pH in Medically Assisted Reproduction Procedures: Its Importance, Measurement and Control

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Summary

The intracellular pH (pH_i) of gametes and embryos is modulated by a number of systems and, in human oocytes and cleavage embryos, is around 7.1. In *in vitro* culture, the extracellular pH (pH_e) of culture media is controlled within a typical range of 7.2 – 7.4. Tight control over this range to avoid pH deviation is prudent. An ideal pH_e is, however, difficult to define due to variations between media formulations, culture conditions, as well as gamete specific or embryo stage specific preferences. In the IVF laboratory, pH_e is generally controlled by adjusting the CO_2 concentration of culture incubators, where it works in combination with media components, like bicarbonate, to reach equilibrium. However, due to varying conditions in individual laboratories, including media type, protein supplement and elevation, there is no single % CO_2 concentration that will give the desired pH_e or the best outcomes in all situations. When handling gametes and embryos outside the incubator, buffers such as HEPES or MOPS are typically used to control pH_e.

Testing of pH_e of culture media presents some technical difficulties but is recommended to verify culture conditions and aid in quality control and troubleshooting.

Introduction

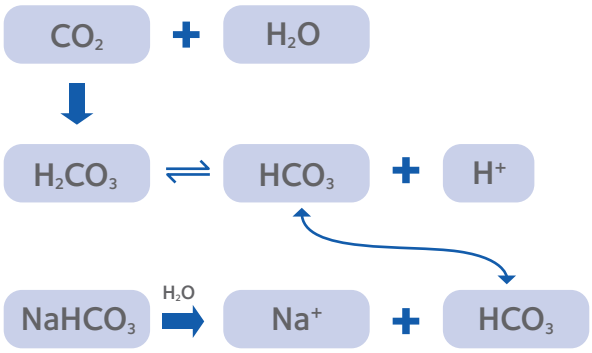
The importance of pH in gamete and embryo biology is axiomatic and, in order to optimize embryo viability and reproductive outcomes, it is essential that pH be properly monitored and controlled. This applies equally (or more) to *in vitro* systems than *in vivo*. It is therefore essential that we appreciate the role of pH at various stages of the IVF process and understand how we can best control it.

Devised in 1923 by the Danish biochemist, Søren Peter Lauritz Sørensen, pH is a logarithmic scale expressing the 'potential of hydrogen' equal to $-\log_{10}c$ where c is the concentration of hydrogen ions in moles per liter.

It ordinarily ranges between 1 and 10^{-14} hence the scale of 1-14 that we are familiar with. Importantly, as a result of the logarithmic nature of pH measurement, relatively "minor" changes in pH are actually large changes in hydrogen ion concentration.

In biological systems, cells have limited capacity for passive buffering of cytoplasmic pH and thus depend on a number of active transport mechanisms. In IVF systems, control of pH usually utilizes the bicarbonate buffer system (Fig. 1).

Figure 1: The CO_2/ bicarbonate buffering system that is the predominant method for establishing pH_e of culture media.



When considering reproductive cells, mechanisms of pH regulation, such as the HCO_3^-/Cl^- exchanger and Na^+/H^+ antiporter (Phillips *et al*, 2000), have been reported in human embryos. And whilst the focus has perhaps understandably been on embryos, the different requirements of various embryos stages, as well as the specific requirement of oocytes (denuded vs. cumulus enclosed) and spermatozoa, demand that we tailor conditions to meet the requirements of these different cells.

In this paper, we will look at pH in gametes and embryos and how it relates to development and function. Based on these findings, we will then consider how pH in our culture systems is manipulated. Finally, we will examine methods of testing pH to help validate proper and stable culture conditions.

pH in reproductive cells

The intracellular pH (pH_i) of human oocytes and embryos is typically believed to be around 7.0-7.1 [Phillips *et al*, 2000], though a smaller study suggested it was around 7.3 (Dale *et al*, 1998), and is regulated by various mechanisms (for a review, see Swain 2010).

Interestingly, the capacity of oocytes to regulate pH_i has been shown to be switched off between meiotic progression and fertilization, with the role conferred upon the supporting cumulus cells via gap junctions (FitzHarris and Baltz, 2006). This exposes denuded metaphase II oocytes to fluctuations of pH_i when pH_e is poorly controlled, for example during procedures such as intracytoplasmic

sperm injection (ICSI) or oocyte vitrification. Given that pH_i impacts the cytoskeleton and meiotic spindle in rodents (Squirrel *et al*, 2001; Swearman *et al*, 2018), and similar impacts may be present in other mammalian oocytes, poorly controlled pH_e has potential implications for rates of aneuploidy (Swain, 2019).

