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ARTICLE

Implementation of air quality control in reproductive laboratories in full compliance with the Brazilian Cells and Germinative Tissue Directive

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Abstract This article describes how Androfert complied with the Brazilian Cells and Germinative Tissue Directive with regard to air quality standards and presents retrospective data of intracytoplasmic sperm injection (ICSI) outcomes performed in controlled environments. An IVF facility, composed of reproductive laboratories, operating room and embryo-transfer room, was constructed according to cleanroom standards for air particles and volatile organic compounds. A total of 2060 couples requesting IVF were treated in the cleanroom facilities, and outcome measures compared with a cohort of 255 couples treated at a conventional facility from the same practice before implementation of cleanrooms. No major fluctuations were observed in the cleanroom validation measurements over the study period. Live birth rates increased (35.6% versus 25.8%; $P = 0.02$) and miscarriage rates decreased (28.7% versus 20.0%; $P = 0.04$) in the first triennium after cleanroom implementation. Thereafter, the proportion of high-quality embryos steadily increased whereas pregnancy outcomes after ICSI were sustained despite the increased female age and decreased number of embryos transferred. This study demonstrates the feasibility of handling human gametes and culturing embryos in full compliance with the Brazilian directive on air quality standards and suggests that performing IVF in controlled environments may optimize its outcomes.

KEYWORDS: air quality, assisted reproduction techniques, cleanroom, directive compliance, environment, volatile organic compounds

Introduction

Human gametes and embryos cultured *in vitro* are extremely sensitive to oscillations in temperature, humidity, light exposure, contaminants and physical trauma. Several reports suggest that toxic agents, e.g. bacteria, particulate matter, dust and chemicals (volatile organic compounds, VOC), may impact fertilization and embryo development (Boone et al., 1997; Cohen et al., 1997; Esteves et al., 2004; Hall et al., 1998; Little and Mirkes,

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1990; Mayer et al., 1999; Worrilow et al., 2002). Although the need for specific technical requirements regarding air quality in human IVF has been extensively debated, most practitioners acknowledge the importance of more rigorous laboratory management and also that minimum standards towards air quality should be implemented (Hartshorne, 2005; Kastrop, 2003; Mortimer, 2005; Von Wyl and Bersinger, 2004). It is still a matter of debate how high the standards for laboratory air quality should be, but animal experiments suggest that embryo development is improved by cultivating embryos in cleanroom environments (Kao et al., 2009). In humans, it has been demonstrated that cultivating embryos in cleanroom facilities with strict control of air quality conditions may optimize fertilization and embryo development (Boone et al., 1997; Esteves et al., 2004; Knaggs et al., 2007; Worrilow et al., 2002).

Regulatory agencies in many countries have issued directives which include specific requirements for air quality standards in embryology laboratories (ANVISA, 2006; EUTCD, 2004). In Brazil, these requirements were first issued in 2006 (Anvisa; RDC33) as part of the Brazilian Cells and Germinative Tissues Directive, which is a legal document originated from the Brazilian National Agency for Sanitary Surveillance (ANVISA). The Brazilian Cells and Germinative Tissues Directive sets standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human reproductive tissues and cells within Brazil. Its aims are to safeguard public health preventing transmission of infectious diseases via transplanted tissues and cells, according to the premises of the precautionary principle (Commission of the European Union Communities, 2000). Assisted reproduction technology is considered as covered by this directive and applies to all assisted reproduction units in Brazil (approximately 200). In summary, the Brazilian Cells and Germinative Tissues Directive, which was amended in 2011, aims at increasing quality through mandatory implementation of a quality management system that involves the presence of adequately trained and certified staff, full documentation and formulation of standard operating procedures, quality control and quality assurance at all units performing assisted reproduction. In this sense, the Brazilian directive is similar to the European Union Tissues and Cells Directive (EUTCD, 2004). With respect to laboratory ambient air, the Brazilian Cells and Germinative Tissues Directive dictates that it should be at least equivalent to ISO class 5 (NBR/ISO 14644-1) in the critical areas where tissues or cells are exposed to the environment during processing, and recommends one of the following methods to achieve such conditions: (i) biological safety cabinet class II type A; (ii) unidirectional laminar flow workstation; and (iii) at least equivalent ISO 5 cleanroom. In addition, background air (clean areas for carrying out less critical stages) should be pressurized (outside and total air volume of 15 and 45 m^3/h/m^2 or higher) and filtered for particulates (at least G3 + F8 dust filtration) in cases when biological safety cabins and unidirectional laminar flows are used. Areas in which oocytes/reproductive tissue/spermatozoa are surgically retrieved should also have ambient air pressurized (outside and total air volume of 6 and 18 m^3/h/m^2 or higher) and filtered for particulates (at least G4 class dust filtration). Lastly, ventilation systems should be equipped with filters imbedded with activated charcoal to remove VOC. Table 1 presents the main aspects of the Brazilian directive with

Table 1 Ambient air quality requirements for IVF laboratories operating under regulatory directives in the European Union and Brazil.

	European Union (EU directive 2004/23/EC; 2006/86/EC)	*Brazil (Anvisa RDC33/2006; RDC23/2011)*
Particle filtration	Equivalent to GMP[a] grade A air quality in the critical areas where tissues or cells are exposed to the environment during processing with a background environment at least equivalent to grade D[b]	At least equivalent to ISO class 5 (NBR/ISO 14644-1) in the critical areas where tissues or cells are exposed to the environment during processing
Microbial contamination	Maximum colony forming units (cfu) in grades A and D air quality environments defined as follows: air sample (cfu/m^3: <1 and 200), 90-mm diameter settle plates (cfu/4 h: <1 and 100), 50-mm diameter contact plates (cfu/plate: <1 and 50), 5-finger glove print (cfu/glove: <1 and 'not defined')	Microbiological monitoring required; specifications not defined
Volatile organic compound filtration	Not required	Ventilation systems should be equipped with filters imbedded with activated carbon

GMP = good manufacturing practice.
[a]GMP grades A and D air quality for particulates are equivalent to international standard ISO 14644-1 classes 5 and 8, respectively.
[b]A less stringent environment may be acceptable in the following cases: (i) where it is demonstrated that exposure in a grade A environment has a detrimental effect on the required properties of the tissue or cell concerned; (ii) where it is demonstrated that the mode and route of application of the tissue or cell to the recipient implies a significantly lower risk of transmitting bacterial or fungal infection to the recipient than with cell and tissue transplantation; (iii) where it is not technically possible to carry out the required process in a grade A environment (for example, due to requirements for specific equipment in the processing area that is not fully compatible with grade A).

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regard to air quality control and how it compares to the European directive.

