The effect of light on embryos and embryo culture

Kimball O Pomeroy, PhD[1] and Michael L. Reed, PhD [2]

[1]Florida Institute for Reproductive Medicine, Miami, FL, 33143, USA and Arizona Reproductive Medicine Specialists, Phoenix, AZ, 85016, USA; [2]Center for Reproductive Medicine of New Mexico, Albuquerque, NM, 87106, USA

Abstract
In oviparous animals, the egg hatches outside of the body and is exposed to light; in some cases throughout the development of the fetus. In mammals, fertilization and the growth of embryos in vivo occurs in the dark but in human IVF, these embryos are exposed to variable light sources and intensities. Light can affect embryonic development in some species via either a direct toxic effect on the embryo, or indirectly via photo-oxidation of components in the media or oil. Although data regarding the effect of light on human embryos is lacking, it is prudent to take appropriate steps to minimize the potential harmful effects of both ambient and microscopic light on embryos.

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Correspondence: Kimball O Pomeroy Ph.D., Florida Institute for Reproductive Medicine, Miami, FL, 33143, USA
USA. **Email**: k.pomeroy@ivfmd.com T: +1-305- 662-7901
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Introduction

The natural incubator of the mammalian embryo, the uterus, has been fine tuned for millions of years. Homeostatic measures are in place to provide an environment that changes little, despite an external environment that is often quite variable. IVF laboratories use modern mechanical incubators that have successfully replicated, to some extent, this environment.

The goal of both incubation systems, the natural one and the artificial one, is to protect the embryos from insults that might result in the consumption of the embryo's precious energy and other resources, or could result in the demise of the embryo. To a large part, the focus of environmental control in incubators has been on temperature and pH. In the body, changes in osmolality, pH and temperature are normally small and gradual, but in the IVF system these excursions can be relatively large and rapid. Perturbations in vitro, not normally present in the in vivo culturing in the uterus may result in stress to the embryos. There are other environmental factors that must be taken into consideration when one begins the process of culturing embryos in this artificial environment. This includes exposure to toxicants, the off-gassing of volatile compounds, exposure to electromagnetic fields, the generation of reactive oxygen species and light exposure.

In order to work safely with embryos, monitor embryo quality and manipulate embryos, the embryo must be exposed to light - a potential environmental factor that is not relevant during in vivo culture; specifically, the embryo may not have developed any protective measures during the evolution of the species. During ART procedures embryos, sperm and oocytes are exposed to light during oocyte retrieval, placement of dishes into and out of incubators, exposure during microscopy for ICSI, fertilization checks, morphological assessment and during the embryo transfer.

Although there is some evidence that some mammalian fetuses, and even the late human fetus, may be exposed to low intensities of light in vivo (Jacques et al., 1987), (Del Giudice, 2011), it is not known whether the human embryo has a functioning system to protect it from the effects of light exposure. Further, the oocyte and the embryo may not have retained a

functional system to either protect or repair potential light damage during these steps of in vitro fertilization and so it is possible that irreparable harm is being done.

Photooxidation

Several variables are important when measuring light. They are duration of exposure, intensity of exposure and finally, the wavelength of light. Often, light intensity is measured in lux, but this measurement is the intensity as measured by the human eye and is not suitable for non-visible wavelengths. Lux also is a poor measurement as it does not take into account the length of exposure. A better measure of intensity is the irradiance (w/m2) which, as a measure of power, includes also the measurement of the duration of the exposure. Unfortunately, most of the studies done on the influence of light on culturing of cells do not include this measurement of irradiance and so it is difficult to even determine the amount of light the cells were exposed to.

