediation of the Crabtree and Pasteur effects. Science. 1972;178(57):127–33.
63. Bavister B. Analysis of culture media for in vitro fertilization and criteria for success. In: Mastroianni L, Biggers J, editors. Fertilization and Early Development In Vitro. New York: Plenum Press; 1981.
64. Vasuthevan S, Ng SC, Edirisinghe R, Bongso A, Ratnam S. The evaluation of various culture media in combination with dimethylsulfoxide for ultrarapid freezing of murine embryos. Fertil Steril. 1992;58(6):1250–3.
65. Stachecki JJ, Garrisi J, Sabino S, Caetano JP, Wiemer KE, Cohen J. A new safe, simple and successful vitrification method for bovine and human blastocysts. Reprod Biomed Online. 2008;17 (3):360–7.

66. Quinn P, Kerin JF. Experience with the cryopreservation of human embryos using the mouse as a model to establish successful techniques. J In Vitro Fert Embryo Transf. 1986;3 (1):40–5.
67. Molinia FC, Evans G, Maxwell WM. Fertility of ram spermatozoa pellet-frozen in zwitterion-buffered diluents. Reprod Nutr Dev. 1996;36(1):2129.
68. Bagger PV, Byskov AG, Christiansen MD. Maturation of mouse oocytes in vitro is influenced by alkalization during their isolation. J Reprod Fertil. 1987;80(1):251–5.
69. Byrd SR, Flores-Foxworth G, Applewhite AA, Westhusin ME. In vitro maturation of ovine oocytes in a portable incubator. Theriogenology. 1997;47(4):857–64.
70. Bhattacharyya A, Yanagimachi R. Synthetic organic pH buffers can support fertilization of guinea pig eggs, but not as efficiently as bicarbonate buffer. Gamete Res. 1988;19(2):123–9.
71. Behr BR, Stratton CJ, Foote WD, Knutzen V, Sher G. In vitro fertilization (IVF) of mouse ova in HEPES-buffered culture media. J In Vitro Fert Embryo Transf. 1990;7(1):9–15.
72. Hagen DR, Prather RS, Sims MM, First NL. Development of one-cell porcine embryos to the blastocyst stage in simple media. J Anim Sci. 1991;69(3):1147–50.
73. Ozawa M, Nagai T, Kaneko H, Noguchi J, Ohnuma K, Kikuchi K. Successful pig embryonic development in vitro outside a CO2 gas-regulated incubator: effects of pH and osmolality. Theriogenology. 2006;65(4):860–9.
74. Ali J, Whitten WK, Shelton JN. Effect of culture systems on mouse early embryo development. Hum Reprod. 1993;8(7):1110–4.
75. Butler JE, Lechene C, Biggers JD. Noninvasive measurement of glucose uptake by two populations of murine embryos. Biol Reprod. 1988;39(4):779–86.
76. Lee MA, Storey BT. Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. Biol Reprod. 1986;34(2):349–56.
77. Walker SK, Lampe RJ, Seamark RF. Culture of sheep zygotes in synthetic oviduct fluid medium with different concentrations of sodium bicarbonate and HEPES. Theriogenology. 1989;32 (5):797–804.
78. Geshi M, Yonai M, Sakaguchi M, Nagai T. Improvement of in vitro co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with beta-mercaptoethanol. Theriogenology. 1999;51(3):551–8.
79. Iwasaki T, Kimura E, Totsukawa K. Studies on a chemically defined medium for in vitro culture of in vitro matured and fertilized porcine oocytes. Theriogenology. 1999;51(4):709–20.
80. Liu Z, Foote RH, Simkin ME. Effect of amino acids and alpha-amanitin on the development of rabbit embryos in modified protein-free KSOM with HEPES. Mol Reprod Dev. 1996;45(2): 157–62.
81. Jones RC, Foote RH. Effect of osmolality and phosphate, 'tris', 'tes', 'mes', and 'hepes' hydrogen ion buffers on the motility of bull spermatozoa stored at 37 or 5 degreesC. Aust J Biol Sci. 1972;25(5):1047–55.
82. Lepe-Zuniga JL, Zigler Jr JS, Gery I. Toxicity of light-exposed Hepes media. J Immunol Methods. 1987;103(1):145.
83. Zigler Jr JS, Lepe-Zuniga JL, Vistica B, Gery I. Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. In Vitro Cell Dev Biol. 1985;21(5):282–7.
84. El-Alamy MA, Foote RH. Freezability of spermatozoa from Finn and Dorset rams in multiple semen extenders. Anim Reprod Sci. 2001;65(3–4):245–54.
85. El-Danasouri I, Selman H, Strehler E, De Santo M, Sterzik K. Comparison of MOPS and HEPES buffers during vitrification of human embryos. Hum Reprod. 2004;14:i136.
86. Wersinger C, Rebel G, Lelong-Rebel IH. Characterisation of taurine uptake in human KB MDR and non-MDR tumour cell lines in culture. Anticancer Res. 2001;21(5): 3397–406.
87. Stellwagen NC, Bossi A, Gelfi C, Righetti PG. DNA and buffers: are there any noninteracting, neutral pH buffers? Anal Biochem. 2000;287(1):167–75.