The impact of applying cleanroom air quality standards to assisted conception facilities has been debated with regard to its feasibility and effectiveness and no consensus has yet been reached. Some authors argue that implementation of strict air quality control, as required by regulatory agencies, is likely to have negligible impact on the risks of culture contamination and operator infection, but would severely compromise the ability to maintain gametes and embryos under optimum environmental conditions (Bhargava, 2005; Mortimer, 2005; Pope and Saunders, 2005), while others suggest that compliance with air quality standards is feasible (Hartshorne, 2005) and with no detrimental impact on IVF clinical results (Knaggs et al., 2007).

This article describes how air quality control was implemented in an embryology laboratory and related areas in full compliance with the Brazilian Cells and Germinative Tissue Directive. Also presented are results from monitoring air quality within the cleanroom areas and retrospective data from handling and culturing human embryos in the cleanroom facilities.

Materials and methods

Implementation of cleanroom areas

Configuration of the air-handling ventilation and filtration system

In order to comply with the Brazilian directive on air quality requirements (Table 1) the concept of cleanrooms was used in the areas that gametes and embryos are handled. This included not only the embryology laboratory but also associated areas (oocyte/sperm retrieval room, embryo-transfer room, sperm processing room). The system is designed to supply pressurized air, which is cleaned by chemical and particulate filters, with adequate heating, cooling and humidification capacity to meet daily needs. At the end of the construction, there was a 3-month waiting period to allow off-gassing before first occupation of the new facilities. During this period, the air-handling ventilation unit, described below, was set up to continuously bring in fresh air. After this period, testing was carried out by independent companies to confirm that the areas had been built according to the design of the engineers and met the standards required by regulatory authorities.

Construction details

The cleanroom facilities have been designed and constructed according to the standards of ISO 14644-4 (International Organization for Standardization, 2001). Construction materials, including internal finishes, doors, air vents/diffusers and floor and ceiling elements, have been selected based on their cleanability, durability and maintainability. Specifically, exposed materials are suitable for effective and frequent cleaning. All surfaces, including ceilings, walls and floors, are made of smooth, impervious and non-shedding materials that offer no surface asperities or porosity which might allow retention of particulate and chemical contamination or the development of microbiological contamination. Walls surfaces are covered with low-odour epoxy-based paint, and floors are made of sheet vinyl with heat-welded seams and a coved base, with the exception of the embryology laboratory in which polyurethane-based coatings were used for walls and floor finishes. The junctures of the ceiling to the walls are coved. Lighting fixtures are flush mounted within the ceiling and sealed, and there are no sinks or floor drains in the cleanroom areas. Additionally, the study centre selected materials with reduced VOC off-gassing potential in accordance to the US Environmental Protection Agency specifications on the environmental impact of materials (EPA 01120). For example, fibreglass, wood and plastic-based materials were not used, and prefabricated site-assembled construction materials were avoided. Instead, in-situ wet construction with applied surface finishes was preferred. Stainless steel and anodized aluminum were used in doors, windows, air vents and diffusers, as well as in workstations. Water-based low-VOC adhesives were used when needed.

Air-handling ventilation unit room

The air-handling ventilation unit room (2.1 m width × 3.9 m length × 2.5 m height; 20.5 m^3) includes a roof-top air-handling unit (model UAECA-300; Veco, Campinas, Brazil) that draws outside air through coarse (G4) and charcoal prefilters before it enters the main ventilation unit. A free-standing main ventilation unit (model UVCA-3000; Veco) pulls prefiltered outside air and the cleanrooms' return air through coarse (G3) filters (first-stage filtration), past a 16-unit pelletized coconut shell-based activated carbon impregnated with potassium permanganate filters (second-stage filtration) and then through fine (F8) dust filters (third-stage filtration). Lastly, filtered air enters the cleanrooms through high-efficiency particulate air (HEPA) filter diffusers (Fig. 1). Floor- and ceiling-level vents in the cleanrooms' return air to the main ventilation unit, to be remixed with the existing air.

Filter beds (593 × 593 × 22 mm; mesh size 4 × 8) containing potassium permanganate-impregnated zeolite plus activated carbon were utilized. Chemical filters, located downstream of the cooling and heating coils, were arranged in a Z configuration, with the air flow nearly perpendicular through each bed. Nominal bed residence times of chemical air cleaners are 0.082 s. Activated carbon life-time estimates were determined by sampling in service filters at 3-month intervals over a 1-year period by the carbon tetrachloride activity method (American Society for Testing and Material, 2009). A reduction in 50% of the original filter activity was observed after 12 months and determined the filter's working capacity. The replacement schedule is set at 6–8 month intervals. This estimation is considered to be adequate to avoid reaching breakthrough capacity due to the moderate polluted urban non-industrial area where the facility is located. Filters type G3 are primary filters that collect coarse dust with a dust spot efficiency of 80–90%, while type F8 are secondary filters that collect and retain small particle dust with a spot efficiency of 90–95%.

Embryology laboratory

The cleanroom embryology lab (3.5 m width × 3.9 m length × 2.5 m height, 34.1 m^3) has two ceiling HEPA-filter air diffusers and two wall-mounted HEPA-filter diffusers

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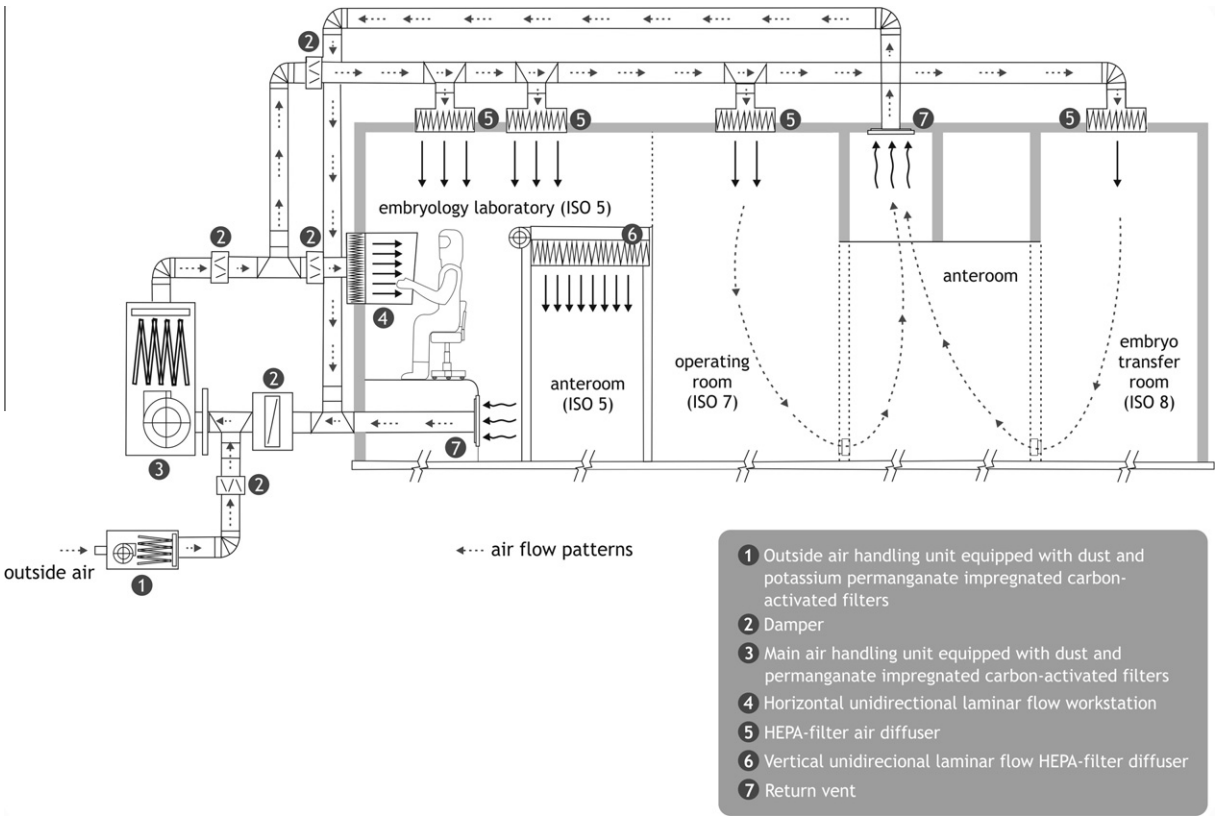

