Среды
для культивирования эмбрионов in vitro:
история создания и компонентный состав

Среда определенного химического состава
(CDM - chemically defined medium):

• может быть приготовлена по прописи в любой лаборатории
• можно изменять содержание каждого компонента (оптимизация среды)
• отсутствуют незапланированные биоактивные вещества (ферменты, гормоны, ростовые факторы и т.п.)

Пути разработки CDM

Эмпирический подбор компонентов для получения наибольшего успеха (максимальный % развития *in vitro*):

Принцип “let the embryo choose ”

Подпор компонентов на основе изучения состава среды, в которой эмбрион развивается in vivo:

Принцип “back-to-nature”

Создание сред для эмбрионов

Эмбрионы мыши

1956: Whitten – культивирование эмбрионов мыши с 8-и клеток до бластоцисты (8 компонентов, растворенных в воде)

1957: Whitten – эмбрионы развиваются в культуре при замене хлорида кальция на лактат кальция (но развития с зиготы не получилось).

1958: Ann McLaren и John D Biggers культивировали на этой среде эмбрионы с 8-ми клеток, пересадили суррогатной матери и родились мышата

1963-66: Ralph Brinster, цикл работ - исследование изменения состава среды на развитие эмбрионов (воздействие источников энергии (содержание глюкозы и лактата); воздействие осмолярности среды, влияние газовой фазы (кислород).

Ralph Brinster 1967 – для развития эмбриона с зиготы необходимо присутствие в среде пирувата (сейчас все сред для развития эмбрионов млекопитающих содержат пируват, в т.ч. и среды для культивирования эмбрионов млекопитающих.

John D Biggers - серия работ по улучшению состава, в т.ч. исследование влияния концентрации различных ионов на успех культивирования; использовал методы математического моделирования для оптимизации сред.

Принцип “let the embryo choose ”

Среда Бринстера
для культивирования эмбрионов мыши

Component	g/l	mM
NaCl	5.546	94.88
Sodium Lactate	2.253	25.00
Sodium Pyruvate	0.028	0.25
KCl	0.356	4.78
$CaCl_2$	0.189	1.71
KH_2PO_4	0.162	1.19
$MgSO_4 .7H_20$	0.294	1.19
$NaHCO_3$	2.106	25.00
Glucose	1.0	5.56
Bovine Serum Albumin	1.0	—

Used in an atmosphere of 5% carbon dioxide with balance air, pH=7.4.

TABLE 1. Composition of medium M16 (Whittingham [10]).

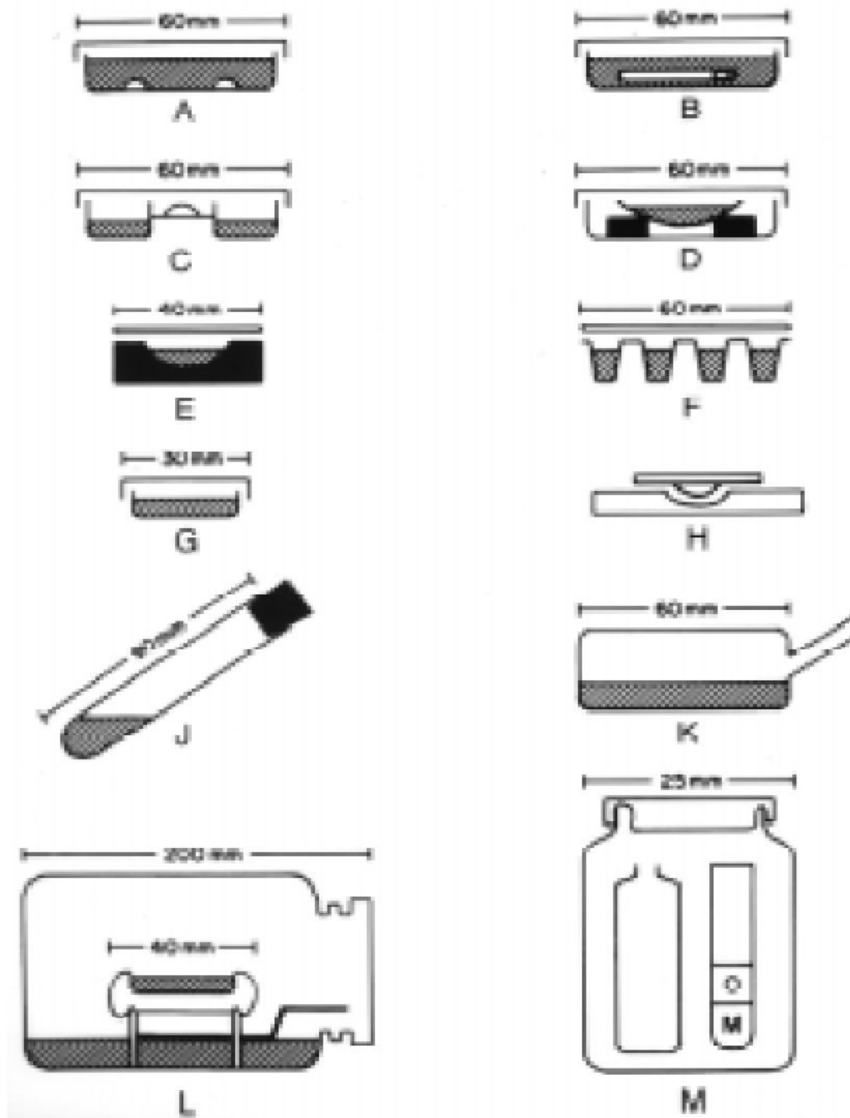
Component	Concentration (mM)
NaCl	94.70
KCl	4.78
KH_2PO_4	1.19
$MgSO_4$	1.19
Lactate	23.30
Pyruvate	0.33
Glucose	5.56
BSA*	4.00
$NaHCO_3$	25.00
$CaCl_2$	1.71

