Effects of oxygen concentration in IVM/IVF on marmoset monkey oocyte maturation and embryo development

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Abstract

The effect of oxygen (O_2) concentration on *in vitro* development of the oocytes from antral follicles of naturally cycling common marmosets (*Callithrix jacchus*) has been investigated. Different O_2 concentrations during *in vitro* maturation (20 or 8%) and during *in vitro* fertilization (20 or 5%) have been applied in three combinations: 8-5, 20-20, 20-5 (maturation and fertilization, respectively). Additionally, 8-20 conditions were tested in a pre-study, but since no fertilization occurred, this test group was excluded from further experiments. Oocyte maturation rate was significantly higher after IVM in 20% than in 8% O_2 (MI: 91% in 20-20 *vs*. 78% in 8-5; MII: 69 *vs*. 45% in 20-5 and 50% in 8-5, $P < 0.05$ for all). The lowest rate of embryo development occurred after maturation under 8% O_2 (59% in 8-5 *vs*. 95% in 20-20 and 83% in 20-5, $P < 0.05$ for all), strongly suggesting this low tension may produce hypoxic conditions during maturation, which damages the oocytes. In conclusion, 20% O_2 was shown to be significantly superior to 8% O_2 for marmoset oocyte maturation, and 5% O_2 was to be preferred over 20% for IVF, based on a trend for better embryo morphology and rates of progression.

Keywords: embryo, gas environment, *in vitro* fertilization, *in vitro* maturation, marmoset monkey, oocyte, oxygen.

Introduction

Optimal gas environment is an important physical factor influencing the success of oocyte and embryo culture. While reduced oxygen (O_2) concentration has been shown to be beneficial for embryo culture for a range of species (Bavister, 1995; Gelatt *et al*., 2001; Karja *et al*., 2004; Kovacic and Vlaisavljevic, 2008; Meintjes *et al*., 2009), O_2 environment for *in vitro* maturation (IVM) and fertilization (IVF) reported as optimal for different species under various conditions is more variable. Protocols for IVM/IVF of various animal species, including human, which are currently applied by different laboratories, are based on both high and low oxygen tension for oocyte maturation and/or fertilization (Piotrowska-Nitsche *et al*., 2009; Koyama *et al*., 2014; Coticchio *et al*., 2015; Sanfins *et al*., 2015). Published reports on IVM and IVF of primate (rhesus and marmoset) oocytes also vary in regard to gaseous environment, with either high oxygen for both IVM and IVF (Grupen *et al*., 1995; Gilchrist *et al*., 1997; Zheng *et al*., 2001; Schramm *et al*., 2003) or low for both these stages (Tomioka *et al*., 2012).

Although *in vivo* data on this issue is limited, especially concerning O_2 tension in follicular fluid (FF), in general different stages of development from maturation of the oocyte within a follicle through its fertilization and early cleavage in the oviduct and finally to embryo implantation in uterus, occur under progressively reducing O_2 tensions (Fischer and Bavister, 1993; Van Blerkom *et al*., 1997). To our knowledge, the only primate species for which follicular fluid (FF) O_2 concentration has been published is human. Van Blerkom *et al*. (1997) showed that in human preovulatory follicles (18-22 mm in diameter) O_2 content varied from <1 to 5.5%.

Van Blerkom (1998) also reported that O_2 content in FF is highly variable both between different individuals and among follicles of the same individual and is associated with peri-follicular vascularity. A mathematic model published by Redding *et al*. (2008) suggests that mean levels of O_2 in human FF during late antral and pre-ovulatory phases range between 11 and 51 mmHg (1.5 to 6.7%), and is therefore supportive of the results of Van Blerkom. Additionally, porcine O_2 content in FF has been shown to have a similar level, 51 mmHg (6.7%; O_2 content was adjusted to corresponding percentage in gas by the authors with a correction for water vapor pressure (47 mmHg by 37°C) as follows: O_2 tension (mmHg) was divided by 760-47=713 mmHg and multiplied by 100%; Knudsen *et al*., 1978).