Figure 1 Schematic representation of the cleanroom embryology facility and its associated areas, including air flow patterns and filtration units. The air-handling ventilation unit room has a roof-top air-handling unit that draws outside air through coarse and charcoal prefilters before it enters into the main ventilation unit. A free-standing main ventilation unit pulls prefiltered outside air and the cleanrooms' return air through coarse filters, past a 16-unit potassium permanganate-impregnated pelletized coal-based activated carbon filters and then through fine dust filters. Lastly, filtered air enters the cleanrooms through high-efficiency particulate air (HEPA) filter diffusers. Floor- and ceiling-level vents in the cleanrooms return air to the main ventilation unit to be remixed with the existing air. Differential positive pressure is maintained between rooms. The embryology laboratory/anteroom is positive to the operating room, which is positive to both the embryo-transfer room and the dressing room/hallways.

that provide horizontal unidirectional laminar air flow to workstations for incoming oocytes and outgoing embryos and micromanipulation. Four vents at the floor level return the air to the main air-handling ventilation unit. Access to the cleanroom is made through an anteroom equipped with two ceiling HEPA-filter air diffusers that draw cleanroom air and provide vertical unidirectional laminar air flow to the entire anteroom. The anteroom has a clean closet to store face masks, safety glasses, hoods, coveralls, boots and disposable laboratory supplies and is used as a gowning room. The anteroom is also the pass-through for specimen transfer from the adjacent operating room to the embryology laboratory. The anteroom and cleanroom undergo 499 and 103 air exchanges per hour, respectively.

Operating room

The operating room (4.7 m width × 3.6 length × 2.8 m height, 47.4 m^3) has a unique ceiling HEPA-filter diffuser and a return vent in the wall at floor level (Fig. 1). Using these passageways, the air in the room undergoes 12 exchanges per hour. In addition, the operating room has a portable minihood containing a HEPA filter (model DM-66; Veco). During oocyte and sperm retrieval, the tubing heating system is placed inside the minihood and it is used to improve air quality directly over the area where capping and uncapping of tubes occurs. Access to the operating room is made through an anteroom where personnel perform hand hygiene and complete other high-particulate-generating activities. Also, the anteroom is a transitional area that maintains the air pressure relationship between the operating and gowning rooms, ensuring air flows from clean to dirty areas and reducing the need for the HVAC control system to respond to significant disturbances.

Embryo-transfer room

The embryo-transfer room (3.0 m width × 3.2 length × 2.6 m height, 24.9 m^3), which is adjacent to the operating room, has a unique ceiling HEPA-filter diffuser and a return vent in the wall at floor level. The embryo-transfer room undergoes nine air exchanges per hour (Fig. 1).

Positive pressure

Differential positive pressure is maintained among rooms. The embryology laboratory/anteroom is positive to the operating room (2.1 mm water column differential,

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mmWC), which is positive to both the embryo-transfer room (0.7 mmWC) and the dressing room/hallways (0.5 mmWC).

Andrology laboratory and cryopreservation storage room

Construction details

Similarly to the embryology laboratory, all andrology and cryoroom surfaces are made of smooth, impervious, and non-shedding materials, and the junctures of the ceiling to the walls are coved. Walls are painted with low-odour epoxy paint, and floors are made of sheet vinyl with heat-welded seams and a coved base. Furniture and equipment are non-permeable, non-shedding, cleanable and resistant to frequent cleaning and disinfecting.

Andrology laboratory

The andrology laboratory (3.5 m width × 5.1 length × 2.8 m height, 50.0 m^3) has a roof-top air-handling unit (model UAECA-300) that draws outside air through coarse (G3 and F8) and carbon-activated filters before it enters the unique ceiling HEPA-filter air diffuser that distributes filtered air to the laboratory under positive pressure at 702 m^3/h (Fig. 2). The andrology laboratory has a class II type A1 biological safety cabinet (model Bioseg-09; Veco) where cryopreservation and sperm handling for therapeutic purposes take place. Access to the andrology laboratory is made through an anteroom where personnel dress and perform hand hygiene.

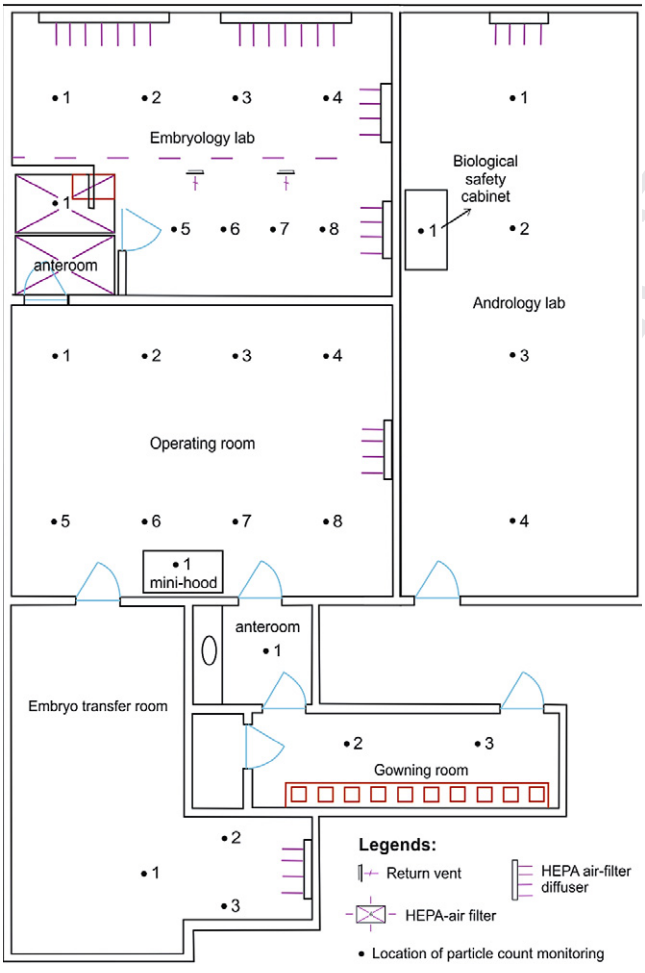

Figure 2 Locations of airborne particle count monitoring within the reproductive facilities and associated areas.

