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# Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development[1]

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## ABSTRACT

**Oxygen is a powerful regulator of preimplantation embryo development, affecting gene expression, the proteome, and energy metabolism. Even a transient exposure to atmospheric oxygen can have a negative impact on embryo development, which is greatest prior to compaction, and subsequent postcompaction culture at low oxygen cannot alleviate this damage. In spite of this evidence, the majority of human in vitro fertilization is still performed at atmospheric oxygen. One of the physiological parameters shown to be affected by the relative oxygen concentration, carbohydrate metabolism, is linked to the ability of the mammalian embryo to develop in culture and remain viable after transfer. The aim of this study was, therefore, to determine the effect of oxygen concentration on the ability of mouse embryos to utilize both amino acids and carbohydrates both before and after compaction. Metabolomic and fluorometric analysis of embryo culture media revealed that when embryos were exposed to atmospheric oxygen during the cleavage stages, they exhibited significantly greater amino acid utilization and pyruvate uptake than when cultured under 5% oxygen. In contrast, postcompaction embryos cultured in atmospheric oxygen showed significantly lower mean amino acid utilization and glucose uptake. These metabolic changes correlated with developmental compromise because embryos grown in atmospheric oxygen at all stages showed significantly lower blastocyst formation and proliferation. These findings confirm the need to consider both embryo development and metabolism in establishing optimal human embryo growth conditions and prognostic markers of viability, and further highlight the impact of oxygen on such vital parameters.**

*blastocyst, culture, early development, embryo, glucose, in vitro fertilization, metabolism, viability*

## INTRODUCTION

From fertilization to implantation, the mammalian embryo in vivo develops in an oxygen concentration below atmospheric level (~20%). Analysis of the environment within the female reproductive tract of several mammalian species has determined the relative oxygen concentration of both the oviduct and uterus to be significantly lower, ranging between 2% to 8% [1–3]. Furthermore, in several livestock and outbred mouse models, preimplantation embryo development in vitro is significantly improved when the culture is performed in reduced oxygen concentrations (5%–7%) [4–8]. In contrast, F1 mouse and human embryos can develop to the blastocyst stage relatively well in either atmospheric or reduced oxygen, which has lead to understandable confusion regarding the relevance of the oxygen concentration when translating from research to clinical in vitro fertilization (IVF). So beyond simply knowing if embryos can develop in atmospheric oxygen or not, it is more important to understand the developmental competency and viability of the resultant embryos.

Recent evidence has confirmed that even a transient exposure to atmospheric oxygen can have a negative impact on mammalian embryo development. Exposure to atmospheric oxygen prior to compaction can induce developmental retardation in mouse embryos, which cannot be rescued by subsequent culture at low oxygen [9]. Other experimental data show conclusively that atmospheric oxygen has a negative impact on mouse blastocyst gene expression [10, 11], causes alterations in the resultant blastocyst proteome [12], and negatively affects pyruvate oxidation at the 2-cell stage [13]. Correlated with these effects are retarded embryo growth as early as the first cleavage division [9] and significant loss of viability at later stages [14]. These negative effects can be alleviated successfully with the use of a reduced oxygen atmosphere throughout the preimplantation period, with evidence from mouse studies showing improvements in subsequent development and viability [14–17]. Reduced oxygen concentrations have also been shown to be beneficial to human embryos [18–22]. In spite of this overwhelming evidence, atmospheric oxygen is still utilized in clinical IVFs and human embryo cultures [23, 24].

Carbohydrate and amino acid utilization have been linked to the ability of the human embryo to develop in culture [25, 26] and to subsequent viability of embryos after transfer [27, 28]. Furthermore, there are distinct differences in embryonic metabolism before and after compaction. Prior to compaction, the pronucleate oocyte and cleavage stage embryo preferentially utilize pyruvate and lactate, together with specific amino acids such as aspartate [29]. Glucose does not become the main energy substrate for the embryo until after compaction. Energy generation through appropriate stage-specific pathways is therefore fundamental to the survival and development of the preimplantation embryo [11, 30], and loss of metabolic regulation is associated with loss of viability [31].

The mammalian preimplantation embryo exhibits plasticity in terms of its ability to adapt its utilization of nutrients to changes in culture conditions, which may explain some of the species differences in response to oxygen. However, these adaptations by embryos to less than optimal culture conditions are not without cost, culminating in incrementally lower rates of development to the blastocyst stage and/or viability postimplantation. Recent studies have shown that exposing the preimplantation embryo to stressors that impair the function of mitochondria not only alters survival to the blastocyst stage and resultant viability, but subsequent fetal growth and health [32–35]. Importantly, mitochondria are intimately involved in the oxygen-dependent conversion of carbohydrates and amino

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acids into energy, which supports differentiation and cell division after compaction. So while embryos may be able to accommodate nonphysiological conditions during preimplantation development, a better understanding of how metabolism, and in particular the utilization of specific substrates, can support specific stages could further improve outcomes for the resultant offspring.

The aim of this study was to determine the effect of oxygen on the utilization of amino acids and carbohydrates at both the cleavage and blastocyst stages. The data reveal that when exposed to 20% oxygen during the cleavage stages, the embryo exhibits significantly greater amino acid utilization and pyruvate uptake than embryos cultured under 5% oxygen. In contrast, postcompaction embryos cultured in 20% oxygen have a significantly lower amino acid use and glucose uptake. Culture in atmospheric oxygen is associated with significant metabolic trauma, and consequently, there is a need to reevaluate data collected from studies employing atmospheric oxygen.