88. Schmidt J, Mangold C, Deitmer J. Membrane responses evoked by organic buffers in identified leech neurones. J Exp Biol. 1996;199(Pt 2):327–35.
89. Jeyendran RS, Graham EF. An evaluation of cryoprotective compounds on bovine spermatozoa. Cryobiology. 1980;17 (5):458–64.
90. Jeyendran RS, Gunawardana VK, Barisic D, Wentz AC. TEST-yolk media and sperm quality. Hum Reprod Update. 1995;1(1): 73–9.
91. Zavos PM, Goodpasture JC, Zaneveld LJ, Cohen MR. Motility and enzyme activity of human spermatozoa stored for 24 hours at +5 degrees C and -196 degrees C. Fertil Steril. 1980;34(6): 607–9.
92. Jaskey DG, Cohen MR. Twenty-four to ninety-six-hour storage of human spermatozoa in test-yolk buffer. Fertil Steril. 1981;35 (2):205–8.
93. Jeyendran RS, Van der Ven HH, Kennedy W, Perez-Pelaez M, Zaneveld LJ. Comparison of glycerol and a zwitter ion buffer system as cryoprotective media for human spermatozoa. Effect on motility, penetration of zona-free hamster oocytes, and acrosin/proacrosin. J Androl. 1984;5(1):1–7.
94. McCoshen J, WA A, Tyson JE. Effectiveness of human semen frozen in TESE-yolk- buffered medium on AID outcome. Fertil Steril. 1984;42:162–3.
95. Bolanos JR, Overstreet JW, Katz DF. Human sperm penetration of zona-free hamster eggs after storage of the semen for 48 hours at 2 degrees C to 5 degrees C. Fertil Steril. 1983;39(4): 536–41.
96. Johnson AR, Syms AJ, Lipshultz LI, Smith RG. Conditions influencing human sperm capacitation and penetration of zona-free hamster ova. Fertil Steril. 1984;41(4):603–8.
97. Yang YS, Rojas FJ, Stone SC. Acrosome reaction of human spermatozoa in zona-free hamster egg penetration test. Fertil Steril. 1988;50(6):954–9.
98. Bielfeld P, Jeyendran RS, Holmgren WJ, Zaneveld LJ. Effect of egg yolk medium on the acrosome reaction of human spermatozoa. J Androl. 1990;11(3):260–9.
99. Carrell DT, Bradshaw WS, Jones KP, Middleton RG, Peterson CM, Urry RL. An evaluation of various treatments to increase sperm penetration capacity for potential use in an in vitro fertilization program. Fertil Steril. 1992;57(1):134–8.
100. Chan SY, Tucker MJ. Comparative study on the use of human follicular fluid or egg yolk medium to enhance the performance of human sperm in the zona-free hamster oocyte penetration assay. Int J Androl. 1992;15(1):32–42.
101. Falk RM, Silverberg KM, Fetterolf PM, Kirchner FK, Rogers BJ. Establishment of TEST-yolk buffer enhanced sperm penetration assay limits for fertile males. Fertil Steril. 1990;54(1): 121–6.
102. Lanzendorf SE, Holmgren WJ, Jeyendran RS. The effect of egg yolk medium on human sperm binding in the hemizona assay. Fertil Steril. 1992;58(3):547–50.
103. Paulson RJ, Sauer MV, Francis MM, Macaso TM, Lobo RA. A prospective controlled evaluation of TEST-yolk buffer in the preparation of sperm for human in vitro fertilization in suspected cases of male infertility. Fertil Steril. 1992;58(3): 551–5.
104. Katayama KP, Stehlik E, Roesler M, Jeyendran RS, Holmgren WJ, Zaneveld LJ. Treatment of human spermatozoa with an egg yolk medium can enhance the outcome of in vitro fertilization. Fertil Steril. 1989;52(6):1077–9.

105. Jacobs BR, Caulfield J, Boldt J. Analysis of TEST (TES and Tris) yolk buffer effects on human sperm. Fertil Steril. 1995;63 (5):1064–70.
106. Syms AJ, Johnson AR, Lipshultz LI, Smith RG. Effect of aging and cold temperature storage of hamster ova as assessed in the sperm penetration assay. Fertil Steril. 1985;43(5):766–72.
107. Molinia FC, Evans G, Maxwell WM. Incorporation of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. Theriogenology. 1994;42(5):849–58.
108. Weidel L, Prins GS. Cryosurvival of human spermatozoa frozen in eight different buffer systems. J Androl. 1987;8(1):41–7.
109. Prins GS, Weidel L. A comparative study of buffer systems as cryoprotectants for human spermatozoa. Fertil Steril. 1986;46 (1):147–9.
110. Swain J, Ord V, Taylor D, Sossamon V, Pool T. Use of Two Zwitterionic Buffers in IVF Handling Media Supports Mouse Blastocyst Development and Normal Human Oocyte Fertilization Following ICSI. in *Proceedings from the 15th Annual World Congress on IVF*. 2009. Geneva, Switzerland.
111. Garcia MA, Graham EF. Development of a buffer system for dialysis of bovine spermatozoa before freezing. III. Effect of different inorganic and organic salts on fresh and frozen-thawed semen. Theriogenology. 1989;31(5):1039–48.