

# Equine intracytoplasmic sperm injection laboratory: an overview of management challenges



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## Abstract

Process of equine in vitro embryo production requires consistent monitoring, controlling, and adjustment of several factors in the laboratory. This is a brief introduction to some of the more important factors.

**Keywords:** Intracytoplasmic sperm injection, equine embryo, laboratory

## Introduction

In vitro embryo production via intracytoplasmic sperm injection (ICSI) is a laboratory-intensive process that involves much more than the procedure of sperm injection. Establishing and maintaining a functional productive environment that is suitable for embryo production and growth has many facets. In utero, oocytes and embryos are in an extremely protected and nourishing environment; however, in vitro they are potentially subject to, and sensitive to, numerous environmental challenges. Although the act of sperm injection is important to be performed well, the real challenge is in the creation and maintenance of a homeostatic 'embryo-friendly' laboratory. The realities of the equine reproduction and embryo transfer industry offer further challenges with planning and communication with multiple parties.

## Air quality

Air, of course, permeates every corner of the laboratory, and although seemingly innocuous, air quality can make or break in vitro embryo production. Laboratory air is evaluated for particulates, toxic gases, and volatile organic compounds (VOCs). Particulates can be a source for contamination of any exposed culture medium but can generally be controlled by HEPA air filtration. The target level for particulates in an in vitro fertilization (IVF) laboratory is < 10,000 particles from 0.5 to 10 microns.<sup>1</sup> Minimizing the number of people in the laboratory and meticulous cleaning can also help keep airborne particulates low. Regardless of the quality of room air and media, culture dishes should be prepared under laminar flow of HEPA filtered air in a laminar flow cabinet (i.e., tissue culture hood).

Although other toxic gases are uncommon in the laboratory, VOCs are a substantial concern, potentially crippling production of viable embryos.<sup>2,3</sup> Volatile organic compounds arise

from several sources including paints, adhesives, upholstery, dry wall, wood composites, plastics, cleaning agents, disinfectants, vehicles, petroleum products, aerosol solvents, and cosmetic products, especially, those with fragrances. The target level for total VOC concentration in an IVF laboratory is < 500 µg per cubic meter (400 - 800 parts per billion, depending on the molecular size) and it is < 5 µg per cubic meter for aldehydes (embryos are particularly sensitive).<sup>1</sup> Precise measurement of airborne VOCs, especially of specific chemicals, is best performed with gas chromatography; however, a single sample may not give a reliable picture of the daily fluctuations produced by human activity and the environment. More commonly, laboratory air can be monitored with handheld photoionization detectors or gas sensitive semiconductors, but these sensors vary in their ability to detect various types of VOCs.<sup>4</sup> These units are also difficult to calibrate for the lower concentrations that are necessary for IVF laboratories. Monitoring is important to identify the source of and amelioration for any surges in VOCs.

Heating, ventilation, and air conditioning (HVAC) system of the laboratory can be a substantial contributor to overall air quality. Laboratory should maintain positive pressure to prevent incursions of contaminants from other rooms in the building. Positive pressure is maintained by increasing the inflow of air from the air handler into the room or limiting the outflow. This may require additional outside air being brought into the system that will generally also decrease VOC concentration. However, at times, external air may be a source of toxicity and has to be completely shut off, such as in periods of high ozone concentrations or VOCs from road work, painting, roofing, or other activities.

Active filtration and removal of VOCs may be necessary in several laboratory locations. This can be provided by filtration through activated charcoal and potassium permanganate filters that are in standalone filtration units or in the HVAC

system. Filters in the HVAC system should be installed on the conditioned side of the air handling unit.<sup>1</sup> Since VOCs become more volatile at higher temperatures, activated charcoal is less effective and could even release VOCs previously absorbed if exposed to increased temperature.

## Toxicity

Toxins present in the laboratory system can harm or stop embryo production and there are many potential sources. Plasticware (petri dishes, centrifuge tubes, culture tubes) and mineral oil, used to cover incubating oocytes and embryos, can be sources of toxins. Plasticware and mineral oil produced specifically for in vitro fertilization use should be screened with mouse embryo assay (MEA) in which mouse zygotes are cultured to the blastocyst stage.<sup>6</sup> Plastics not screened are a risk, as plastics are a common source of VOCs. A good generalization is that harder plastics, such as polystyrene, release less VOCs than softer plastics such as polypropylene. All plasticware should have its packaging wrap opened for 2 - 4 weeks to allow VOCs to 'off-gas' before use.

Mineral oil, a petroleum distillate, is used to overlay micro-drops of culture medium during incubation. Mineral oil provides a barrier to contamination and evaporation. Mineral oil is prone to peroxidation, especially when exposed to light. Being hydrocarbon petroleum distillates, mineral oil or paraffin oil has to be extremely well-refined, screened via MEA, and washed with a fluid containing protein to remove lipophilic and hydrophilic toxins to be considered safe for embryology.