## MATERIALS AND METHODS

### Embryo Culture

Pronucleate oocytes were obtained from (C57BL/6 × CBA/Ca) $F_1$ hybrid mice. Animals were housed in 12L:12 D with food and water ad libitum. Four-wk old virgin females were superovulated with 5 international units (IU) equine chorionic gonadotropin delivered intraperitoneally (Folligon; Intevet) followed 48 h later by 5 IU human chorionic gonadotropin (hCG) (Chorulon; Intervet). Females were mated with F1 males overnight, following hCG administration. The presence of a vaginal plug the following morning (Day 0.5) was used as an indicator of successful mating. All the animal use was approved by the Institutional Animal Ethics Committee.

The composition of culture and handling media used for all the experiments is outlined in Lane and Gardner [36], with glutamine being used instead of alanyl-glutamine. All the media were prepared in house and tested in a proven mouse embryo assay prior to use [37]. Human serum albumin (5 mg/ml) and hyaluronan (0.125 mg/ml; Vitrolife AB) were added to all the culture media. All the chemicals were purchased from Sigma Aldrich unless otherwise noted and were of cell culture grade.

On the day of culture, pronucleate oocytes were collected at 22 h post-hCG in G-MOPS handling medium [36] followed by cumulus removal in G-MOPS containing 300 IU/ml hyaluronidase (bovine testes, type IV; Sigma Aldrich). Pronucleate oocytes were removed from the hyaluronidase once cumulus cells started to detach using a micropipette. Embryos were washed twice in G-MOPS and once in preincubated G1 before being returned to the culture incubator for 48 h in the same medium at 37°C, under a humidified atmosphere of either 6% $CO_2$, 5% $O_2$, and 89% $N_2$ (reduced oxygen) or 6% $CO_2$ in air (atmospheric oxygen, ~20%), followed by 48 h of culture in medium G2. Embryos assigned to a particular oxygen concentration were maintained throughout under that oxygen regime. All the cultures were performed in Petri dishes under a layer of paraffin oil (Ovoil; Vitrolife).

### Analysis of Amino Acid Utilization

In order to quantitate amino acid utilization during the second day of development, 2-cell mouse embryos were cultured in groups of 10 in 2-μl drops of G1 for 24 h until the same time on Day 3 (24-h period). Quantification of amino acid utilization during Day 4–5 (24-h period) was undertaken on another group of early blastocysts cultured in groups of three in 2-μl drops of G2. A positive displacement pipette (eVol; SGE Analytical Science) was used to accurately dispense 2-μl volumes. The precision and accuracy of the incubation drops was evaluated by measuring the dispensed volume by weight. The displacement pipette showed higher precision and greater accuracy than a conventional pipette.

Amino acid analysis was performed using the derivatization-labeling reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Aqc) and the mass detection capabilities of a triple-quadrupole mass spectrometer (LC-QqQ-MS), which enables the concentrations of coeluted fractions of a variety of amines to be resolved and quantitated by comparison against a standard calibration curve [38]. A 2.5-mM stock solution of amino acids was prepared containing the following; lysine, histidine, asparagine, arginine, taurine, serine, glutamine, glycine, aspartate, glutamate, threonine, alanine, proline, cysteine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine, and tryptophan. Calibration standards for these amino acids were then prepared by diluting the stock solution to 150, 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 μM in MilliQ water using volumetric glassware. Norleucine (25 μM) was used as an internal standard in borate buffer (pH 8.8) containing sodium borate (200 mM), the antioxidant ascorbic acid (1 mM) and the reducing agent tris(2-carboxyethyl)phosphine (10 mM).

Following 24 h of incubation, 1-μl aliquots of the spent culture media, including a control drop without embryos, were collected and vacuum dried. Each dried culture media sample was resuspended in 10 μl of MilliQ water. Alongside these, 10-μl aliquots of each amino acid standard were then prepared. To all the standards and samples, 70 μl of borate buffer was then added and mixed by vortex for 20 sec followed by centrifugation (1 min). To a total volume of 80 μl, 20 μl of Aqc (10 mM) was then added, vortexed immediately for 20 sec, and then warmed on a heating block (Thermomixer; Eppendorf) with shaking (1000 rpm) for 10 min at 55°C. The final solution was then allowed to cool to ambient temperature before centrifugation (1 min), followed by liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent 1200 LC-system coupled to an Agilent 6420 ESI-QqQ-MS.

### Analysis of Carbohydrate Levels

Carbohydrate levels in samples of embryo culture (test) and embryo-free (control) media were determined by quantitative microliter-scale fluorimetry using coupled enzyme-based reactions involving production or consumption of the autofluorescent cofactor NAD (P)H [39, 40]. Test values lower or higher than controls represented metabolite consumption or production, respectively. Pyruvate and glucose levels from Day 1 to Day 3 of development (first 48-h period) were obtained by culturing pronucleate oocytes individually in 2-μl drops of G1. Pyruvate and glucose levels from Day 3 to Day 5 (second 48-h period) were determined by culturing postcompaction embryos (8 cells or greater) individually in 2-μl drops of modified G2 medium (0.5 mM glucose). Glucose levels for Day 3–4 and Day 4–5 (sequential 24-h periods) was further determined by culturing postcompaction embryos individually in 1-ul drops of modified G2 medium with the embryos moved to fresh medium for both 24-h periods. Embryos that reached the expanded blastocyst stage, after 96 h of routine culture (10 embryos per 20 μl, first 48 h in G1 and second 48 h in G2), were then cultured for a further 4 h in 1-μl drops of modified G2 medium (0.5 mM glucose, lactate free). Glucose and lactate were then quantitated from split samples of the same medium. Glucose uptake was then expressed as a fraction of lactate production, as an indirect measure of glycolytic activity, based on the assumption that 2 mol of lactate is produced per 1 mol of glucose consumed when metabolized directly in the glycolysis pathway.