In preimplantation embryos, pH controls various cellular functions that influence embryo development. Squirrel and colleagues (Squirrel *et al*, 2001) reported disrupted localization of mitochondrial and actin filaments in mouse embryos when pH_i was raised or lowered compared to controls at pH 7.2. Metabolism is also affected with increased glycolysis and reduced oxidative metabolism in response to rising pH_i in hamster embryos (Lane *et al*, 2000). This is especially important in early cleavage embryo culture: in 1-cell mouse embryos, culture at an inappropriately low pH_i resulted in reduced cell number in blastocysts, higher levels of apoptosis and reduced fetal growth (Zander-Fox *et al*, 2008). In contrast, later stages of embryos appear to regulate pH_i more efficiently which is linked with the acquisition of tight cell junctions (Spindler *et al*, 2000). It is vital, however, to recognize that cryopreserved embryos may have a reduced ability to regulate pH_i for 3-4 hours after warming/rehydration (Lane *et al*, 2000), so extra caution is advisable when handling and/or culturing embryos in these circumstances.

While oocytes and embryos favor mildly alkaline conditions, spermatozoa will function in the same conditions but thrive at higher pH. The pH_i follows pH_e in a quasi-linear relationship in human spermatozoa (Hamameh *et al*, 1996) but capacitation may rely on the selective activation of one major pH_i regulator in the mouse at least (Zeng *et al*, 1996). Achikanu and colleagues (Achikanu *et al*, 2018) reported that higher pH_e led to increased pH_i and intracellular calcium concentration which resulted in hyperactivated motility; higher bicarbonate (HCO_3^-) also drove increased sperm motility. Comparing various formulations of sperm washing media, de Rosa *et al* (2015) demonstrated how the composition impacted sperm function, again showing high pH_e, as result of high HCO_3^- was beneficial. This begins to also demonstrate the difficulty in isolating an ideal pH_e due to the independent actions of both CO_2 and HCO_3^- on various cellular processes (Quinn & Wales 1974, Swain 2012, Swain 2011, Hentemann *et al*, 2011).

Control of pH in IVF systems

For routine culture, the pH_e is determined by the equilibrium between the HCO_3^- in the medium and the level of CO_2 in the incubator (as fig 1). Since most labs have moved away from making their own culture media, the concentration of HCO_3^- is effectively pre-set by the manufacturer, leaving the % CO_2 as the parameter that can be manipulated by the laboratory to set pH_e. Commercial media preparation is performed under tight controls and regulatory oversight, giving a high level of batch-to-batch consistency, and manufacturers test media for pH against a standard % CO_2 which is then reported in the certificate of analysis provided with each new batch. Whilst a useful general guide to show that the medium composition is within specification, there are a number of factors to consider that might lead to variations in pH_e in each user laboratory.

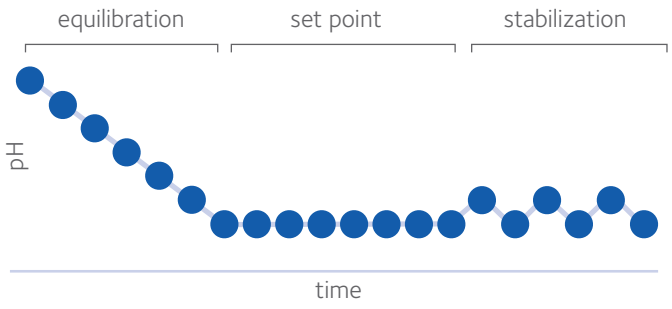
One factor affecting pH_e is altitude: simply, the higher above sea level a laboratory is located, the higher the % CO_2 in the gas mix to achieve the same pH_e. For example, at sea level the standard barometric pressure is typically 101kPa (or 760mm Hg) but at 500, 1000m and 2000m above sea level, this drops to 96kPa, 90kPa and 81kPa respectively. Put another way, only 94%, 89% and 80% of the CO_2 available at sea level is available at these altitudes even though the gas mix (by percentage) is the same (see https://baillielab.net/critical_care/air_pressure/).

It should be noted that the pH_e will also depend on temperature, with pH_e decreasing with increasing temperature, especially in zwitterionic buffered solutions, so stable control of this parameter is equally important.

Additionally, type and amount of protein supplement can affect pH_e especially if added by the laboratory, both via the protein itself, but also via a dilution effect, essentially diluting the bicarbonate concentration v/v (Swain, 2012). Consideration of these factors is imperative in achieving the desired pH_e and maintaining stability in the lab.

For quality control in the IVF laboratory, pH_e can be thought of in 3 phases (Swain, 2010): equilibration, set point and stabilization (Fig. 2).

Figure 2: the three phases of pH in in vitro culture (from Swain, 2010)*



Time for complete equilibration will depend on volume of medium, surface area (influenced by drop size and/or well shape), oil overlay and type of dish used, as well as starting and ending pH_e. Sufficient time should be given for pH to reach equilibrium before exposing gametes or embryos. Steel and Conaghan (2008) suggested a minimum of 10 hours, whether using 50µl or 500µl under oil, though the ideal situation would be to validate equilibration times in each laboratory.