Cryopreservation storage room

Liquid nitrogen tanks containing cryopreserved specimens are stored in the cryopreservation room (2.1 m width × 3.5 length × 3.0 m height, 22.1 m^3). The cryoroom is equipped with an oxygen depletion alarm unit and a ventilation system (model UE 500; Veco) to exhaust ambient air under negative pressure at 150 $m^3/h/m^2$. Access to the cryoroom is made through the andrology laboratory.

Mechanisms to reduce contamination

In addition to the construction details, several measures were taken to reduce contamination. Only the minimum amount of furniture, equipment and supplies were taken into the cleanrooms. Furniture and equipment are non-permeable, non-shedding, cleanable and resistant to frequent cleaning and disinfecting. Personnel access to reproductive laboratories is limited and is made through the anteroom equipped with a gowning room chamber and hand-hygiene area. All personnel entering reproductive laboratories or adjacent areas (operating room, embryo-transfer room) are required to gown up properly. In addition, personnel are required to step on adhesive-covered mats that remove dirt and dust from the soles of shoes. An anteroom between the embryology laboratory and the operating room allows for passage of gametes and embryos between these two locations and minimizes the mixture of air from the embryology laboratory and the adjacent operating room. Embryology laboratory personnel wear non-shedding Dacron coveralls, hoods and shoe covers as well as masks and gloves. Gowning up to the embryology laboratory entrance takes place in the anteroom between the laboratory itself and the operating room. Care is taken to select and use commodity items in the embryology laboratory. Lint-free wipes, cleanroom paper and pencils only are allowed. Many cosmetics contain sodium, magnesium, silicon, calcium, potassium or iron and may emit VOC. These chemicals are banned in the reproductive laboratories. Ultra high-purity (UHP) medical grade compressed carbon dioxide is supplied to incubators, and dedicated gas lines are fitted with particulate and chemical filters (GenX, USA).

Cleaning is an essential element of the contamination control system. A list of cleaning tasks that are performed on a daily basis both in reproductive laboratories and adjacent critical areas includes cleaning of all work surfaces as well as equipment and vents, emptying of trash and waste, cleaning of the doors, door frames and lockers in the pre-staging area and gowning areas using isopropyl alcohol, and mopping all room floors. On a monthly basis, rooms and incubators are 'term-cleaned'. Bi-annually, rooms are sanitized with 2% sodium hypochlorite solution. As part of quality control, the rooms' and incubators' temperature and humidity values are obtained twice a day. Semi-annually, an inhibitive mould agar Petri dish (for moulds/fungi) and a blood agar Petri dish (for bacteria) are labelled with the room, location and date and sent to microbiological analysis.

Air quality monitoring

Initial testing was performed both before occupation (as built) and after 6 months at normal operational state. Assessed parameters included determination of air volume flow rate, air exchange rate, room air pressure differential, filter integrity leak testing, airborne particle cleanliness counts, recovery performance testing, lighting and noise level measurements and temperature and humidity monitoring. Particle counts were performed in various locations within the embryology laboratory and other critical areas. Monitoring was performed in eight locations in the embryology facility, eight locations in the operating room, one location in each anteroom, one location each in the laminar flow cabinets, three locations in the embryo-transfer room, four locations in the andrology laboratory and two in the gowning room (Fig. 2). Ten particle-count cycles were performed at each of the nine sites, and the results were pooled to provide mean counts for different particle sizes (0.3, 0.5 and 5.0 μm) in each site. Determination of VOC concentrations in the embryology laboratory was carried out by active sampling on Tenax TA sorbent, followed by thermal desorption and gas chromatography employing a mass spectrometric detector, in accordance with the US EPA method TO-1 (US Environmental Protection Agency, 1984). Three parallel air samples with different sampling volumes of 1, 3 and 5 l were taken in the centre of the room. The presence of aldehydes was determined by active sampling using adsorbent cartridges coated with 2,4-dinitrophenylhydrazine and subsequent analysis of the hydrazones formed by high-performance liquid chromatography with detection by ultraviolet absorption, in accordance with the ISO 16000-3 standards (International Organization for Standardization, 2001). The embryology laboratory was selected as the appropriate room for sampling because it is the place where gametes and embryos are exposed to ambient air. Incubators were not checked since 90–95% of chamber air consists of ambient air, and the remaining comes from chemical and particulate-filtered CO_2.

After initial validation, subsequent monitoring of the operational state has been carried out by a third-party certification company to prove continued compliance with ISO 14644-1. Schedule of testing to demonstrate compliance with limits of airborne particle concentration, air flow volume, air pressure difference, number of air exchanges per hour, ambient air humidity and room temperature was set at 6-month intervals, in accordance to the ISO 14644 (parts 2 and 3) specifications (International Organization for Standardization 2000, 2005). Physical inspection of the ventilation and filtration mechanical system is also performed. Annually, additional testing within the testing schedule includes HEPA filter integrity leak testing, recovery performance testing, containment leakage and noise levels.

Routine determination of VOC concentrations has not been performed. Instead, the remaining activity of activated carbon filters is checked by the carbon tetrachloride method at every other carbon replacement to determine whether the schedule of filter changing is adequate for safe operating life cycles. Filters attached to incoming CO_2 gas lines are replaced at 3-month intervals in accordance with the manufacturer's specifications (GenX). Temperature and humidity levels of incoming air are checked on a daily basis and are kept within the limits of 22–25°C and 40–60% relative humidity, respectively, as they may interfere with filter deabsorption capacity (Worrilow et al., 2001). In addition, chemical filters are inspected monthly for plugging of activated carbon pellet beds due to particulate matter.