It must be noted that O_2 concentration in biological liquids or in culture medium may be expressed either as actual concentration of dissolved O_2 molecules in this liquid (vol%), or, conventionally, as a partial pressure of O_2 in a gas which would create such O_2 concentration in a liquid (usually expressed in

mmHg; Silbey *et al*., 2005). However, in the literature it is usual to present O_2 content in the medium as vol% of O_2 in a gas phase above this medium rather than as vol% O_2 in the liquid itself; e.g. O_2 concentration in pure water produced at 37°C and at 21vol% (159 mmHg) O_2 in air, is only up to 0.6 vol% (Weiss, 1970). Therefore, the values (%) of O_2 tension (or concentration) in the present manuscript actually mean vol% of oxygen in the gas phase.

After ovulation, the matured oocyte surrounded by the expanded cumulus mass is drawn into the ampular end of the oviduct, where fertilization takes place. The only published O_2 measurements for the reproductive tract of a primate species, is in rhesus monkey (Fischer and Bavister, 1993). They showed that during ovulation and in the early post-ovulatory phase, O_2 concentration in the rhesus oviduct was around 56 mmHg (8%; similar in ovulatory and non-ovulatory oviducts); but was much lower in the uterus, around 13 mmHg (1.8 %). The same study provides information about O_2 in rabbit and hamster reproductive tract (species with a bircornate uterus and different implantation/placentation strategies). It showed no reduction of O_2 tension from oviduct to uterus in hamster (both 5.9%) and only slight reduction in rabbit (7.4 and 5.9% in oviduct and uterus respectively). These species-specific differences strongly suggest that it would be advisable to measure O_2 tension in both follicular fluid and the reproductive tract in the same species. Additional factors, such as uterus form (unicornuate *vs*. bicornuate) and implantation type (centric, eccentric or interstitial), may be associated with different oxygen tension patterns.

In most of the studies investigating O_2 tension during IVM (with the only one study carried out in a primate species; Yeoman *et al*., 1999), effects are first observed at the early cleavage stage or even later – with alterations in blastocyst quality. Thus, O_2 tension may be affecting some cytoplasmic factors and/or embryo genome activation; but this effect differed with species. For example, reduction of IVM O_2 concentration from 20 to 5% improved subsequent blastocyst formation and/or quality in mice (Adam *et al*., 2004; Banwell *et al*., 2007; Preis *et al*., 2007) and porcine (Kikuchi *et al*., 2002; Iwamoto *et al*., 2005; Park *et al*., 2005), but negatively affected bovine oocyte and embryo development (Pinyopummintr and Bavister, 1995; Watson *et al*., 2000).

To our knowledge, to date, the only investigation of the effects of O_2 on oocyte maturation in a primate species has been performed by Yeoman *et al*. (1999) for squirrel monkey. They evaluated the effect of an O_2. reduced environment and glucose on IVM outcome (in the presence of serum, gonadotrophins, pyruvate and glutamine). The present work, is the second involving a new world primate species and was performed as a part of a larger project to improve efficiency of normal mature oocyte production *in vitro*. With this aim, we evaluated the reasons for failure at each stage of culture to provide a background for improvement of *in vitro* conditions at next stages of experiments. Here we provide the first

information on the effects of different O_2 concentrations on IVM and IVF in the marmoset monkey, and is the first data of its type for any primate species.

Materials and Methods

All procedures were carried out according to German Animal Experimentation Law (Animal Experiment Permission #33.42502/08-01.03) and animals were housed and surgery procedures carried out according to standard German Primate Centre practice for this species (Gilchrist *et al*., 1997; Isachenko *et al*., 2002).

Materials and Methods applied in the present paper have been previously published in full in Tkachenko *et al*. (2010) and therefore are described here as briefly as possible.

Animals

The females were housed with castrated partners and allowed to cycle naturally with ovarian cycles monitored by plasma progesterone levels to evaluate the mean length of luteal and follicular phase for each animal. Standard methods developed by Heistermann *et al*. (1993) were used. Animals were injected with prostaglandin F2α analogue (PGF-2α, Estrumate, Essex Tierarznei, Munich, Germany) only on the cycle of the ovarectomy, in order to induce luteolysis and time the surgery. The females were operated 1-2 days before the expected day of ovulation.