There are several ways that light might affect a cell. There may be a direct effect where light "stresses" the cell, activates stress genes or even damages DNA directly via ionization. Light may also indirectly affect cells by oxidation of components in the media or oil, changing a neutral or even beneficial component into a toxicant. This indirect method can occur via photooxidation - a chemical reaction between light and components of culture medium and oil. Light has been implicated in the oxidation of oil used in the culture of human embryos (Otsuki et al., 2009). The mechanisms involved in photooxidation of media components and oil may also work in photooxidation of sperm and oocyte membranes (which are lipids), producing changes in these membranes that could potentially inhibit fertilization. Light has also been shown to induce production of hydrogen peroxide, a substance toxic to cells, when media containing HEPES and riboflavin are exposed to light (Hill Jr. et al., 1960a; Stoien and Wang, 1974; Wang and Nixon, 1978; Wang, 1975; Zigler et al., 1985).

Ultraviolet B radiation in the 290-320 nm wavelength can result in DNA damage and oxidative stress of sea urchin embryos (Lesser et al., 2003). It can damage both proteins and membrane lipids (Halliwell, 2006). The intensity of light that can damage sea urchin embryos is in the range of what sea urchins are naturally exposed to during embryonic growth in tide pools. When the DNA of sea urchin embryos are damaged by light it can result in delays in cell division and consequent developmental delays while the cells are retained in the G1/S phase of the cell cycle to repair the damaged DNA. If this damage cannot be repaired, the sea urchin cells will undergo apoptosis and cell death. DNA damage can occur not only due to the direct effect of light on DNA but also indirectly via reactive oxygen species (Lesser et al., 2003). Sea urchin embryos have developed systems to protect them from damage due to light exposure - the expression of superoxide dismutase for example, an antioxidant produced by the embryo. Sea urchin embryos can also increase the expression of both p53 and p21 genes, genes that play a role in apoptosis or cell arrest which can help to either repair damage or eliminate damaged cells. p53 is also a cell checkpoint gene that allows the repair or removal of cells in response to DNA damage, hypoxia, reactive oxygen species (ROS) or mutagens.

Sea urchins have developed these repair mechanisms to light damage in response to selection over millennia. Although humans have the same type of repair machinery and cell cycle checkpoints, it is not known whether these systems are functional and whether they can repair any potential damage from light exposure encountered during in vitro culture.

Light toxicity, tissue cultures, and non-primate embryos

One of the first observations to indicate that light could be toxic to cells was the report of Raab (Hill Jr. et al., 1960b) that light exposure killed protozoans placed in an acridine dye solution. This was not a direct effect of light but was due to light modifying the chemical properties of acridine orange that resulted in cessation of growth, decreased protein synthesis and inhibition of replication. Since then, studies have shown that light can also indirectly damage mammalian cells via photooxidation (Stoien and Wang, 1974;(Wang, 1975). In one study, photooxidation of media components (riboflavin, tryptophan, and tyrosine) resulted in the production of toxic hydrogen peroxide. The addition of N-2-

hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) to the culture medium RPMI 1640 markedly increased the production of cytotoxic products during exposure of the medium to visible light (Zigler et al., 1985).

There are also several examples where light has been implicated in harming either gametes or embryos. The first observation of the direct effects of light on mammalian embryos was by Daniel (Daniel, 1964). He observed that rabbit ova exposed to light for up to 12 hours displayed retarded cleavage rates. Even after using various light filters, cleavage rates were still affected; red filters seemed to ameliorate this cleavage inhibition the most. Others have shown (Schumacher and Fischer, 1988) that earlier stage embryos are more sensitive to light than later stages and that as little as one hour exposure to light (of day-1 rabbit embryos) can result in decreased thymidine incorporation, a measurement of cell proliferation. It should be noted though, that at least one study (Bedford and Dobrenis, 1989) reported no negative effects on embryonic development or implantation in rabbit embryos exposed to 3250 lux of fluorescent light.