Case series

Between 2002 and 2010, 2060 consecutive intracytoplasmic sperm injection (ICSI) cycles involving fresh embryo transfers were performed in the cleanroom facility described above. The outcome measures were compared with a historical cohort of 255 consecutive ICSI cycles performed by the same staff in an older facility within the same institution between 1999 and 2001 prior to the implementation of air quality control standards (group 2). The conventional embryology facility and its associated areas (oocyte retrieval room and embryo-transfer room) operated without air filtration for particulates and VOC. Indications for ICSI were in accordance with the guidelines of the II Brazilian Consensus of Male Infertility even if the indication of IVF was a female factor (Marinelli et al., 2003). Cycles involving egg donation were excluded. Ovarian stimulation, oocyte and sperm retrieval, sperm processing and sperm injections were carried out as previously reported (Verza and Esteves, 2008; Esteves et al., 2007, 2009; Esteves and Agarwal, 2011). Fertilization was considered normal when oocytes with 2PN and 2 polar bodies were observed 16–18 h after ICSI. Fertilized oocytes were cultured until embryo transfer to the uterine cavity, which was guided by abdominal ultrasound on day 3 of embryo culture. Embryos were graded morphologically using a light inverted microscope 48 and 72 h after ICSI. High-quality embryos had 3 or 4 and 7 or 8 symmetrical blastomeres on days 2 and 3 of culture, respectively, with no multinucleation, grade 1 or 2 fragmentation or zona pellucida abnormalities. Clinical pregnancy was confirmed by a gestational sac with an embryo showing cardiac activity on ultrasound at weeks 6–7. Miscarriage was considered when nonviable clinical pregnancy was noted on ultrasound follow up.

Statistical analysis

The qualitative variables are expressed as both absolute (n) and relative (%) frequencies; the quantitative variables are mean ± standard deviation. The Kolmogorov–Smirnov test was applied to check the normal distribution through numeric variables. The relationship among the variables was evaluated by the chi-squared test. The Student t-test and analysis of variance for one factor (one-way ANOVA) were used for the comparison of quantitative variables when there was a normal distribution of each variable. Differences were analysed by the Tukey multiple comparisons test. For the variables without normal distribution, comparisons were performed by the Kruskal–Wallis test and the differences were compared using the Dunn multiple comparisons test. A P-value <0.05 is considered significant.

Ethical approval

This study was exempted of IRB approval according to the Brazilian legislation since it involved the analysis of existing

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records involving established clinical practices (Conselho Nacional de Saúde, 1996). The primary goal of the investigation was to provide technical details of how air quality control was implemented in these facilities in compliance with the Brazilian Cells and Germinative Tissue Directive and to monitor outcomes by comparing the current clinical practice with a historical cohort. Nevertheless, it is this study centre's policy to obtain signed informed consent from all patients undergoing IVF treatment to use their data for analysis with guarantees of confidentiality.

Results

Particle count monitoring and other validation measurements

Air quality validation testing results, performed both before and after 6 months of normal operation, confirmed that the cleanroom facility was built according to design and in compliance with regulatory agency requirements. Total VOC concentrations, defined as the sum of all compounds expressed in toluene equivalents that appear in the gas chromatogram between and including n-hexane and n-hexadecane, were below 2 $\mu g/m^3$ of air. Aldehyde concentrations were below the detectable limit of 1 $\mu g/m^3$.

Results of air quality monitoring within cleanrooms and associated areas are shown in Table 2. There was a significant location effect for each of the three particle sizes ($P = 0.001$). In the 0.5- and 5.0-μm particle groups, counts for the embryology facility and its anteroom were not different from one another while the 0.3-μm particle group was lower in the latter ($P = 0.0008$). Mean particle counts (sizes 0.3, 0.5 and 5.0 μm) differed for the embryology facility and associated areas ($P < 0.001$). The cleanest locations were both the embryology facility and its anteroom, followed by the operating room and then the embryo-transfer room, and lastly the andrology laboratory. No major fluctuations were observed in the validation measurements which included air particle count, air volume flow rates, number of air exchange per hour, ambient air humidity, room temperature and noise levels. In addition, the number of personnel members performing activities did not change during the period of study.

Clinical results of handling gametes and culturing human embryos in cleanroom areas

From January 1999 to December 2010, 2315 consecutive ICSI cycles and fresh embryo transfers were performed at the study institution. Of these cycles, 2060 were carried out after the implementation of air quality control in the embryology facility and associated areas while a cohort of 255 cycles were performed at a conventional facility from the same practice before implementation of cleanrooms. Over this same period, there was no considerable difference in embryo culture techniques. Furthermore, the reasons that patients underwent ICSI did not significantly change after the installation of the cleanrooms. The proportions of patients undergoing ICSI for male and female factor infertility were 28.6% and 33.3%, respectively, in group 1 compared with 28.2% and 34.6% in group 2. Male and female factor infertility combined represented 38.1% and 37.2% in groups 1 and 2, respectively. The mean female patient age was significantly lower in the group treated in the IVF facilities without air quality control (30.2 ± 5.2 years) than in the group treated in the cleanroom facilities (34.0 ± 5.1; $P < 0.001$).

Sperm injection outcomes are presented in Table 3. There was no statistically significant difference in the total number of retrieved and mature oocytes between the groups. The mean rate of normally fertilized oocytes was also similar. The proportion of high-quality embryos was significantly higher in group 1 (48.3% versus 36.4%; $P < 0.001$). The mean number of transferred embryos was significantly lower in the group treated in the cleanroom facilities than in the standard IVF group ($P < 0.001$). The clinical pregnancy and live birth rates per transfer were higher in group 1 while the miscarriage rate was lower in this same group, although differences were not statistically significant.

Stratified data analysis of sperm injection cycles by time periods is presented in Fig. 3. We noted improved clinical pregnancy (44.8% versus 36.2%; $P = 0.03$) and live birth (35.6% versus 25.8%; $P = 0.02$) rates and a decreased miscarriage rate (28.7% versus 20.0%; $P = 0.04$) in the first triennium after installation of the cleanrooms. Embryo development also improved significantly ($P < 0.001$) over the same periods while fertilization rates were not different. These results were achieved by transferring similar numbers of embryos, despite the fact that female age was lower ($P = 0.001$) in the group of patients treated before the implementation of cleanrooms. A non-statistically significant decrease in live birth rates occurred in the second and third triennium after the implementation of the cleanrooms, while miscarriage rates remained unchanged. During these same years, the mean number of embryos transferred significantly decreased (3.4 versus 2.3; $P < 0.001$) while the mean age of females who sought IVF increased (34.4 versus 32.8 years; $P = 0.01$). The proportion of cleavage-stage embryos classified as having high quality at the day of transfer steadily increased after the implementation of the cleanrooms ($P = 0.007$). A significantly higher proportion of embryos were classified as having 8-cell stage and grades 1–2 cytoplasmic fragmentation on day 3 of embryo culture after cleanroom implementation ($P = 0.01$; Table 4).

Discussion

This article describes in detail how an embryology laboratory and related areas with air quality control were implemented in full compliance with the Brazilian Cells and Germinative Tissue Directive and presents the results of monitoring air quality within the cleanroom areas. The cleanliness of our facilities was periodically validated and no major variation was noted over a 9-year period. Furthermore, retrospective data of sperm injection cycles performed in the IVF facilities are given for before and after the implementation of the cleanrooms. The results show that it is not only feasible to implement air quality standards but also possible to operate and comply with such standards while maintaining sustainable results of an ongoing assisted reproduction programme.