Seven unstimulated, naturally cycling female marmosets were used for the present experiment. However, oocytes from one out of the seven animals demonstrated low maturation and failed to fertilize with a sperm of a proven male. This was assumed to be an animal-specific difference, and the data obtained from that animal has, therefore, been excluded from this study, with exception of the spindle defect data for the oocytes of that animal. The age of the six experimental animals varied from 26 to 30 months (mean age 29 ± 2.5 months), weight – from 340 to 492 g (396 ± 59 g) and ovary volume – from 0.06 to 0.31 cm^3 (mean 0.20 ± 0.096 cm^3; Ovary volume [cm3] = length [cm]×width [cm]×height [cm]). The animal excluded from the general statistics was 26 months old, 532 g (the youngest and heaviest individual), had mean ovary volume 0.18 cm^3 and number of recovered COCs – 39. Between 17 and 68 COCs (mean 39 ± 18 COCs) suitable for culture were recovered from 1 full + $^2/_3$ ovaries of the six experimental animals, resulting in total 226 harvested COCs.

Experimental design

Three experimental groups were compared, each with a different combination of O_2 concentrations during maturation and fertilization time: 8-5 (8% O_2 in maturation and 5% in fertilization); 20-5 (20% in maturation and 5% in fertilization) and 20-20 (20% in maturation and 20% in fertilization). As a part of a preliminary study we had tested maturation under 8%

and fertilization under 20% O_2 (8-20 group). This additional group was included for first two animals, but excluded later from the main study in order not to sacrifice more animals due to failed fertilization under 8-20 conditions.

Material from each animal was processed individually with oocytes divided randomly and as equally as possible into the 3 treatment groups to equalize animal differences across the treatment groups. Oocytes from two out of six animals were divided into 4 treatment groups, including the 8-20 group, however, oocytes and embryos from different animals were never physically pooled.

Media and culture plates

All media, plate preparations and culture procedures have been performed as described in Tkachenko *et al*. (2015). IVM medium was supplemented with 1 μg/ml β-estradiol, as this concentration has been shown to be optimal according to the study cited above (Tkachenko *et al*., 2015).

COCs recovery and IVM

Recovered ovaries were carefully dissected into small pieces and antral follicles were punctured with forceps and 26G needles in ovary collection medium. Recovered COCs were immediately transferred into 4-well plates with holding medium under oil and left in the incubator with controlled humidified atmosphere of 5% CO_2 in air, 38.3°C until the dissection of all ovary pieces was completed. Thereafter, COCs were randomly taken from all holding plates and equally divided between experimental groups with 5-7 COCs per well of IVM medium. After washing COCs were finally transferred into maturation wells (25 μl) and matured in humidified atmosphere of either 5% CO_2 in air (~20% O_2) or 5%CO_2, 8% O_2, 77% N_2 at 38.3°C for 28-29 h.

Sperm collection and preparation

Detailed procedures for sperm collection, preparation and IVF have been described previously in full (Valle *et al*., 2008; Tkachenko *et al*., 2015). For IVF sperm from donors with proven fertility was used. Each time, semen samples were collected from three sperm donors and the highest quality sample was chosen for IVF, based on sperm viability, motility and concentration.

IVF

One hour prior to IVF (28-29 h after IVM start) cumulus expansion of COCs was evaluated. Then after washing COCs were transferred into final drop of IVF medium (25 μl) and 2 μl of diluted sperm suspension with the final concentration of 20,000-30,000 sperm/μl was added to each drop. Oocytes were cultured with sperm for 15-16 h air at 38.3°C under one of the two experimental conditions - either in a gas mixture of 5%CO_2, 5% O_2 and 90% N_2 or in 5%CO_2 in air (~20% O_2). The day of IVF was called embryo day (ED) 0.

Embryo culture

After IVF and fertilization status assessment performed as described in Tkachenko *et al*. (2015), fertilized oocytes/zygotes were cultured in a gas mixture of 5%CO_2, 5% O_2 and 90% N_2 with the half of the medium changed every 48 h. Embryos were fixed in the case of no progress in the last 72 h or if visible signs of degeneration occurred, such as dark cytoplasm or loose cell contacts.