Hamster embryos are also quite sensitive to light. In fact, the first births of hamsters using ICSI occurred only when the microscopic light source was filtered using red light in a darkened room (Yamauchi et al., 2002). One hour exposure of hamster oocytes to fluorescent lighting inhibited normal meiotic development (Hirao and Yanagimachi, 1978). The most common defects resulting from light exposure were failure of the chromosomes to either develop properly after metaphase or the formation of numerous small pronuclei. In most cases, the second polar body was not extruded. Wavelengths in the 470-480 nm range (blue-violet-ultraviolet) were the most harmful and a red filter was protective. Sunlight, UV light and fluorescent lamps were more detrimental than incandescent lamps. It appears that at least in the hamster, short wavelengths of light can disrupt the spindle apparatus. In another study, light was shown to affect the early embryonic development of hamster embryos (Umaoka et al., 1992). As little as 30 minutes of exposure to light (380-760 nm) blocked the development of embryos to the 2-cell stage. Embryo development was also inhibited when 2-cell and 8-cell embryos were exposed to as little as 5 minutes of light.

In another study, hamster 2-cell embryos were exposed to light of different intensities and different wavelengths (Oh et al., 2007). Early embryonic development, concentrations of toxic reactive oxygen species (ROS), and heat shock protein 70, an inducible cell stress gene, were measured. Embryonic development to the blastocyst stage was inhibited by exposure to 900 lux when compared to 200 lux. Wavelengths in the 445 to 500 range (visible blue) also reduced development to the blastocyst stage, increased expression of HSP70 and resulted in higher levels of ROS compared to embryos exposed to 620-750 nm light (visible orange/red).

Besides rabbit and hamster embryos, light can affect the development of mouse embryos (Takenaka et al., 2007) although to a lesser degree. Early embryonic development is not affected by exposure of 15 minutes to cool white fluorescent light at 1200 lux, but increased internal levels of ROS were detected in mouse embryos exposed to this level. The concentration of light-induced ROS was also less in mouse embryos compared to hamster embryos. Mouse embryos exposed to cool white light had a higher number of apoptotic cells in blastocysts than embryos shielded from light. Implantation of normal blastocyst transferred into pseudo-pregnant females was also lower in mouse embryos exposed to light compared to shielded embryos (66% vs. 42%).

Another study examined the effect of light on the in vitro production of bovine embryos (Korhonen et al., 2009). Embryos were exposed to either filtered light (498 nm to 563 nm) or unfiltered light (halogen lamp) while being examined with a microscope. No differences were detected in embryo development or in the number of cells in blastocysts, but unfiltered-exposed bovine embryos had higher levels of the inducible stress protein, HSP70. It appears then that although light does not appear to overtly affect early embryonic development of mouse or bovine embryos, there is a toxic effect as measured by induction of ROS, HSP70 or impairment of implantation (in the mouse).

Light exposure in the human IVF laboratory

In 1998 (Edwards, RG 1998), Robert Edwards commented on his concerns regarding handling of gametes and embryos in vitro, and exposure of embryos to light:

"Light has also been one of my major concerns ever since IVF began. We were aware of the many papers on mammals published by embryologists on the evolution of reactive oxygen species in response to light exposure, and its deleterious effects on embryo growth. We could not afford any risks with human embryos to be replaced into the mother, so we used green filters routinely to remove some of the light radiation, lower the light intensity and produce a more acceptable colour for the eye by modifying the harsh artificial light from the microscope. The potential effects of light concerned me in another way. During transfer, gynaecologists often used an intense operating theatre light to shine on the cervical os. Yet this was where the embryo is passed during the transfer process. At the last moment, after hormone stimulation, oocyte collection, fertilization and cleavage in vitro, these precious embryos could be exposed to an intense light which might impair their ongoing development. We therefore dimmed this light during transfer to avoid any damage to the embryos in the last stage of their ex-utero existence. Several investigators have disparaged my attitude, and they may even be right when they claim that human embryos can tolerate this degree of intense light exposure. But I have never seen any evidence on this point from these investigators, and it is surely better to be safe than sorry. So I still use many of these precautions."