### Statistics

All the data was checked for Gaussian distribution. For both oxygen conditions, metabolism data did not meet the assumption of Gaussian distribution and so underwent nonparametric analysis using the Mann-Whitney test and were expressed as median values. Chi-square tests were used for proportion data. All the analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software).

## RESULTS

Embryo development across the whole preimplantation period was altered in response to differences in oxygen level in the culture environment. Culturing embryos in 5% oxygen resulted in fewer embryos arresting and a greater number of embryos developing to the blastocyst stage (Table 1). Furthermore, these resultant blastocysts had significantly more cells compared to blastocysts grown in atmospheric oxygen (Table 1).

TABLE 1. Effect of oxygen concentration during culture on embryo development rates to Day 5.

| Oxygen concentration | No. of pronucleate oocytes | Development | | Total cell number[a] |
|---|---|---|---|---|
| | | ≥2 cells | Blastocyst | |
| 5% | 397 | 98% | 91% | 112.3 ± 6 |
| 20% | 440 | 94%* | 75%* | 80.8 ± 5* |

[a] Cell numbers ± SEM.

* Significantly different from 5% oxygen; $P < 0.05$.

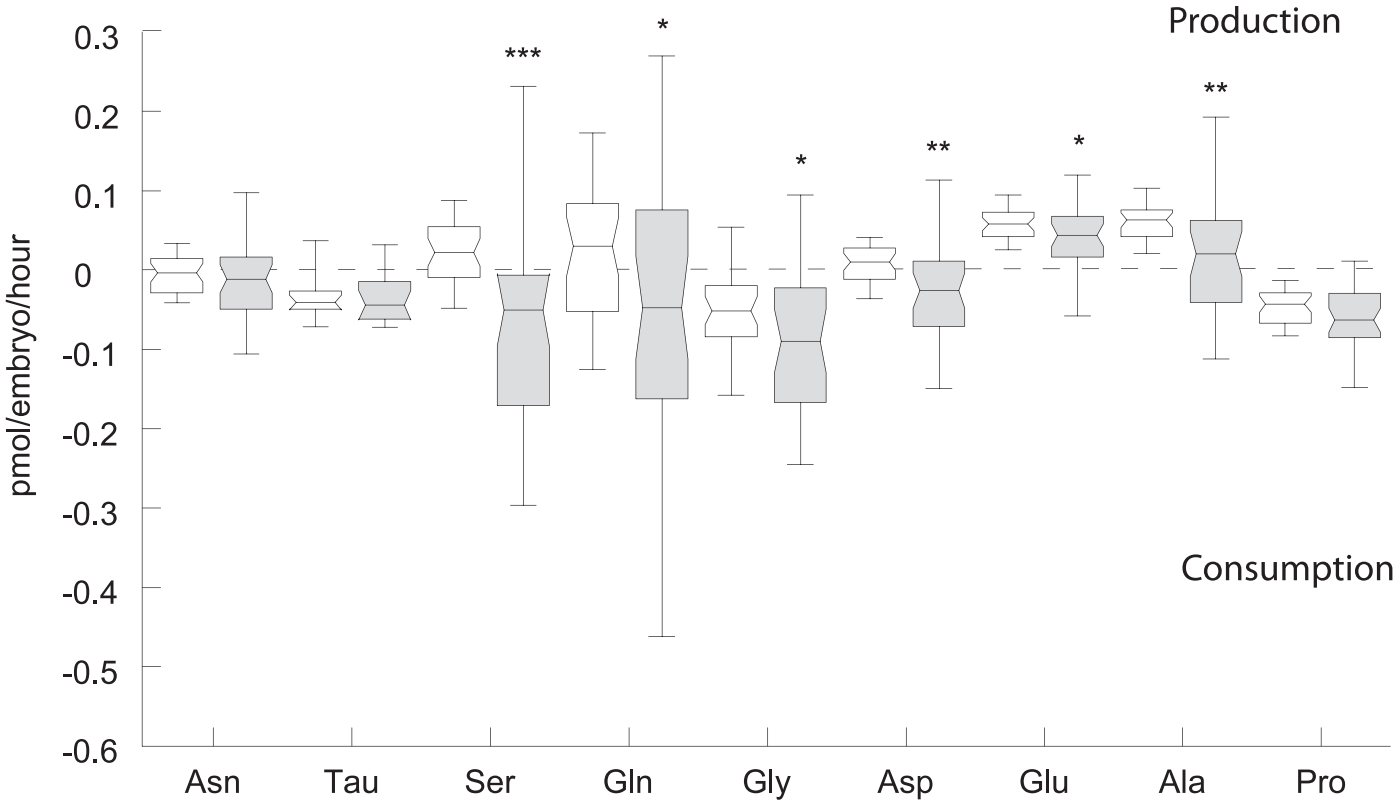


FIG. 1. Turnover of individual amino acids by precompaction embryos from Day 2–3. Open box, 5% oxygen; gray box, 20% oxygen. (Thirty-three replicates, groups of 10, n = 330 embryos per treatment.) Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the median uptake or release. Significantly different from embryos cultured in 5% oxygen: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Alongside this dramatic developmental effect, differences in amino acid profiles were also observed in response to culture in low versus atmospheric oxygen. The amino acid profile for precompaction embryos cultured in both oxygen concentrations is shown in Figure 1. During the precompaction stages, asparagine, taurine, glycine, and proline were consumed, and both glutamate and alanine were produced, regardless of the oxygen concentration. In contrast, in the presence of 5% oxygen, serine, glutamine, and aspartate were all found to be released into the culture medium, whereas in atmospheric oxygen, all three amino acids were consumed. For embryos cultured under atmospheric oxygen, there was a significant increase in the overall utilization of glutamine, glycine, and glutamate ($P < 0.05$); alanine ($P < 0.01$); and serine and aspartate ($P < 0.001$). Furthermore, the variation in utilization for each amino acid appeared larger when embryos were cultured in atmospheric oxygen (Fig. 1). When total amino acid turnover (sum of amino acid consumption and production) was calculated, embryos cultured in atmospheric oxygen displayed greater overall amino acid utilization than embryos culture in 5% oxygen ($P < 0.001$) (Fig. 2). This difference could be attributed primarily to the consumption of amino acids, with embryos cultured in atmospheric exhibiting a 2.6 fold increase relative to embryos cultured at 5% oxygen ($P < 0.01$).