Immunocytochemical procedures

All oocytes arrested at different stages of maturation as well as arrested or degenerating embryos were fixed and stained for the evaluation of the final developmental stage and quality. Immunostaining procedures were carried out according to Delimitreva *et al*. (2006). Briefly, microtubules were stained using monoclonal anti-α-tubulin mouse antibody (MP Biomedicals Germany GmbH, Eschwege, Germany 69-125, 1:50) and fluorescein isothiocyanate (1:100 FITC-goat anti-mouse immunoglobulin G F(ab`) 2Frag. Affin.). DNA was counterstained with Hoechst 33258 (50 μg/ml), added to the reaction mixture with FITC. Additionally, in embryos actin was stained with phalloidin-TRITC (1 μg/ml) added to the same reaction mixture. FITC: emission 525 nm, excitation 490 nm, Hoechst: emission 480 nm, excitation 365 nm; TRITC: emission 570 nm, excitation 544 nm.

Evaluation of the results

Cumulus expansion was evaluated after the end of maturation, before the COCs were transferred into fertilization medium, using 4 grades: 1) attached cumulus, 2) partially attached and expanded cumulus, 3) expanded cumulus with non-radial expansion, 4) cumulus with radial expansion (Tkachenko *et al*., 2010).

Developmental stage of arrested oocytes fixed after co-incubation with sperm was evaluated after fluorescent stain of microtubules and chromosomes. Oocyte stage was referred to as: germinal vesicle (GV), metaphase I (MI), metaphase II (MII) or fertilized (only MII oocytes with evidence of fertilization, manifested by presence of 2 pronuclei and two distinct or fragmented polar bodies were counted as fertilized). Embryos were defined by the presence of at least two nucleated cells. Developmental rates were calculated both as progressive (% from the previous stage) and from total number of non-degenerated oocytes. Embryo progression rate in the group was evaluated as a proportion of the embryos reached 2-3; 4-5; 6-8; 10-12 cell and morula stage. Embryo morphology on ED2 for analysis of differences between the groups was evaluated by uniformity of cell size and visible nuclei distribution.

Final evaluation of embryo progression and quality was also performed after fluorescent staining. Normality of embryos was estimated by following criteria: all blastomeres have clear borders and

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cytoplasm, and single nucleus with normally dispersed interphase chromatin or mitotic chromosomal configuration, with less than 30% cytoplasmic fragmentation. Otherwise embryo was referred to as multinucleated or degenerated.

Statistics

Chi-square without Yates' correction or Fisher-exact tests (SigmaStat 3.0) were used to assess the differences between experimental groups. P-value less than 0.05 was considered significant.

Results

Cumulus expansion

Cumulus expansion was compared for the oocytes matured under 8% O_2 and 20% (groups 20-20 and 20-5 were pooled for this analysis). Only COCs with visible oocytes connected to surrounding cumulus cells were analyzed (64 and 134 COCs matured at 8 or 20%, respectively, were evaluated). There was no difference in the rate of any expansion grade (Table 1).

Table 1. Effect of O_2 concentration during IVM on cumulus expansion (data from 6 animals).

O_2 concentration at maturation	Expansion grades % ± SD (n)				Total COCs, n
	attached (non-expanded)	partially attached and expanded	non-radial expansion	radial expansion	
8%	56.3 ± 6.20 (36)	37.5 ± 6.05 (24)	3.1 ± 2.17 (2)	3.1 ± 2.17 (2)	64
20%	44.0 ± 4.29 (59)	39.6 ± 4.22 (53)	10.4 ± 2.64 (14)	6.0 ± 2.05 (8)	134

Maturation and degeneration

Since maturation status could be evaluated only after fertilization, the fact that some oocytes might have finished their maturation later, while being in fertilization medium, had to be taken into account. Therefore, maturation rate was evaluated separately in all 3 groups, without pooling together both groups matured under 20% O_2. MI progression rate in 20-20 group was significantly higher (91%, 64/70) than in 8-5 group (78%, 50/64), P < 0.05 (Fig.1). The MI rate in 20-5 group was 89% (57/64). Contrastingly, the rates of progression towards MII stage, were significantly higher in 20-20 group (69%, 48/70) than both in 20-5 (45%, 29/64) and in 8-5 (50%, 32/64) groups (P < 0.05).