Several IVF clinics providing assisted reproduction treatment with in-vitro manipulation of gametes and embryos

Table 2 Validation testing results for reproductive laboratories and associated critical areas.*

Facility	*ISO 14644-1 cleanroom classification*	*Air particle count (μm/m³)*[a]			*Ambient air humidity (%)*	*Room temperature (°C)*	*Noise level (dBA)*[b]	*Air volume flow rate (m³/h)*	*No. of air exchanges per hour*	*No. of personnel members in daily activities*
		0.3	*0.5*	*5.0*						
Embryology	ISO 5	2767 ± 1231	621 ± 299	0 ± 0	45.0 ± 6.9	23.5 ± 0.5	63 ± 11	3523 ± 201	102 ± 4	2
Embryology anteroom	ISO 5	1329 ± 1105	527 ± 507	0 ± 0	NR	NR	60 ± 6	1583 ± 112	489 ± 16	1
Operating room	ISO 6	98,231 ± 26,607	1567 ± 496	81 ± 59	46.1 ± 5.8	23.8 ± 0.8	65 ± 22	589 ± 96	12 ± 2	4
Operating room anteroom	ISO 7	80,042 ± 11,822	2008 ± 412	2221 ± 116	NR	NR	67 ± 12	NR	NR	1
Embryo transfer	ISO 7	83,261 ± 10,023	1213 ± 782	1711 ± 601	49.0 ± 4.9	24.2 ± 1.6	59 ± 9	234 ± ± 27	9 ± 1	3
Andrology	ISO 7	NR	312,812 ± 38,175	332 ± 80	47.3 ± 6.9	23.1 ± 1.9	69 ± 14	721 ± 88	14 ± 2	2
P-value	—	0.001	<0.001	<0.001	0.23	0.34	0.04	0.003	<0.001	—

Values are mean ± SD or *n*. Validation measures were obtained 'at operation'. Data were pooled from semi-annual validation testing performed by a third-party company (CCL, Campinas, Brazil) from 2002—2010.
NR = not reported.
[a]Air particle counts pairwise comparisons not significant for: operating room anteroom versus embryo transfer at 0.3 μm/m³; embryology versus embryology anteroom; and operating room versus embryo transfer at 0.5 μm/m³.
[b]Noise level pairwise comparisons significant for only andrology versus embryo transfer.

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Table 3 Patient characteristics and main outcome measures for intracytoplasmic sperm injection cycles performed in cleanroom facilities (group 1) and in conventional IVF facilities (group 2).

	Group 1	*Group 2*	*P-value*
No. of cycles	2060	255	–
Oocytes retrieved (*n*)	10.3 ± 7.0	10.8 ± 6.9	NS[a]
MII oocytes (*n*)	8.5 ± 5.8	8.9 ± 5.6	NS[a]
2PN fertilization (%)	66.9 ± 34.1	69.4 ± 25.3	NS[a]
High-quality embryo (%)	48.3 ± 33.1	36.4 ± 29.2	<0.001[a]
Embryos transferred (*n*)	2.6 ± 1.1	3.3 ± 1.8	<0.001[a]
Clinical pregnancies/transfer	779/1967 (39.6)	87/240 (36.3)	NS[b]
Miscarriages	167 (21.4)	25 (28.7)	NS[b]
Live births (%)	612 (31.1)	62 (25.8)	NS[b]

Values are mean ± SD, *n*/total (%) or *n* (%).
[a]Unpaired Student's t test.
[b]Pearson's chi-squared test.

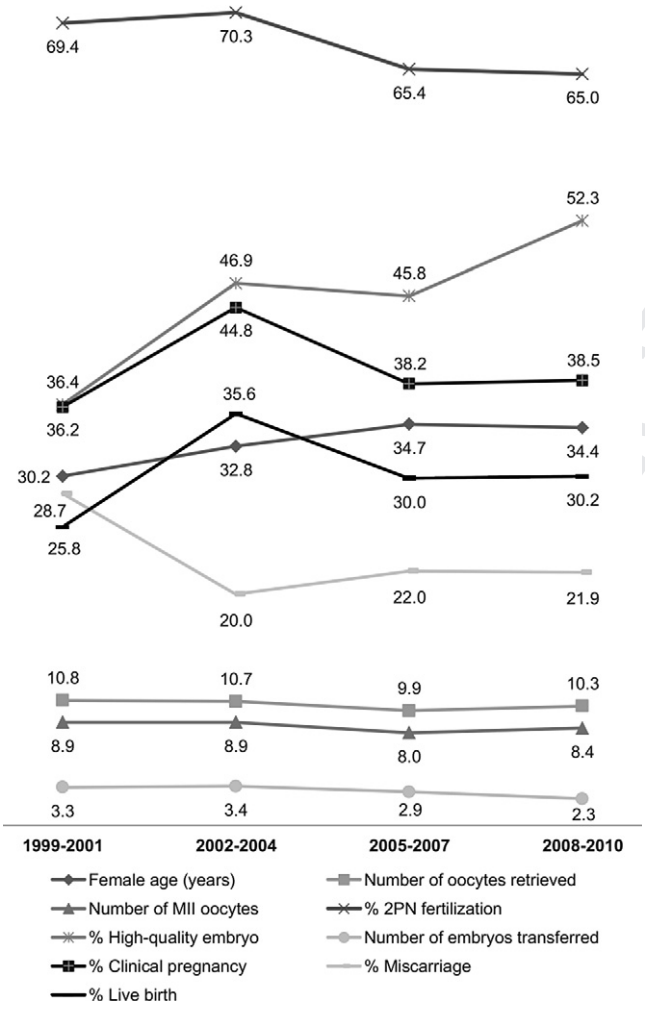

Figure 3 Stratified data analysis by triennium periods of intracytoplasmic sperm injection cycles performed at standard IVF facilities (1999–2001) and cleanroom facilities (2002–2010). Values are expressed as means. MII = metaphase II; 2PN = two pronuclei.

are now obliged to comply with specific regulatory requirements, as defined by standards of quality and safety for the donation, obtaining, testing, processing, preservation, storage and distribution of human tissues and cells. In Europe, for instance, the European Union issued the Tissues and Cells Directive in 2004 (Commission of the European Parliament, 2004) while in Brazil the National Agency for Sanitary Surveillance passed a similar regulatory directive in 2006, subsequently amended in 2011 (ANVISA, 2006).