Set point is influenced primarily, but not exclusively, by CO_2 and HCO_3^- with other components of the medium also having an impact. As already discussed, the pH_i of oocytes and embryos is around 7.1. In embryo culture, it is crucial to avoid inducing acidosis and driving pH_i below this figure and so the generally accepted lower limit for pH_e is 7.2 to avoid acidification by intracellular metabolic processes.

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The upper limit is typically 7.4 since some reports (see Gatimel *et al*, 2020) suggest poorer outcomes at pH_e >7.4. There may be some value in targeting pH_e nearer 7.2 but it is impossible to give an ideal pH_e as media composition also affects pH_i (Swain 2010; Swain, 2012; Gatimel *et al*, 2020). Bearing in mind that a move from pH 7.2 to pH 7.4 represents a very significant 60% increase in hydrogen ions, it makes sense to attempt to stabilize pH_e in our culture systems within a much narrower band. Various methods can be employed to aid in this stabilization.

Stabilization is the degree to which the pH_e is unperturbed, taking precautions such as limiting the frequency of incubator doors being opened, using oil overlay or gassed isolettes or zwitterionic buffered media (with temperature considerations). Zwitterionic buffered media are modified media to provide a stable pH_e under ambient atmosphere. These tend to have reduced HCO_3^- levels and the addition of a buffer, such as HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) or MOPS (3-morpholinopropane-1-sulfonic acid), and allow longer handling times without pH_e changes, which is especially useful for ICSI or embryo biopsy for example, (for review, see Swain & Pool, 2009; Will *et al*, 2011). A combination of buffers (eg HEPES + MOPS) offers possible benefits in terms of optimizing pKa (the acid dissociation constant equating to the pH where buffering capacity is maximal) and reducing individual concentrations and hence possible risk of toxicity (Will *et al*, 2011).

An additional consideration is to avoid pH_e perturbation is during dish preparation or culture. Poor technique during preparation of dishes for microdrop culture or extended periods in a non-humified environment without proper precautions can cause media evaporation (Swain *et al*, 2012). As a result, the solute concentration of the medium, including bicarbonate, increases. This results in an increase in pH_e.

In summary, it is essential to select an appropriate set point for pH_e, then ascertain what % CO_2 is needed to generate this set point in the specific circumstances of each individual lab. Thereafter, one must implement adequate controls across the whole system to maintain the set point and minimize potential for fluctuations and/or drift. Allied to this, a proportionate and targeted system of testing pH_e should be introduced.

Testing and internal quality control (IQC)

A recurring question is how and when the pH_e of culture media should be tested. In the first instance, it is worth acknowledging the technical difficulties that many IVF labs encounter with implementing a robust methodology for pH_e testing. These issues generally arise due to limited sample volume or other factors, but with emerging technology, can be addressed.

A reliance on accurate % CO_2 has serious limitations but may have to be adopted as a proxy measure of pH_e and incubator function if the latter cannot be reliably determined using a suitable pH meter. Whilst the use of incubator displays and fyrite should not be heavily relied upon (Pool, 2004), pre-certified gas mixes or appropriately calibrated external CO_2 probes can provide some reassurances.

That said, provided it is performed well, direct pH testing should remain the gold standard. Accuracy and precision of pH measurement is reliant upon using an appropriate probe which is properly calibrated and used at the correct temperature (Swain, 2010). As well as solution temperature effects discussed above, there may also be electrode temperature effects so appropriate temperature compensation is needed; ideally pH measuring systems should be used *and* calibrated at the temperature a medium is used, usually 37°C. A very useful alternative is a hand-held blood gas analyzer (Gatimel *et al*, 2020) since this can test small volumes and has internal calibration, though systems should still be validated (Swain, 2013; Diaz de Pool *et al*, 2018; Cairo Consensus Group, 2020).

Though each has its pros and cons, none of these systems exactly match the culture system used in treatments and offer only snapshots of the pH. Systems are available, however, that provide real-time monitoring that can lend valuable insight into equipment function and impact of daily workflows or other lab environmental occurrences that may impact pH_e. Systems such as SignipHy™ (https://fertility.coopersurgical.com/equipment/g210-invicell/#signiphy), may offer a more robust approach.

Figure 3: SignipHyTM system for continuous real-time pH monitoring

A: sv2 sensor: single-use sensor measures pH continuously over 7 days
B: TrakPod: one per incubator; controls frequency of measurements
C: qc2 alignment tool: one per lab; part of QC testing for the system
D: TrakStation: data logging and user alarms for up to 8 TrakPods/incubators

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