The effect of oxygen concentration on pyruvate and glucose uptake from individually cultured precompaction embryos is shown in Figure 3. There was a small but significant increase in pyruvate uptake during the precompaction stage in embryos cultured in atmospheric oxygen compared to 5% oxygen. Similar to the amino acid data, variation in pyruvate utilization by embryos cultured in atmospheric oxygen was larger. Glucose consumption did not differ between the two groups.

Blastocyst amino acid utilization with respect to oxygen concentration is shown in Figure 4. There were significant differences in the utilization of asparagine, glutamate, tryptophan, and lysine ($P < 0.05$) and significantly greater utilization of threonine, tyrosine, methionine, valine, isoleucine, leucine, and phenylalanine, ($P < 0.01$) between the two oxygen concentrations. Consumption for each of these amino acids was

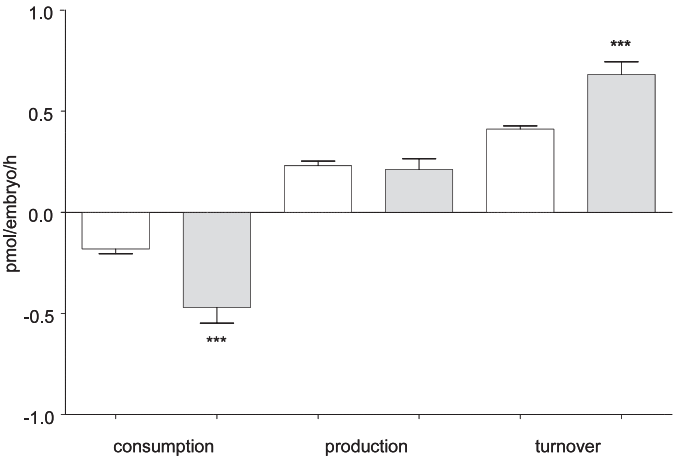


FIG. 2. Total amino acid consumption, production, and turnover by precompaction embryos from Day 2–3. Open box, 5% oxygen; gray box, 20% oxygen. Significantly different from embryos cultured in 5% oxygen: ***$P < 0.001$.

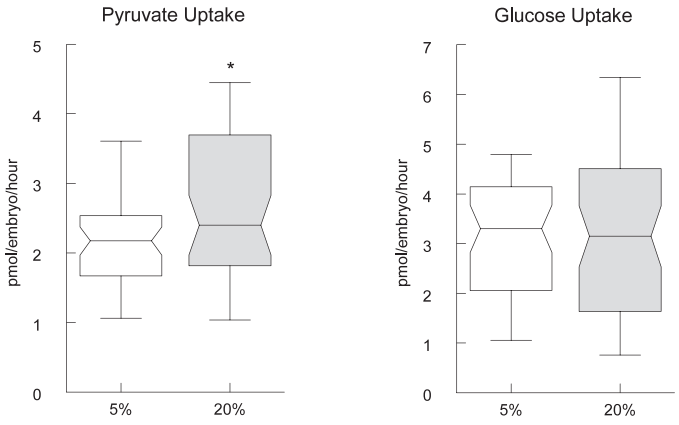


FIG. 3. Carbohydrate turnover by precompaction embryos for 48 h from Day 1 to Day 3. Open box, 5% oxygen; gray box, 20% oxygen (n = at least 45 embryos per treatment). Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the median carbohydrate consumption. Significantly different from embryos cultured in 5% oxygen: $^{*}P < 0.05$.

greater at 5% oxygen than atmospheric oxygen, and overall amino acid utilization was higher at 5% oxygen compared to 20% oxygen ($P < 0.05$, Fig. 5).

Pyruvate and glucose uptake profiles from postcompaction embryos cultured individually are shown in Figure 6. There was no difference in pyruvate uptake between the two oxygen concentrations. In contrast, embryos cultured in 5 % oxygen had a significantly higher glucose uptake during the second 48-h period. Figure 7 shows the postcompaction glucose uptake measured for sequential 24-h periods: Day 3–4 and Day 4–5. Consistent with the 48-h data, embryos cultured in 5% oxygen had a significantly higher glucose uptake compared to embryos cultured in atmospheric oxygen for both periods. The distribution of glycolytic activities for blastocysts cultured in either 5% or 20% oxygen concentration is shown in Figure 8. Glycolytic activity of blastocysts ranged from 13% to 187% (median of 37%) for embryos cultured in 5% oxygen compared to 18% to 264% (median of 62%) for embryos cultured in atmospheric embryos. The mean rate of glycolysis was significantly lower in 5% oxygen (49.6 ± 6.0) compared to 20% oxygen (78.1 ± 8.7; $P < 0.01$), indicating that more glucose was oxidized at low oxygen.