One of the main challenges of these directives is the need to control air quality, as they require air quality equivalent to ISO 5 or even higher in cleanrooms where gametes are handled. A cleanroom is defined by ISO 14644-1 as 'a room in which the concentration of airborne particles is controlled, and which is constructed and used in a manner to minimize the introduction, generation, and retention of particles inside the room and in which other relevant parameters, e.g. temperature, humidity, and pressure, are controlled as necessary' (International Organization for Standardization, 1999). In reproductive laboratories, particles of interest measure between 0.1 and 10 μm. Because bacteria and other contaminants can attach themselves to particles, a decrease in particles equates to an increase in air quality. The ISO 14644-1 establishes standard classes of air cleanliness for airborne particulate levels in cleanrooms and clean zones. Removal of airborne particulates involves the forced movement of air using positive air pressurization through a series of filters of increasing efficiency. Filter efficiency is achieved by decreasing the diameter of the pores' membranes. First, air filtration eliminates larger particles such as dust, and subsequently, high-efficiency particulate air (HEPA) or ultralow penetration air (ULPA) filters trap small particulates, fungi, spores and bacteria, thus decreasing microbiological contamination (National Environmental Balancing Bureau, 1998). HEPA air filters have 99.97% minimum particle-collective efficiency for particles as small as 0.3 μm. In the current model, particle count monitoring is lower in the embryology laboratory because it is the most critical area for gamete and embryo handling. Particle count increases as air passes from the embryology laboratory to the operating room and increases further inside the embryo-transfer room. This

Table 4 Embryo development characteristics of intracytoplasmic sperm injection cycles.

	1999–2001	2002–2004	2005–2007	2008–2010	P-value
No. of embryos	1862	2479	3522	6797	–
Embryos/cycle[a]	5.9 ± 4.2	6.0 ± 3.9	6.1 ± 3.8	6.1 ± 3.7	NS[e]
Embryos of 4-cell stage on D2	2.7 ± 2.7	3.5 ± 2.5	3.0 ± 2.9	3.1 ± 2.7	NS[e]
Grades 1–2 cytoplasmic fragmentation on D2[b]	50	71	66	65	NS[f]
Embryos of 8-cell stage on D3[c]	2.3 ± 2.2	3.2 ± 2.7	2.8 ± 2.5	3.0 ± 2.1	0.01[e]
Grades 1–2 cytoplasmic fragmentation on D3[b,d]	52	78	72	69	0.02[f]

Values are mean ± Sd or %. Data for fresh embryo transfer. Cycles prior to 2002 were performed in a conventional IVF facility. D2 = second day of embryo culture; D3 = third day of embryo culture; NS = not significant.

[a]

[b]Based on the total number of embryos.[c]1999–2001 versus all others, $P = 0.01$; 2002–2004 versus all others, NS.[d]1999–2001 versus all others, $P = 0.02$; 2002–2004 versus all others, NS.[e]Kruskal–Wallis test.

[f]Pearson's chi-squared test.

shift towards increased airborne particles is expected, since the cleanroom facilities were designed so as to have the cleanest air inside the embryology laboratory and operating room, and then efficiency decreased in the locations not so critical to the process. It should be noted, however, that all locations exhibited markedly low particle counts in operation conditions.

Nonetheless, other filtration mechanisms rather than particle elimination alone through coarse, fine and HEPA and/or ULPA filtration are needed to control contamination in the assisted reproduction environment. In this sense, it has been suggested that removal of VOC that are constantly being generated by materials and cleaning agents used inside the laboratory is also essential (Cohen et al., 1997; Hall et al., 1998). As such, this study proposes that a better definition for assisted reproduction technology cleanrooms would be 'a room in which the concentration of airborne particles and VOC is controlled, and which is constructed and used in a manner to minimize the introduction, generation, and retention of particles and VOC inside the room, and in which temperature, humidity and pressure are controlled'.

Organic chemical compounds are everywhere in both indoor and outdoor environments because they have become essential ingredients in many products and materials. VOC are organic chemical compounds whose composition allows them to evaporate under normal indoor atmospheric conditions of temperature and pressure. Indoor VOC react with the indoor ozone, and the chemical reactions produce submicron-sized particles and harmful by-products that may be associated with adverse health effects in some sensitive populations. An early study conducted in seven assisted reproduction clinics showed that air quality deteriorated with regard to VOC contamination as it passed from the exterior of the buildings into the embryology laboratory and deteriorated further inside incubators. The numbers ranged from an average of 533 $\mu g/m^3$ outside-air VOC to an average of 2769 $\mu g/m^3$ in the incubators, representing a 5-fold increase in VOC concentration (Cohen et al. 1997). VOC have been linked to reduced outcomes in assisted reproduction technology (Cohen et al., 1997; Little and Mirkes, 1990; Racowsky et al., 1999; Schimmel et al., 1997; Worrilow et al., 2002; Esteves et al., 2006). In the assisted reproduction setting, benzene can be found in CO_2 gas cylinders, while ethylbenzene and benzaldehyde are emitted from plastic ware. Elevated concentrations of other VOC, such as toluene, formaldehyde coming from insulation used in air-handling systems, refrigerant gases, isopropyl alcohol fumes and aliphatic hydrocarbons have also been described in assisted reproduction laboratories. Laboratory cleaning agents and writing instruments generally produce VOC.

VOC, which are 100–1000-times smaller than the effective pore size of HEPA filters, are not trapped by HEPA air filtration. Removal of VOC is achieved by potassium permanganate-impregnated pelletized coal or coconut shell-based activated carbon filters. The spaces between the carbon particles contain a cloud of delocalized electrons that acts as electronic glue, thus forcing the chemical contaminants to bind to the carbon. Coconut shell activated carbon is now preferred over coal-based activated carbon because it has higher density and purity and is virtually dust free. Also, the pore structure of coconut shell-based carbons is finer thus resulting in a higher retention rate (Chiang et al., 2001). However, alcohols and ketones are not easily removed by carbon, but they can be oxidized, and thereby detoxified, by potassium permanganate (Hall et al., 1998).

VOC can be measured by adsorption from air on Tenax TA, thermal desorption, gas chromatographic separation over a 100% nonpolar column (dimethylpolysiloxane) or mass spectrometry (Hall et al., 1998). A common method is the use of Summa canisters to capture air samples for VOC followed by a gas chromatography/mass spectrometry (GC/MS). While the cost is relatively high, the sampling is simple and captures the common VOC at a concentration of 1 $\mu g/m^3$ (Cohen et al., 1997). However, GC/MS requires sophisticated equipment and lacks the prospect for rapid real-time monitoring. Alternatively, VOC can be detected based on different principles and interactions between organic compounds and sensor components. There are electronic devices that can detect parts per million (ppm) concentrations and predict with reasonable accuracy the molecular structure of the VOC in the environment or enclosed atmospheres (Martinez-Hurtado et al., 2010).