## Incubator

The incubator is the heart of an embryo culture system, a complex system that provides appropriate temperature, humidity, and atmosphere. Embryo culture media use a bicarbonate-based buffer system that balances bicarbonate and CO<sub>2</sub> to arrive at the target pH. Oxygen is reduced to 5% to mimic oxygen tension in the reproductive tract and minimize oxidative damage. To produce this environment, carbon dioxide and oxygen sensors control and balance carbon dioxide and nitrogen gas supplies. These sensors can be calibrated to standard gases; however, drift over time will require periodic monitoring and adjustment, and oxygen sensors are particularly prone to failure without warning, making monitoring even more imperative. The incubator can also be a source of VOCs and new incubators must be 'burned in' through a high temperature disinfection cycle and run for several weeks to 'off-gas' harmful VOCs.

Temperature maintenance is another function of the incubator. Digital thermometry has very small drift, so once calibrated it should maintain accuracy. Equine embryos can handle a range of temperatures,<sup>6</sup> but blastocyst production should be the best in the center of that range. Since most incubators have multiple shelves there is an overall temperature difference of up to 0.1 - 0.2°C between the top and bottom shelves. This difference can affect blastocyst production<sup>7</sup> and the overall incubator temperature should be adjusted so that all shelves are as close to the ideal production temperature as possible.

## Culture medium

Culture medium provides the developing embryo, electrolytes, carbohydrates (pyruvate, lactate, and glucose for energy), amino acids, vitamins, nucleic acid precursors, chelators, antioxidants, antibiotics, proteins, and other growth factors. Culture media for equine embryos have not received the research and development as several other species. However, generally they are developed based on information derived from those species. The first reliable medium for in vitro equine blastocyst production was a complex tissue culture medium, DMEM/F12 that demonstrated the early equine embryo's tolerance and the early blastocyst's affinity for a high glucose concentration (17 mM). Although a commercial equine medium is now available, most laboratories still prepare their own. Some use sequential media, starting with a relatively low glucose medium the first 4 - 5 days of culture and then move to a higher glucose, usually more complex medium. Nonsequential media are used by some to avoid the stress of change.

In vitro culture of most mammalian embryos has moved away from using serum in the culture medium, largely because it is somewhat unpredictable in its composition from batch-to-batch, and so also its function in the medium. Addition of another protein source (e.g., bovine serum albumin, and several cytokines) has generally replaced the functions of serum and frequently improved results in other species. However, this has not been the case with equine culture media. Most equine labs still use fetal bovine serum in equine embryo culture, if not during the first days, at least starting at day 5. Despite the inconsistency in batches, it still provides useful growth factors and cytokines.

Early embryos have limited ability to control their internal pH, so maintaining a desirable pH of the culture medium is essential. Equine ICSI embryos tolerate a fairly wide range of pH; however, maximum embryo production generally occurred optimally within a pH range between 7.25 and 7.35.<sup>6</sup> This is maintained by a balance between the bicarbonate in the medium and the concentration of CO<sub>2</sub> in the incubator. Most media are designed to maintain an appropriate pH at 5 or 6% CO<sub>2</sub>, although a higher CO<sub>2</sub> concentration is required as height above sea level increases. The pH should be monitored in medium equilibrated in the incubator at regular intervals and with any change in culture medium lot or formulation. The pH is then adjusted by either regulating the CO<sub>2</sub> concentration or by the addition of acid or base to the medium.

## Light

Light exposure had deleterious effects on embryos, especially certain wavelengths.<sup>8,9</sup> Farther towards the ultraviolet spectrum the more harmful light is to embryos. Mouse zygotes were exposed to 15 minutes of cool white light, 15 minutes of warm white light, or 1 minute of sunlight; of these, 73% of controls, 58% of those exposed to warm white fluorescent light, 44% of those exposed to cool fluorescent light, and 25% of those exposed to sunlight developed to term.<sup>9</sup> It is imperative that laboratory light should be in the 'warm' spectrum, kept at low levels, and oocyte and embryo exposure to light should be minimal.

## Communication

The equine ICSI laboratory has unique communication challenges. There are usually many parties involved in the entire process that all must be either kept informed of progress and/or consulted. First there is the mare owner who not only wants to be updated at each stage, and also has to plan ahead, knowing which semen to use. Laboratory has to know from the owner where to send the embryos produced, how many to send, etc. Veterinarian or facility collecting the oocytes has to know the progress, as this might affect planning for the next cycle of the mare. Stallion/semen owner has to be contacted to give permission to use stored semen or to supply semen. Stallion owner will also like to know the outcome. The facility receiving embryos has to know the progress periodically so that they can prepare suitable recipient mares. Conversely, not every curious person that might call wanting to know what is going on with a mare's oocytes has the right to know, so dispensing information properly can be challenging. To handle all of these needs, a laboratory should have a defined communication plan or protocol.

## Conflict of interest

None to declare.

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