In the context of minimizing additive or interactive stressors in vitro, effects of lighting may be added to temperature, pH, ionic, metabolic, and other environmental excursions that influence developmental competency. There are no well-designed studies utilizing human gametes and embryos in vitro to evaluate the impact, if any, of the type of lighting, duration of exposure, or exposure to specific wavelengths; rather the information available on this topic is derived from animal model studies mentioned previously where there are variable tolerances to alterations in wavelength (nm) and intensity of light exposure according to developmental stage, species, and environmental temperature.

In the early years of IVF, laboratories were adapted from rooms originally tasked for other procedures, ranging from closets with no lighting to operating theaters with variable sources of light, rooms with incandescent lighting that might have variable intensity controls, and rooms with only standard fluorescent cool wavelength lights; so the type, quality, and intensity of lighting was not often taken into consideration (see Table 1). In fact, there were few to no standards or laboratory prototypes available, nor was there consensus as to what constituted the 'ideal' laboratory environment.

Table 1: Typical sources of visible light in the ART laboratory

Windows	
	Filtered, curtained, shades, blinds
Ceiling lights	
	Fluorescent; cold white, warm white
	Incandescent; dimmable and single intensity
Lamps	
	Floor, desktop
	Hoods/cabinets
Microscopes	
	Inverted
	Dissection
	Time-lapse
Direct effects of light	
	Mineral oil overlay
	Culture medium components
	Plasticware
Indirect effect of light	
	ROS
	Gene transcription
	Culture medium breakdown products
Egg retrieval	
	Direct and indirect room lighting, hoods,
	Other surgery lamps and microscope
Manipulation and routine handling	
	Direct and indirect room lighting, hoods,
	Other microscope
Embryo transfer	
	Direct and indirect room lighting, hoods,
	Other microscopes
	Headlamps, floor lamps, surgery lamps

With regard to human clinical IVF, there is one early publication discussing changes made in the laboratory design, prospectively, to improve the laboratory environment; two

parameters were considered, oxygen tension and lighting in the laboratory (Noda et al., 1994). While this study did not follow through to embryo transfer and gestational parameters, supernumerary spare embryos were allowed to develop under 1) low oxygen conditions (5%) and 2) low intensity lighting (20 and 100 lux, as measured from ambient and microscope lights, respectively). The authors noted increased development to the blastocyst stage in the low oxygen tension and low light conditions compared to culture under room air and standard lighting conditions.

A landmark study (Ottosen et al., 2007) provided measured, replicated baseline data for light intensity and duration of exposure to light under working clinical IVF laboratory conditions; a laboratory with windows, a laboratory without windows, application of various wavelength filters to microscopes, and importantly, technician preferences for microscope light intensity settings. The following should be taken under consideration when trying to determine the role that light might have in embryo development: 1) blue light wavelengths of 400 to 500 nm (near-ultraviolet) are considered to be potentially damaging to cells (gametes and embryos), 2) visible white light ranges from near UV (400 nm; short wavelength) to the near infrared spectrum (700 nm; longer wavelength), 3) cool fluorescent bulbs, common in many labs, emit wavelength spectra across 540 to 590 nm, while the newer generation warm fluorescent bulbs emit wavelength spectra across 540-560 and 620-640 nm, 4) incandescent lighting emits wavelengths across 620-640nm, and finally 5) halogen bulbs, common in microscopes emit light similar to other incandescent tubes, except that often they are coated with glass or a filter to filter out unintentional UV light. Mercury arc lamps are also used in some microscopes. These lamps emit light in the 300 to 600 nm range.

Is it practical or possible to reduce the wavelengths in the critical spectra- 400 to 500 nm, without compromising patient and technician safety? Under variable, but working lighting conditions, these authors demonstrated 1) that plastic polystyrene dishes did reduce or absorb small, but not significant amounts of energies in the 400 to 500 nm spectra, and 2) short wavelength filters applied to halogen bulb microscopes and room (background fluorescent and natural lighting) significantly reduced the light energies in this critical spectral range. Duration and intensity of light exposure were also modeled according to each technician preference, and were considered important; but the authors concluded that reducing ambient light intensity (while maintaining light intensity comfortable for the technicians), reducing duration of light exposure to gametes and embryos, and specifically targeting microscope lighting with addition of filtration medium (e.g. red or amber filters) would provide the most benefit.