Degeneration rates observed after 28 h of maturation did not differ significantly among the groups (14%, 10/74; 16%, 12/76 and 8%, 6/76 in 8-5, 20-5 in 20-20 groups respectively.
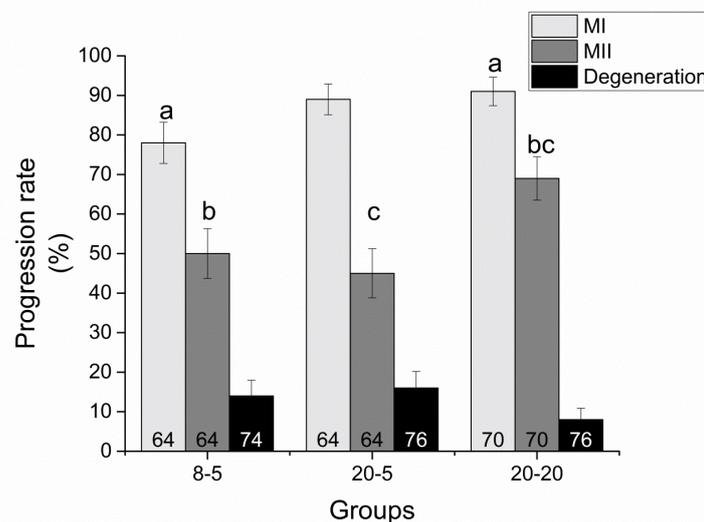
Figure 1. Maturation to MI (light grey bars), MII (dark grey bars) and degeneration (black bars). Numbers on the bars for MI and MII rates are total numbers of non-degenerated oocytes, and for degeneration rates - total number of all oocytes selected for IVM for each experimental group respectively. Group abbreviations (8-5; 20-5; 20-20): first figure is O_2 tension at maturation, second - at fertilization. [a,b,c]Differences between columns with the same subscripts are significant (P < 0.05).

Fertilization

In 20-5 group, fertilization occurred in 62% (16/29) of the 29 oocytes which had reached MII, out of a total of 64 oocytes receiving this treatment. In the 20-20 group, out of 48 oocytes reaching MII from a total of 70 oocytes, 44% (21/48) fertilized. It shows that despite the high maturation rate the 20-20 group had lower proportional productivity in fertilization. In the 8-5 group, out of 64 oocytes total, only 32 matured and 17 fertilized (53%). Importantly, rates of fertilization calculated from total initial oocyte number were similar for all three groups and ranged between 27 and 30% in spite of differences in maturation rates.

Contrastingly, oocytes matured under 8% O_2 and co-incubated with sperm under 20% O_2 failed to fertilize [data obtained with 13 oocytes from two animals: MI: 12/13 (92%); MII: 8/13 (62%)], although oocytes from these same two animals fertilized at the normal rate under the other conditions, indicating this was not an animal specific difference. Analysis of spindles in this group was performed (see below).

Spindles

Spindle shape was analyzed in 129 (both MI and MII) oocytes from 7 animals which failed to fertilize or failed to cleave after fertilization. These include also the data from the animal with total failure of fertilization, which did not demonstrate a difference in the pattern of spindle shape distribution compared with the other animals, and among all animals, there were no significant differences. Data on distribution of spindles into different classes is presented in Table 2.

Bipolar spindles with properly organized poles, well orientated microtubules and aligned chromosomes were referred to as apparently normal. However, this did not rule out possible minor abnormalities or chromosomal problems. Among the pooled MI+MII arrested oocytes, the highest rate of apparently normal spindles was observed in 20-20 groups (68 *vs*. 38% and 33% in 8-5 and 20-5 groups, respectively, P < 0.05). Although, no statistically significant difference was observed for comparisons between MI and MII oocytes from respective groups due to low numbers, this difference must be introduced by MI oocytes, for which normal spindle shape rate was 40% in 20-20 group *vs*. 14 and 17 in two others, respectively. Additionally, analysis of spindles of the 8 oocytes from 8-20 group was performed, and high rates of abnormal spindles indicated development of cytoskeletal disturbances under these conditions (MI: 100% abnormal (2/2); MII: 67% abnormal (4/6)). Other 4 MI/MII oocytes from this group were lost during processing.

Table 2. Analysis of spindle shape in MI and MII arrested oocytes from different groups (data from 7 animals*).