## DISCUSSION

The present study confirms that atmospheric oxygen (~20%) not only has a negative impact on embryo development in culture, but that oxygen regulates both amino acid and carbohydrate utilization during the preimplantation period. Furthermore, the embryo responds to oxygen in a stage-dependent manner. When embryos are cultured in what is considered to be a more physiological oxygen concentration (5%), they exhibit lower rates of pyruvate uptake and amino acid turnover during the precompaction period compared to embryos cultured in atmospheric oxygen (Figs. 1–3). The increase in amino acid turnover by the cleavage stage embryos cultured in atmospheric oxygen could mainly be attributed to higher amino acid consumption. Conversely, during the postcompaction period, embryos cultured in a physiological oxygen concentration (5%) exhibited higher rates of glucose uptake and a greater uptake of most amino acids (Figs. 4–7). Glycolytic rates also differed between the resultant blastocysts, with embryos cultured in 5% oxygen exhibiting lower rates of glycolysis than their counterparts in atmospheric oxygen (Fig. 8).

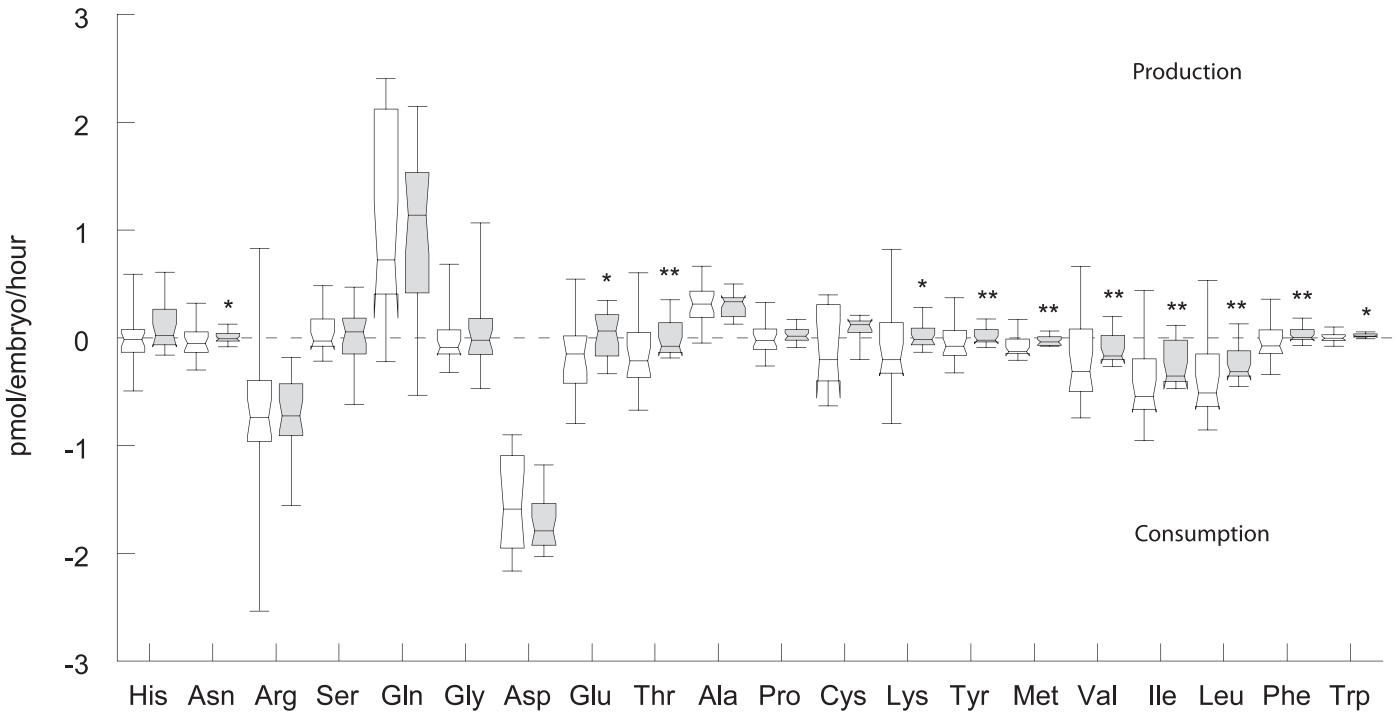


FIG. 4. Amino acid utilization for postcompaction embryos from Day 4–5. Open box, 5% oxygen; gray box, 20% oxygen. (Twenty-five replicates per treatment, groups of 3, n = 75 embryos.) Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the median uptake or release. Significantly different from embryos cultured in 5% oxygen: $^{*}P < 0.05$ and $^{**}P < 0.01$.