One simple solution to reduce the exposure of embryos to light from a microscope may be to use a green bypass filter (such as the Schott VG-9) until the effect of light on human embryos is better understood. It can protect embryos from both the deleterious blue and near infra-red wavelengths. A green filter will work better than a red one as the eye is more sensitive to green than red (Parr, 2001).

From a practical standpoint, lighting in the laboratory should be sufficiently bright for personnel safety, and to ensure the safety of the gametes and embryos being handled in the laboratory; blue wavelength filters create a visible spectrum perceived as amber to yellow light; color labels can be washed out or altered in this environment, pH monitoring via phenol red in culture medium can be compromised, and the filters reduce the intensity of available lighting, making it necessary in some laboratories to employ additional incandescent lamps.

Reactive oxygen species

Two general methods are proposed to reduce the potential negative effects of light on ova and embryos 1) reduce exposure to light via filtration with filters on microscopes and light fixtures to remove the blue spectrum (mentioned above) and 2) mitigate damages from light-induced ROS by adding antioxidants to our media.

Reactive oxygen species (ROS) are generated as an intrinsic consequence of cell metabolism, e.g. mitochondrial function, where the potential damaging nature of these molecules is balanced by the inherent antioxidant capacity of the cells. When this balance is interrupted, an overabundance of the more potent radicals (hydroxyl and peroxide

compounds) can damage cells, specifically targeting lipids and DNA. Gametes and embryos in vitro are exposed, transiently or continuously to supra-physiological oxygen, temperature and pH excursions; exposure to various light energies can be responsible for adding to the total oxidative load to cells in vitro. Male and female reproductive cells are sensitive to increased ROS exposure in vivo and in vitro, and in some cases, impairment of reproductive potential is possible (Agarwal and Allamaneni, 2004; Agarwal et al., 2006).

The target for damaging ROS compounds in vitro has been debated. In regard to mammalian sperm, plasma membrane lipids can be damaged following light-induced increased ROS production. Oxygen radicals were produced in significantly greater quantities following exposure of sperm membranes to blue (400-505nm) spectra compared to red (600-800nm) spectra; the authors speculate that the mechanism directly involves membrane lipids, and not an external catalyzed process (Lavi et al., 2012). Ram sperm were exposed to white light (400-800nm), red light (660nm), blue light (360nm) and UV light (294nm) in vitro. Processed, light-exposed sperm were then used for in vitro fertilization. Motility of ram sperm was compromised by exposure to white, blue and UV spectra, and embryo viability after IVF was highest for sperm exposed to control (darkness) and red spectra. Additionally, lower ROS concentrations were generated by exposure to the longer (red) spectral wavelengths (Zan-Bar et al., 2005).

As has been mentioned previously, light has been linked to increased production of ROS and to DNA damage. Human embryo fragmentation (Yang et al., 1998) has been linked to apoptotic processes, which may be affected in vitro by ROS production, and further, ROS production increases as oxygen tension increases; the detrimental effect of increased ROS may manifest at the mitochondrial level; antioxidative capacity of the culture medium and the embryo itself may counter some of these negative ROS effects (Catt and Henman, 2000; Guerin, P. et al., 2001). It is not impossible then to conclude that ROS production is additive, for example, combining non-physiological oxygen tension with ambient light exposure which might reduce the development of human embryos in vitro (Noda et al., 1994).