	MI				MII				MI+MII pooled		
Group	Spindle shape, % ± SD (n)		Total	Group	Spindle shape, % ± SD (n)		Total	Group	Spindle shape, % ± SD (n)		Total
	Normal	Abnormal			Normal	Abnormal			Normal	Abnormal	
8-5	14 ± 7.6 (3)	86 ± 7.6 (18)	21	8-5	62 ± 10.6 (13)	38 ± 10.6 (8)	21	8-5	38 ± 25.6 (16)[a]	62 ± 25.6 (26)	42
20-5	17 ± 6.9 (5)	83 ± 6.9 (25)	30	20-5	75 ± 12.5 (9)	25 ± 12.5 (3)	12	20-5	33 ± 19.9 (14)[a]	68 ± 19.9 (28)	42
20-20	40 ± 12.6 (6)	60 ± 12.6 (9)	15	20-20	73 ± 8.1 (22)	27 ± 8.1 (8)	30	20-20	62 ± 11.4 (28)[b]	38 ± 11.4 (17)	45

*Oocytes from the animal excluded from the main analysis due to failed fertilization were included into this analysis. 8-5; 20-5; 20-20: in group abbreviations first figure is O2 tension at maturation, second – at fertilization.
[a,b]Different subscripts indicate significant (P < 0.05) differences.

First cleavage

Significantly lower proportion of fertilized oocytes achieved first cleavage in 8-5 group (59%, 10/17, P < 0.05) compared with 20-20 group (95%, 20/21), and 83% (15/18) of fertilized oocytes cleaved in 20-5 group. No difference was observed in total embryo production assessed based on the proportions calculated from total initial numbers of oocytes per group (Fig.2).
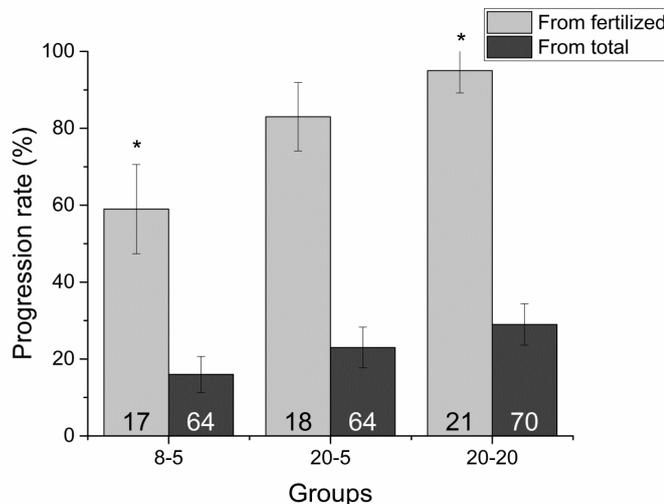
Figure 2. Cleavage rate from fertilized oocytes (light grey bars) and from total non-degenerated (dark grey bars). Numbers on the bars are the totals from which each rate has been calculated (fertilized or total non-degenerated, respectively). Group abbreviations (8-5; 20-5; 20-20): first figure is O_2 tension at maturation, second - at fertilization. *The asterisk marks significant differences between the columns (P < 0.05).

Embryo progression and embryo quality

There was a consistent pattern suggesting better quality of embryos in the groups matured under high oxygen. Analysis of embryo morphology on ED2 (when embryos normally have 4 cells) demonstrated a trend for better embryo morphology in groups matured under high oxygen (both 60%: 9/15 and 12/20 in 20-5 and 20-20 groups, respectively). Examples of good *vs*. bad morphology embryos are shown in Tkachenko *et al*. (2010). We have, however, noted a trend towards better progression of 4-cell embryos to morula stage in 20-5 group (30%, 3/10 *vs*. 12.5% in both other groups: 2/16

in 20-20 and 1/8 and in 8-5 groups) compared to 8-5 group (only 20% of embryos had normal morphology, 2/10). Example of an individual embryo progression to compact morula is shown in Fig.3. One morula from 20-5 group and one from 20-20 group have demonstrated evidence of early antrum formation, however, they arrested at this stage (see these early blastocysts in Fig.4).