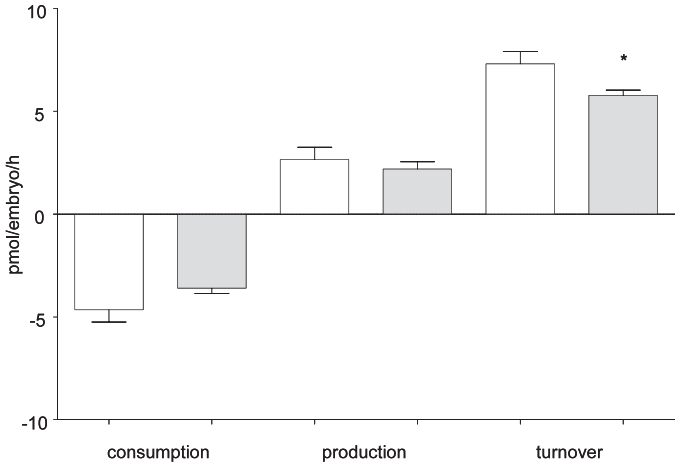


FIG. 5. Total amino acid consumption, production, and turnover by postcompaction embryos from Day 4–5. Open box, 5% oxygen; gray box, 20% oxygen. Significantly different from embryos cultured in 5% oxygen: $*P < 0.05$.

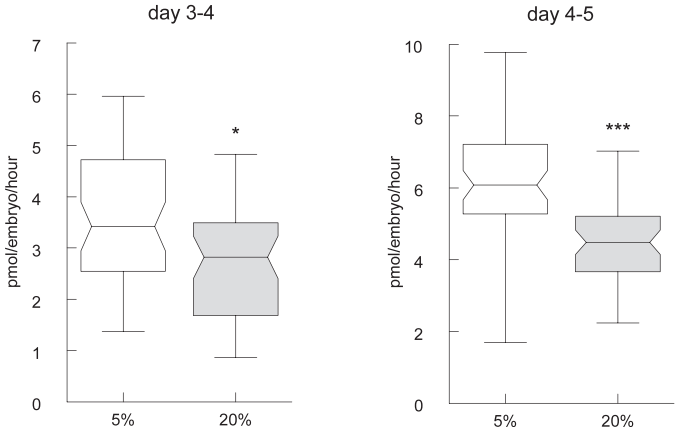


FIG. 7. Sequential glucose turnover by postcompaction embryos for 24 h period Day 3–4 and Day 4–5. Open box, 5% oxygen; gray box, 20% oxygen (n = at least 47 embryos per treatment). Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the median carbohydrate consumption. Significantly different from embryos cultured in 5% oxygen: $*P < 0.05$ and $***P < 0.001$.

Culture of the cleavage stage embryo under atmospheric oxygen was associated with an increase in the uptake of six amino acids (alanine, aspartate, glutamine, glutamate, glycine, and serine). Interestingly, three of these amino acids (aspartate, glutamine, and serine) were released into the medium by the embryo in 5% oxygen, rather than being consumed, as was the case with atmospheric oxygen, indicating that a major shift in the utilization of these three amino acids had occurred. Aspartate has been implicated in the regulation of carbohydrate metabolism by the preimplantation embryo via its own metabolism through the malate-aspartate shuttle (MAS) [29]. The switch from aspartate production to aspartate consumption in 20% oxygen could reflect an increase in MAS activity, plausibly driven by the increase in glycolysis reported at 20% oxygen [29], which in turn would need higher MAS activity to generate the $NAD^+$ required. Glutamine in embryo culture media has been demonstrated to have beneficial effects on mouse cleavage stage embryos, with alleviation of the so-called 2-cell block in some strains [41]. The increased utilization of glutamine by the cleavage stage embryo in response to culture in atmospheric oxygen may reflect an adaptive increase in the oxidative metabolism of this amino acid, given the results of a previous study showing that atmospheric oxygen impairs the oxidation of pyruvate by 2-cell mouse embryos measured using a radiolabeled substrate [13]. Serine is a nonessential amino acid and can be synthesized from a number of pathways. The synthesis and breakdown of serine shares common intermediates with glycine and cysteine and almost all can be biosynthesized from one another. The role of serine within the cleavage stage embryo and its apparent pathway switch in response to the oxygen concentration employed warrants further investigation.

Determination of total amino acid turnover prior to compaction revealed that under stress-inducing conditions, that is, 20% oxygen, turnover was significantly increased. Consequently, the data indicate that when embryos are cultured under conditions that support viability, that is, 5% oxygen,

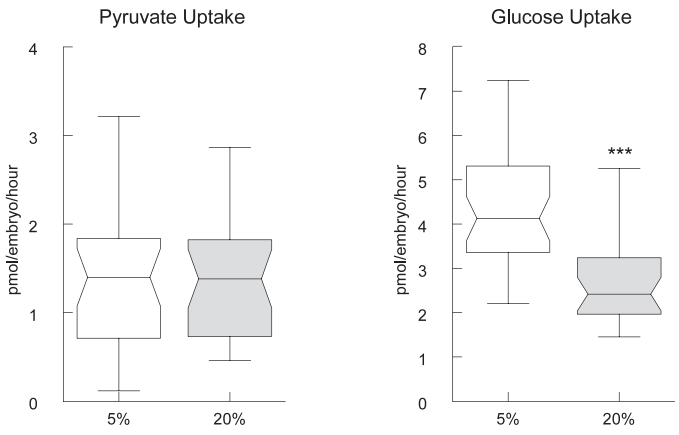


FIG. 6. Carbohydrate turnover by postcompaction embryos for 48 h from Day 3 to Day 5. Open box, 5% oxygen; gray box, 20% oxygen (n = at least 27 embryos per treatment). Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the median carbohydrate consumption. $***P < 0.001$, significantly different from embryos cultured in 5% oxygen.