Oocytes and embryos have some capacity for intrinsic DNA repair however the sperm cell does not (Menezo et al., 2007; Ménézo et al., 2010). Protecting cells in vitro - sperm, oocytes, and embryos - involves minimizing environmental stressors; the culture environment contributes to oxidative damage, and inclusion of antioxidants in the medium may provide additional protection (Bavister, 2000; Guerin, P. et al., 2001; Martín-Romero et al., 2008; Moshkdanian et al., 2011; Orsi and Leese, 2001). Altering the ability of the oocyte and embryo to repair DNA damage, for example by exposure to increased ROS can lead to altered transcription of repair mechanisms, and potentially reduced fertility (El-Mouatassim et al., 2007).

Therefore the design of the culture system should focus on minimizing opportunities for additional ROS production. A systematic approach to designing an embryo culture system includes choosing from the many commercial culture media available (though some clinics still manufacture their own culture media in-house); this is one of the more critical aspects of human clinical IVF. Culture media for human embryos have evolved significantly over the years, yet there are unsubstantiated and often misinterpreted concepts regarding culture medium components and phototoxicity that have been perpetuated over the years, stemming from several early tumor cell culture studies. For example, Spierenburg et al (Spierenburg et al., 1984), found that cell culture media (not formulated for use with mammalian embryos in vitro) containing HEPES and riboflavin compounds (B_2 vitamins), but not pyruvate, were found to be vulnerable to fluorescent light-induced peroxidation in the presence of oxygen. Current culture media for human and other mammalian embryos typically contain pyruvate, some contain some or all essential and nonessential amino acids, but they do not usually contain riboflavin compounds which are responsible for light-induced phototoxicity (Edwards and Silva, 2001); however significant ROS generation may still occur (Martín-Romero et al., 2008) simply by warming the medium to incubator temperatures.

An additional important and often overlooked component of the culture system is the quality of the oil used to overlay culture medium; a commercial oil product may arrive sterile from the manufacturer with a valid certificate of

analysis, but improper transportation temperature, exposure to sunlight and/or visible light, or improper storage may result in adulteration of the oil. Peroxides can form in a cascade reaction as a result of exposure to light and/or heat, resulting in transfer of water soluble contaminants to the culture drops (Otsuki et al., 2009, 2007; Provo and Herr, 1998).

It is clear that ROS generation in vitro is unavoidable; addition of exogenous antioxidants (e.g. ascorbic acid), and support of gamete and embryonic health to drive intrinsic antioxidant capabilities may counter the more damaging effects of ROS exposure. Technologies already exist to limit external stressors, for example supraphysiological oxygen concentrations can be avoided by using specific incubator designs to reduce oxygen concentrations, and pre-mixed low oxygen concentration gasses are available for use with mini-incubators and enclosed containers. Blue wavelength light energies can be avoided altogether by selection of lighting sources and/or light filtration devices and limiting exposure of gametes and embryos to any light energies by limiting the time that these cells are maintained outside of the incubator. Choice of embryo culture media and oil for overlays, in addition to maintaining supplies and reagents properly can contribute to reduction of ROS exposure.

Conclusion

No conclusive data exists to indicate that light is harmful to human gametes or embryos, but there is substantial evidence that light can be harmful to non-primate mammalian gametes and embryos. It is also known that light can affect the quality of oil and culture media, including buffers such as HEPES. Light in the blue visible and ultraviolet spectrum (<500nm) appears to have the highest potential for harm. There may be ways to reduce the effects of light on embryos and media by the inclusion of antioxidants or the exclusion of photooxidative media components. Light exposure can also be reduced by reducing the amount of harmful wavelengths in our laboratories via limited exposure to any light, use of ambient light filters, and avoidance of most fluorescent lighting.

Embryology laboratories should not be located in areas where direct sunlight might damage them. Care should be taken with hood lights, ambient lights, headlamps and microscope lamps. A green bypass filter may be prudent when viewing gametes and embryos. With new methods for determining embryo quality and suitability for transfer with frequent monitoring using in-incubator optics, it is prudent to try to understand more regarding the role light might play in the production and growth of human embryos.

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