*BSA concentration is in mg/ml. Penicillin and streptomycin were also added at concentrations of 100 U/ml and 50 μg/ml, respectively.

Среда M16 (Whittingham, 1971)
для культивирования эмбрионов мыши

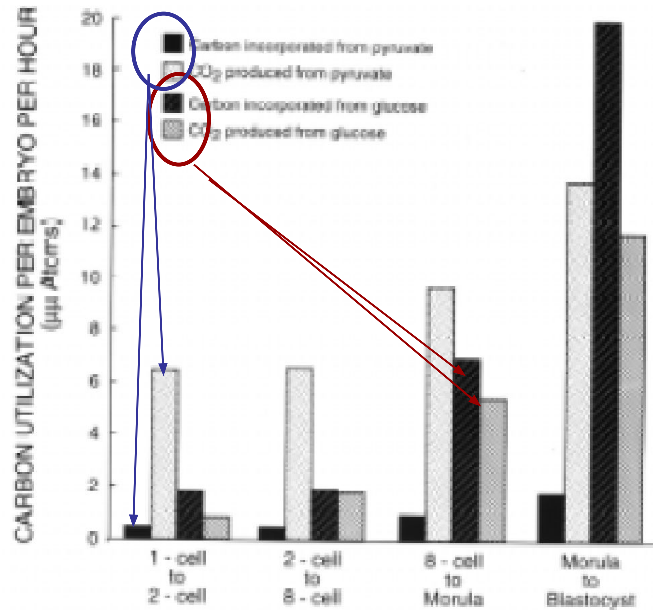
Принцип “let the embryo choose ”