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Holographic sensors, for example, can give a direct reading of the analytic concentration as a colour change. The main limitation of using sensors to measure VOC concentrations is related to their lower detection limits. Devices usually detect VOC as ppm which may be inadequate to measure individual harmful VOC present in much lower concentrations in the IVF setting. Measurement devices with lower detection limits as parts per billion would be more adequate for monitoring VOC concentrations in reproductive laboratories (Hall et al., 1998). As such, it is important to understand that measurement for VOC in indoor air is highly dependent on how they are measured. All available measurement methods are selective in what they can measure and quantify accurately, and none are capable of measuring all VOC that are present. For example, benzene and toluene are measured by a different method than formaldehyde and other similar compounds. The range of measurement methods and analytical instruments is large and will determine the sensitivity of the measurements as well as their selectivity or biases. This is why any statement about VOC that are present in a given environment needs to be accompanied by a description of how the VOC were measured so that the results can be interpreted correctly. It is the opinion from this study that VOC-reducing technology, such as the incorporation of commercial filters imbedded with activated carbon and potassium permanganate in the air ventilation system, offers a more practical solution compared with the expensive and labour-intensive VOC testing as currently performed. The efficiency of these filters in eliminating VOC has been validated (Cohen et al., 1997; Hall et al., 1998). Despite that, the limitations of incorporating chemical filters to air filtration systems without periodic testing for indoor VOC concentrations is acknowledged. Ideally, IVF cleanliness with regard to VOC should be evaluated periodically by quantitative measurements as part of the laboratory quality control/quality assurance programmes. Direct determination of air quality by GC/MS will provide quantifiable verification of the facility's status.

With the publication of the aforesaid directives, a worldwide debate started among practitioners, with several of them challenging their feasibility and effectiveness and supporting the idea of a likely adverse impact of applying cleanroom air quality standards to IVF laboratories (Bhargava, 2005; Mortimer, 2005; Saunders and Pope, 2005). In brief, these authors postulated that laminar flow cabinets do not provide optimized conditions for the control of both temperature and pH that are crucial in IVF procedures. In addition, the vibration from laminar flow cabinets would greatly compromise micromanipulation of the gametes, and the high volume air flow would create a cooling effect that would be difficult to counter with microscope warm stages. Lastly, large oscillations in temperature and humidity due to the air flow would jeopardize embryo development because of the need to remove them from incubators for grading purposes. Despite the debate, regulatory authorities maintained the requirements for air quality control in the environments in which gametes and embryos are handled, based on the premises of the precautionary principle to safeguard public health preventing the transmission of infectious diseases via transplanted tissues and cells. Regulatory agencies apply the precautionary principle when measures are needed in the face of a possible danger to human health and when scientific data do not permit a complete evaluation of the risk (Commission of the European Union, 2000).

In fact, few studies exist addressing the impact of performing IVF in cleanroom facilities. In a retrospective cohort study, Boone et al. (1997) observed that the construction of a class 100 cleanroom improved air quality and IVF rate and increased the number of high-quality embryos available for transfer. Esteves et al. (2004) reported the results of ICSI cycles performed in an unselected IVF population and showed that better outcomes were achieved in an IVF facility equipped with cleanrooms with strict air quality control for particles and VOC. Knaggs et al. (2007) rebuilt their IVF facilities as cleanrooms, in accordance to the EU directive, and reported that the overall clinical pregnancy rate for the 6-month period after moving into the new laboratory was significantly higher than the 6-month period in the old laboratory (42.6% versus 30.6%). The current study's IVF facilities were also built as cleanrooms and are in full compliance with the Brazilian Cells and Germinative Tissues Directive. We observed that there was no detrimental effect of operating under cleanroom and good manufacturing practice conditions (European Commission Guide to Good Manufacturing Practice, 2003). On the contrary, this study has observed that operating under such stringent air quality standards is not only feasible but also associated with better embryo development and pregnancy outcomes in the first triennium after moving to the new cleanroom facilities.

While this study reports a large cohort of couples treated in cleanroom environments and includes a group treated in standard IVF facilities, there are several limitations to this large observational experience. Outcomes for the triennium of 1996–1998 were not available for comparison because data were not systematically collected. Moreover, due to the retrospective design of this analysis the possibility of inherent bias exists, although the non-selected patient population was representative of the therapeutic profile observed in current clinical practice. Outcomes have been analysed on an overall basis before and after the implementation of cleanrooms and thereafter stratified by different time periods. This stratification was important to appreciate the overall impact of air quality control in the face of important changes that occurred over time. For example, the mean age of women who sought IVF at this institution increased steadily while the mean number of embryos transferred significantly decreased. In addition to air filtration, strict rules and procedures were followed to control or eliminate particle sources whenever possible. They included proper cleanroom design and construction as well as cleaning procedures and personnel training. It is therefore difficult to ascertain as whether the air quality control has been the primary cause for this sustainable reproductive outcomes. Nevertheless, there was no considerable difference in embryo culture techniques, culture media, catheters and other disposable products used in the laboratory. Embryologists and physicians performing procedures were practically unchanged over these time periods. The mean number of oocytes retrieved, mature oocytes and normal fertilization rates after ICSI were unaltered. Furthermore, the reasons that patients underwent ICSI did not significantly change after installation of the cleanrooms. These

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factors did not seem to have impacted on the events, although other uncontrolled factors rather than air quality may have influenced IVF outcomes. After 2004, the study centre experienced a decrease, albeit of non-statistical significance, in the pregnancy and live birth rates. A plausible reason for this observation was a change of practice in terms of the number of embryos transferred to the uterine cavity. The policy of transferring up to four embryos regardless of female age was changed to a maximum of two in the younger group (34 years or less). Later in 2010, this practice became enforced by law in Brazil. As a result, the mean number of embryos transferred significantly decreased (3.4 versus 2.3; $P < 0.001$) while the mean age of females who sought IVF increased significantly during these same years (34.4 versus 32.8 years; $P = 0.01$). In spite of that, the proportion of cleavage-stage embryos classified as having high quality at the day of transfer steadily increased over all periods after implementation of cleanrooms.

Despite the limitations discussed above, this study's main strength is to present a long-term experience of operating an IVF facility in full compliance with air quality standards directives and to demonstrate that such operation is both feasible and not detrimental to IVF outcomes. First, the cost for implementing the air ventilation and filtration system was US$ 150,000. Operational costs, which include filter changing, certification, maintenance and purchase of cleanroom disposable supplies, are approximately US$ 15,000 per year. Considering that the programme performed 2060 fresh IVF cycles and embryo transfers from 2002 to 2010, the additional cost per cycle to pay off the investment and cover maintenance costs over this period was US$ 131.00. The retrospective data suggest that performing IVF in controlled environments is beneficial to embryo development. However, randomized controlled trials would be necessary to adequately evaluate whether performing IVF in cleanroom facilities will improve assisted reproduction outcomes.

In conclusion, implementation of cleanroom standards to reproductive laboratories, which include air quality control through filtration of airborne particles and VOC and the adoption of good laboratory practices, offers adequate conditions for contamination control and risk management. The data demonstrate that it is feasible to handle human gametes and to culture embryos in cleanroom environments in full compliance with air quality standards directives, such as the one imposed by the Brazilian regulatory authorities, and suggest that performing IVF in controlled environments may optimize its outcomes.

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