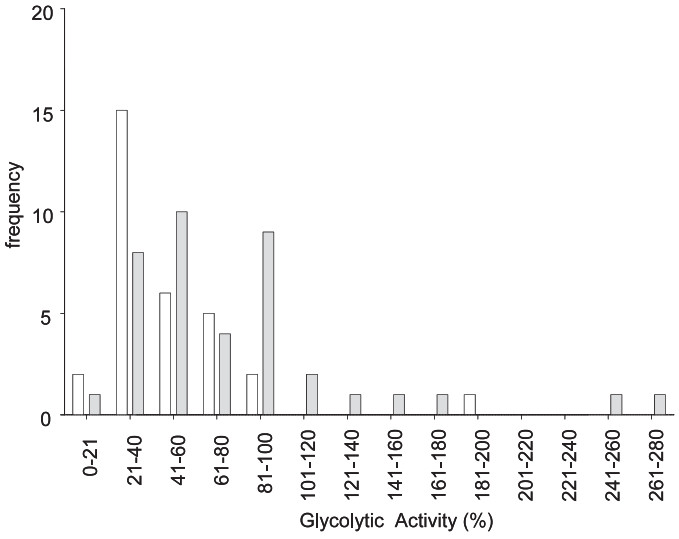


FIG. 8. Distribution of glycolytic activity in blastocysts cultured in either 5% (open box) or 20% (grey box) oxygen conditions (n = at least 31 blastocysts per treatment).

amino acid turnover is lower. Conversely, when the physiology of the embryo prior to compaction is stressed, there is a greater utilization of amino acids. These data are, therefore, consistent with the data of Houghton et al. [26] who showed that amino acid turnover by the cleavage stage human embryo is significantly increased by embryos that subsequently fail to reach the blastocyst stage.

Analysis of carbohydrate uptake by the cleavage stage embryo at the two oxygen concentrations showed that pyruvate was significantly increased in the presence of atmospheric oxygen, even though previous work has indicated that pyruvate oxidation is reduced at this level of oxygen [13]. In contrast, oxygen had no effect on glucose uptake at the same stage. It has been established that oxygen impairs the viability of the preimplantation mammalian embryo [14, 17] and that the embryo is most sensitive to oxygen damage during the cleavage stages [9]. Plausibly higher rates of reactive oxygen species, generated through oxidative metabolism, could be a major source of the cellular stress. Traditionally, pyruvate is thought of as a metabolic substrate; however, it has been demonstrated that pyruvate can also function as a powerful antioxidant [42], with an ability to cause decreases in hydrogen peroxide levels within the preimplantation embryo [43, 44]. Hence, the increased consumption of pyruvate by the cleavage stage embryo in response to atmospheric oxygen may reflect an increase in the requirement for antioxidant protection. Furthermore, high oxygen may shift the cytoplasmic redox balance, which has been shown to affect the metabolism of pyruvate via lactate dehydrogenase at cleavage stages in the mouse [45].

Of interest, two clinical studies have examined the relationship between pyruvate uptake by the cleavage stage human embryo and subsequent outcome posttransfer [46, 47]. In both studies, in which human embryo culture and or analysis occurred in an atmosphere of 5% $CO_2$ in air (20% oxygen), it was observed that those embryos with a pyruvate uptake rate distributed in the mid- to lower range had greater viability postembryo transfer. As yet, it is not known what level of nutrient utilization for cleavage stage embryos correlates with viability under physiological oxygen conditions (i.e., 5% oxygen). In this study, cleavage stage embryos cultured in reduced oxygen display relatively small heterogeneity in pyruvate uptake. In comparison, the heterogeneity within the data from embryos cultured in atmospheric oxygen conditions is large, which further suggests that atmospheric oxygen is imposing a stress to which the embryo population is displaying a plasticity of response. Within such a cohort, it is predicted that those embryos that can deal with stress, that is, the ones able to maintain a more in vivo like metabolism, similar to those embryos cultured at 5% oxygen, will be the most viable. This concept goes some way to explaining the data of Conaghan et al. [46], Turner et al. [47], and Houghton et al. [26], who presented data in which those embryos cultured, or analyzed, at atmospheric oxygen that went on to develop in vitro or posttransfer exhibited lower rates of nutrient utilization than those that failed to grow or survive.

In contrast to the cleavage stage embryo, the postcompaction embryo response to atmospheric oxygen is switched such that amino acid utilization and glucose uptake were higher in 5% oxygen. Following compaction, only two amino acids were produced by the embryo, alanine and glutamine, irrespective of the oxygen concentration. It has been suggested that pyruvate, after transamination to alanine, may be used as an ammonium sink, thereby preventing the build-up of ammonium ions in the culture medium [48, 49]. However, the results of Orsi and Leese [49] did not demonstrate a dose-dependent increase in alanine production when bovine embryos were exposed to increasing concentrations of ammonium, which could suggest that the transamination pathway is saturated at low concentrations of ammonium. Several bovine studies have reported glutamine production at the blastocyst stage [49, 50] and the production of glutamine has also been reported in murine embryos on Day 4 of development [51] and in expanded porcine blastocysts [52]. Glutamate metabolism, catalyzed by glutamine synthetase, may be implicated in removing free ammonium ions through its conversion to glutamine [49]. Therefore, the production of both alanine and glutamine by the postcompaction embryos in this study could reflect the embryo's ability to remove ammonium, whose production by the embryos does increase significantly at this time [53].