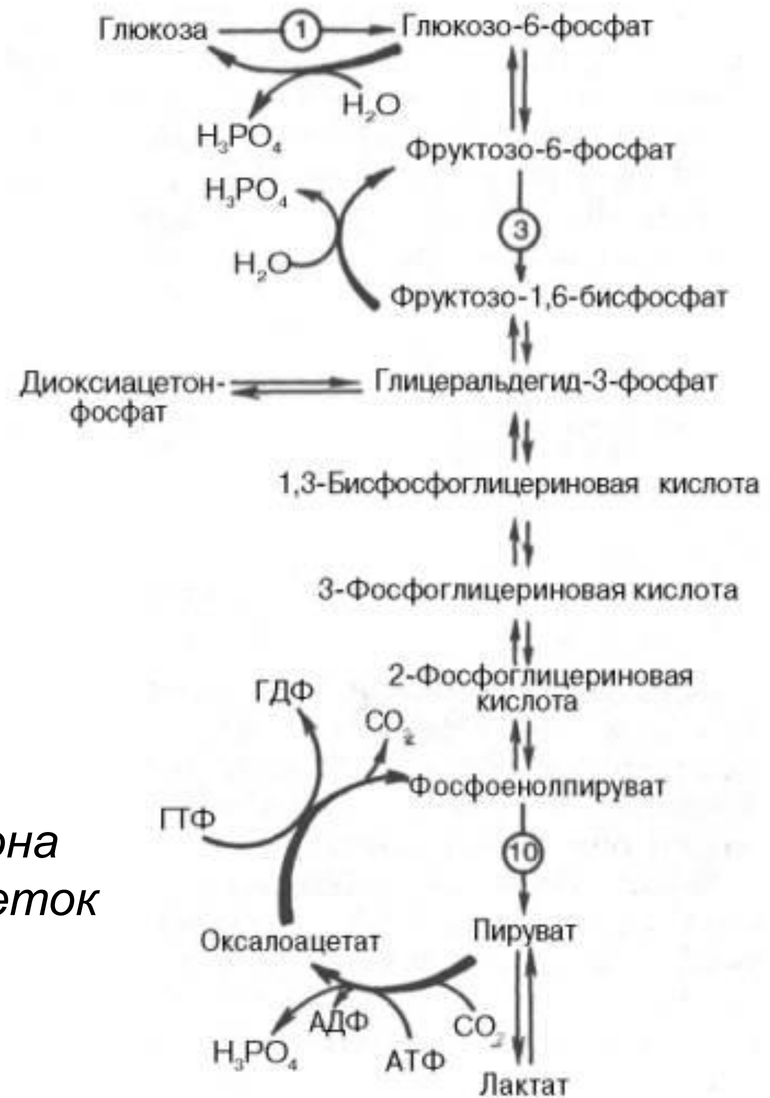
Ralph Brinster – в какой системе культивировать эмбрионы?



В настоящее время используются только вариант А – культивирование с микрокаплях под маслом и вариация варианта С – культивирование в 4-х луночной плате

Глюкоза и пируват могут быть утилизированы клетками эмбриона (в результате- энергия для эмбриона + CO2)