Results of evaluation of micronucleation in fixed embryos of all stages have shown a similar pattern. The highest rate of apparently normal embryos (50%, 7/14) was in 20-5 group the lowest - in 8-5 group (22%, 2/9) (Table 3).
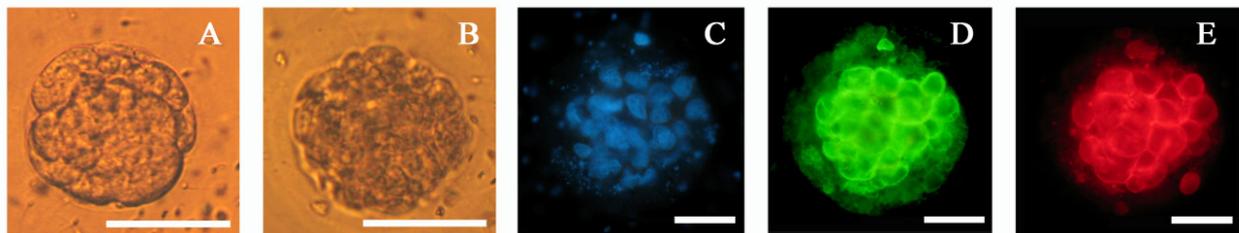
Figure 3. An example of an embryo developed to morula stage on embryo day (ED) 8 (A) and ED 9 (B). This embryo was from the group matured at 20% O2 and fertilized at 5% O_2 Nuclei of approx. 25 cells are visible. Stained (after fixation on ED 11) with Hoechst (B), tubulin (C) and actin (D). Strong localization of actin and tubulin at cell junctions indicates that compaction has occurred. Scale bars are 70 µm (A,B) and 50 µm (C,D,E).
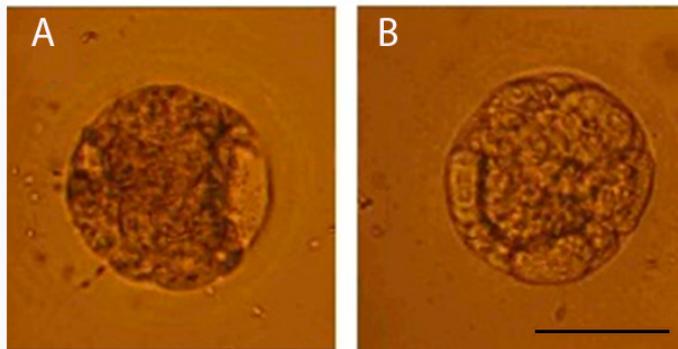
Figure 4. Early blastocysts developed in culture (embryo day 9) after IVM under 20% and IVF under 5% O2 (A), and after IVM under 20% and IVF under 20% O2 (B). Scale bar is 70 um.

Table 3. Multinucleation in embryos. Embryos from 4 animals were stained for tubulin and chromatin visualization.

Embryo quality	Rates of embryos with different quality, % ± SD (n)		
	8-5	20-5	20-20
Apparently normal	22 ± 13.8 (2)	50 ± 13.4 (7)	41 ± 11.9 (7)
Multinucleated	33 ± 15.7 (3)	43 ± 13.2 (6)	41 ± 11.9 (7)
Degenerated	45 ± 16.5 (4)	7 ± 6.8 (1)	18 ± 9.0 (3)
Embryos total, n	9	14	17

8-5; 20-5; 20-20: in group abbreviations first figure is O_2 tension at maturation, second - at fertilization.

Discussion

The impact of O_2 concentration during IVM and IVF on oocyte maturation, fertilization, and subsequent embryo development has been investigated for marmoset monkey oocytes, and this provides the first information of this kind for this species.

The present study demonstrates better results after maturation under 20 than 8% gas phase O_2. This is similar to the results obtained for other species (Pinyopummintr and Bavister, 1995; Watson *et al*., 2000; Kikuchi *et al*., 2002; Oyamada and Fukui, 2004;

Iwamoto *et al*., 2005; Park *et al*., 2005; Banwell *et al*., 2007; Mingoti *et al*., 2009), including the only other comparable study for primates (squirrel monkey) published by Yeoman *et al*. (1999).

These findings seem surprising, since culture under atmospheric O_2 would actually result in O_2 tension in culture drops significantly higher than the natural oocyte environment during periovulatory maturation within the follicle: reported values in human and porcine FF correspond to about 6% O_2 in the gas phase (Knudsen *et al*., 1978; Fischer and Bavister, 1993; Van Blerkom *et al*., 1997; Redding *et al*., 2008).