Previous work on postcompaction amino acid turnover has demonstrated that the relative concentration of amino acids present in the culture/incubation medium can affect the turnover rate of individual amino acids [51, 54]. Hence, due to the different concentrations of amino acids in the different media used, a direct comparison between the rates of turnover reported in this study with other studies is difficult. Lamb and Leese [51] collected in vivo derived embryos on the morning of Day 4 and incubated them in medium containing either a combination of essential and nonessential amino acids at concentrations ranging from 0.1 to 2.0 mM, based on the composition of amino acids detected in rabbit oviduct and uterine fluids [55], or amino acids that were all present at 0.1 mM. When embryos were incubated for 4 h on Day 4 of development regardless of the amino acid concentrations, aspartate and arginine were consumed the most. This study produced similar results, with arginine and asparate being preferentially consumed over the second 24-h incubation period (Day 4–5) under both oxygen tensions. Of note, when MAS is inhibited at the blastocyst stage, subsequent viability and fetal growth are significantly compromised [35]. Given that the levels of aspartate amino transferase increase at the blastocyst stage [56], the data could well reflect an increase in the significance of aspartate metabolism to the postcompaction embryo.

Embryos cultured in 5% oxygen displayed significantly increased glucose uptake at the postcompaction stage. These data are consistent with reports that higher glucose uptakes by the embryo on both Days 4 and 5 in the human [28] and Day 5 in the mouse [57] are associated with a positive pregnancy outcome and improved fetal development. The depressed glucose uptakes associated with culture in atmospheric oxygen may consequently reflect a lower viability of those embryos [14]. It is plausible that the increase in glucose uptake following culture at 5% oxygen was a result of the up-regulation of glucose transporters. However, Kind et al. [58] did not observe oxygen-regulated expression of GLUT-1 or GLUT-3 when embryos were cultured at 7% oxygen. They did, however, observe an increase in the GLUT expression when embryos were cultured in 2% oxygen, although embryo development was compromised at this oxygen level. The effects of 5% oxygen on GLUT expression warrants further consideration.

The lower rates of glycolysis from embryos cultured under reduced oxygen reported in this study are consistent with the data generated by Lane and Gardner [13], who determined that mouse blastocysts with a lower glycolytic activity ($<88\%$) were more viable than those embryos that had high glycolytic rates ($>160\%$) after transfer. Few embryos cultured under 5% oxygen had a glycolytic rate over 80% in the present study. In contrast, those embryos cultured in atmospheric oxygen exhibited an increased range for glycolytic activity (Fig. 8).

A similar pattern of increased population range as a result of embryos being cultured in atmospheric oxygen was reported by Wale and Gardner [9], where continuous assessment of embryo development using time-lapse microscopy demonstrated a 6 h increase in the range over which cleavage to the 8-cell stage occurred when embryos were cultured in atmospheric oxygen [9].

Taken together the data presented on amino acid and glucose utilization by the postcompaction embryo indicate that culture in 5% oxygen is associated with a significant increase in nutrient utilization and supports a more oxidative metabolism, in stark contrast to the effects of oxygen on the cleavage stage embryo. In support of this, Khurana and Wales [59] demonstrated that culture of mouse embryos at 5% oxygen resulted in higher levels of glucose oxidation, compared to embryos cultured at 20%. Consequently, higher rates of oxidative metabolism and greater nutrient utilization are associated with a viable metabolism at the blastocyst stage.

In conclusion, this study demonstrates that atmospheric oxygen is a powerful regulator of carbohydrate and amino acid utilization, altering not only the pattern of utilization but the rate of turnover. Furthermore, the precompaction stage embryo displays greater sensitivity to high oxygen as a possible toxicity effect. Recently it was demonstrated that the detrimental effects of atmospheric oxygen on the precompaction embryo were irreversible and that replacing the embryo to low oxygen atmosphere for the postcompaction period was not able to alleviate the developmental perturbations induced earlier on [9]. The precompaction embryo appears to have limited mechanisms for managing suboptimal culture conditions such as these. This is contrary to current hypotheses that extended culture results in increased stress on the embryo; the results of this and other studies confirm that the first stages of precompaction development are actually more sensitive than the latter stages [11, 34]. Leese [60] proposed that the viability of early mammalian embryos is associated with a metabolism that is quiet rather than active, with embryo viability being inversely related to nutrient utilization. As discussed, data for this hypothesis were drawn from measurements of amino acid turnover [26] and pyruvate consumption [46, 47] in studies where the embryos were either cultured or measured in atmospheric oxygen and where consequently the embryos were experiencing significant metabolic stress, compromised gene expression [10, 11] and proteome alterations [12]. Leese et al. [61] suggest elsewhere that the metabolism of early embryos may be up-regulated in response to different environments, one of which was oxygen concentrations above those present physiologically (e.g., under air, i.e., 20% $O_2$, verses 1%–5% $O_2$ in the female reproductive tract). Data from the present study confirm this and suggest that the so-called quiet hypothesis is largely based on collective data from embryos experiencing culture-induced stress as a result of atmospheric oxygen, which is more likely to induce active metabolism at the cleavage stage. The data herein presented also indicate that it is difficult to create a unifying hypothesis for the entire preimplantation period, given that postcompaction the embryo exhibits a very different response to oxidative stress and an up-regulated metabolic activity is associated with successful development and viability posttransfer [28]. The optimum range of amino acid and carbohydrate utilization, which reflects developmental potential of the mammalian embryo therefore needs to be established under physiological oxygen conditions for each successive stage of development, particularly for more reliable inferences to the improvement of human embryo culture conditions and for the development of appropriate biomarkers for embryo selection at transfer.

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