However, there are several factors of the culture environment which may either actually lower the amount of O_2 available for the oocytes or protect them from excessive oxidation. First, O_2 consumption by cumulus cells may act as a natural buffer protecting the oocyte from too high O_2. Cumulus complex has been estimated to reduce O_2 level at the oocyte surface to 5-15% lower than in surrounding FF (Clark *et al*., 2006). Additionally, when culture is performed in microdrops under mineral oil, the amount of O_2 available in a culture drop might be further slightly reduced due to slower O_2 diffusion into medium and geometry of the drop (Stokes, 2009). There are also other factors, such as culture medium components, which may mitigate effects of high O_2 on oocyte metabolism. All this transforms what first appears to be a non-physiologically high O_2 (20%) into one closer to physiological at the surface of the oocyte in our culture conditions. It is therefore logical that a lower O_2 in the gas phase (e.g. 8%), could produce hypoxic conditions in the culture drop through the same mechanisms, and damage the oocyte similar to the changes occurring in oocytes in atretic follicles with inadequate vascularity. This idea is further supported by *in vivo* data demonstrating that healthy preovulatory follicles have higher O_2 content in FF compared to smaller or less well vascularised follicles (Van Blerkom *et al*., 1997). This study further showed that oocytes from follicles with O_2 content less than 3% appeared to possess high rates of abnormal chromosome organization and alignment (Van Blerkom *et al*., 1997). Our analysis of spindle organization in arrested oocytes indirectly supports this finding.

Further, we observed the highest rate of normal spindles in the group matured and fertilized under 20% oxygen, which exceeded the rate in 20-5 group. In our study maturation status of oocytes could be evaluated only post-IVF, once cumulus cells were removed. Therefore by the time of assessment, mature oocytes could have included those which completed polar body extrusion in fertilization medium. We found here a significant difference in MII progression rates (69 *vs*. 45%) between two groups cultured under 20% O_2 during IVM and either 20 or 5% O_2 during IVF. The effect of gas during fertilization must be attributed to the completion of maturation among those oocytes which had not fully matured in IVM medium, which also explains the higher rate of MII progression in the 20-20 group compared to the 20-5 group. Apparently, drastic reduction in oxygen tension (for example from 20 down to 5%) impairs late maturation causing disturbances in cytoskeletal organization. In support of this, negative effect was also observed for the group matured under low and fertilized under high oxygen, evidenced by high rates of spindle abnormalities and absence of fertilization.

In this study we have achieved the aim of improving early stages on *in vitro* culture of unstimulated marmoset oocytes, obtaining high rates of maturation, fertilization and early cleavage. However, the next stages of the experiments need to focus on improvements of the progressive embryo development

using our best maturation and fertilization conditions and varying the embryo culture media in a focused experimental design. It should be noted that production of blastocysts from immature oocytes collected from unstimulated marmosets has been only reported by two groups so far. Tomioka et al. obtained 13 morulas from starting number of 428 oocytes (12 animals), while Gilchrist reported 31 morulas from 135 oocytes (3 animals; (Gilchrist *et al*., 1997; Tomioka *et al*., 2012). In this study we started with 226 oocytes from 6 animals, and obtained in total 6 morulas. However, unlike Tomioka et al., and similar to Gilchrist et al. (who used the same embryo culture system as in this paper), progression of morulas to early blastocysts in the present study was low. Porcine culture medium used by Tomioka *et al*. for embryo culture definitely supports morula to blastocyst transition better than our culture medium, and analysis of these two culture approaches might provide valuable information on marmoset embryo requirements.

In summary, in terms of oocyte maturation, early embryo development and embryo quality, 20% O_2 during IVM of marmoset monkey oocytes provided better results than 8% O_2 under the present culture conditions, while lower O_2 seemed to be preferable during IVF based on embryo quality compared with 20% even though 20% O_2 appears to have allowed late maturing oocytes to complete the process in the fertilization medium. These results provide the first information of its kind for this species, and are an important step in the process of developing an optimized IVM-IVF-embryo development system for marmoset monkey, but further studies are required.

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