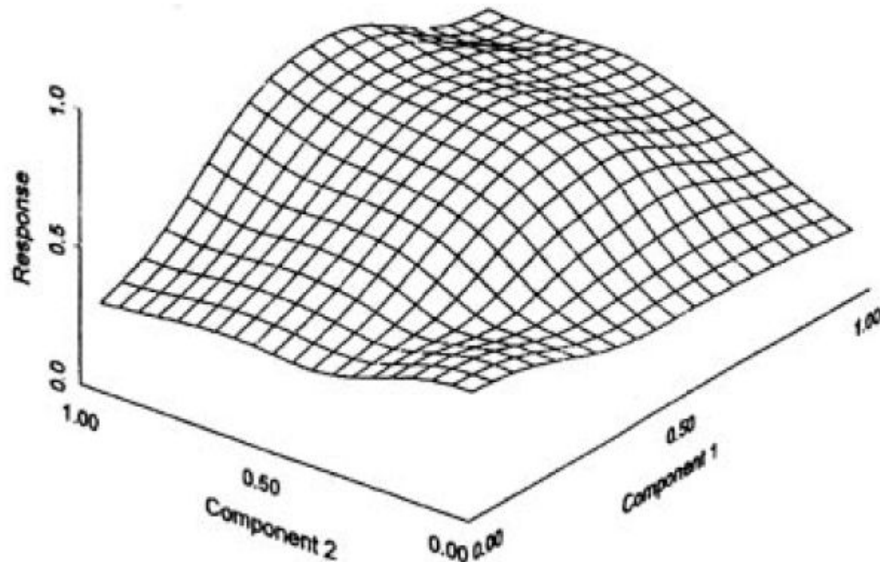


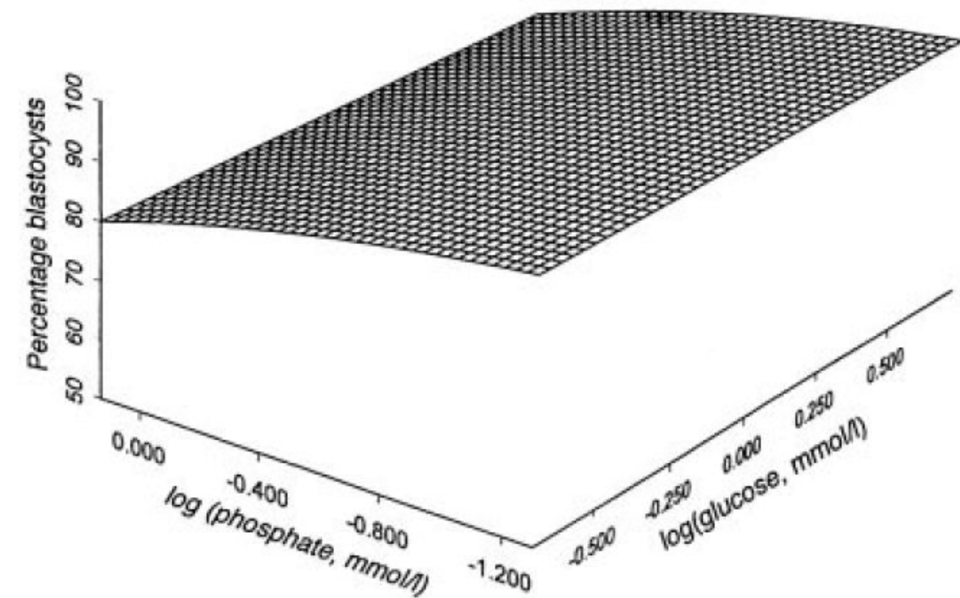
Бринстер, работы 1967-69 гг. (микроанализ утилизации энергетических субстратов):

1. *Метаболическая активность эмбриона резко возрастает со стадии 8-16 клеток*
2. *Только с этой стадии начинается эффективная утилизация глюкозы*

Проблема при разработке сред:

Как оценивать взаимодействие компонентов в среде при изменении их концентраций?





“Response surface model”
-геометрическое представление этой проблемы.

В данном случае представлена оценка совместного действия 2-х компонентов в зависимости от их концентраций в среде при помощи этой модели: 3-х мерная модель.

При изменении N компонентов среды – нужна n+1 - мерная модель

Принцип “let the embryo choose ”

Блок развития при культивировании с зиготы: для решения проблемы были использованы математические программы и компьютерные модели

J. Reprod. Fert. (1991) **91**, 543–556

Printed in Great Britain
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Optimization of mouse embryo culture media using simplex methods

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Summary. Culture media were developed for pronuclear-stage mouse embryos using simplex optimization, which has the benefit of being able to optimize several components simultaneously. Initially, several different media were generated. All media contained the same components, yet each medium was characterized by having a different component at a high concentration. The simplex procedure identified 4 components (NaCl, pyruvate, KH_2PO_4 and glucose) which at high concentrations were detrimental to embryo development, compared to the other components tested. For example, all embryos cultured in a medium with high NaCl blocked at the 2-cell stage. The optimization method then adjusted each medium by lowering the concentration of the component or removing it entirely, which resulted in a significant increase in development. In an experiment comparing 8 media generated from the simplex optimization, along with 7 other media, removal of KH_2PO_4 resulted in the largest increase in development; 88% of embryos were ≥4 cells on Day 3 after hCG, and 53% developed into blastocysts by Day 5.

Another experiment compared 4 of the best media generated from the simplex optimization. In 3 out of the 4 media, 90% or more of the embryos were ≥4 cells on Day 3. In 3 of the media, approximately 60% or more of the embryos developed into blastocysts. The simplex optimization procedure is an efficient method for developing culture media and determining requirements for development *in vitro*.

Keywords: Simplex; culture; mouse; embryo

Sequential simplex optimization

SOM - simplex optimization method

Среда KSOM

Liwitts, Biggers:
“…. Two computer programs are now commercially available to control the experimental program—COPS from Elsevier Scientific Software, New York, USA, and SIMPLEX-V® from Statistical Programs, Houston, Texas, USA.
We have adapted the COPS program to optimize 10 components in a medium for the culture of mouse preimplantation embryos. Our method takes into consideration the biological variation associated with culture systems…. “

Sequential simplex optimization

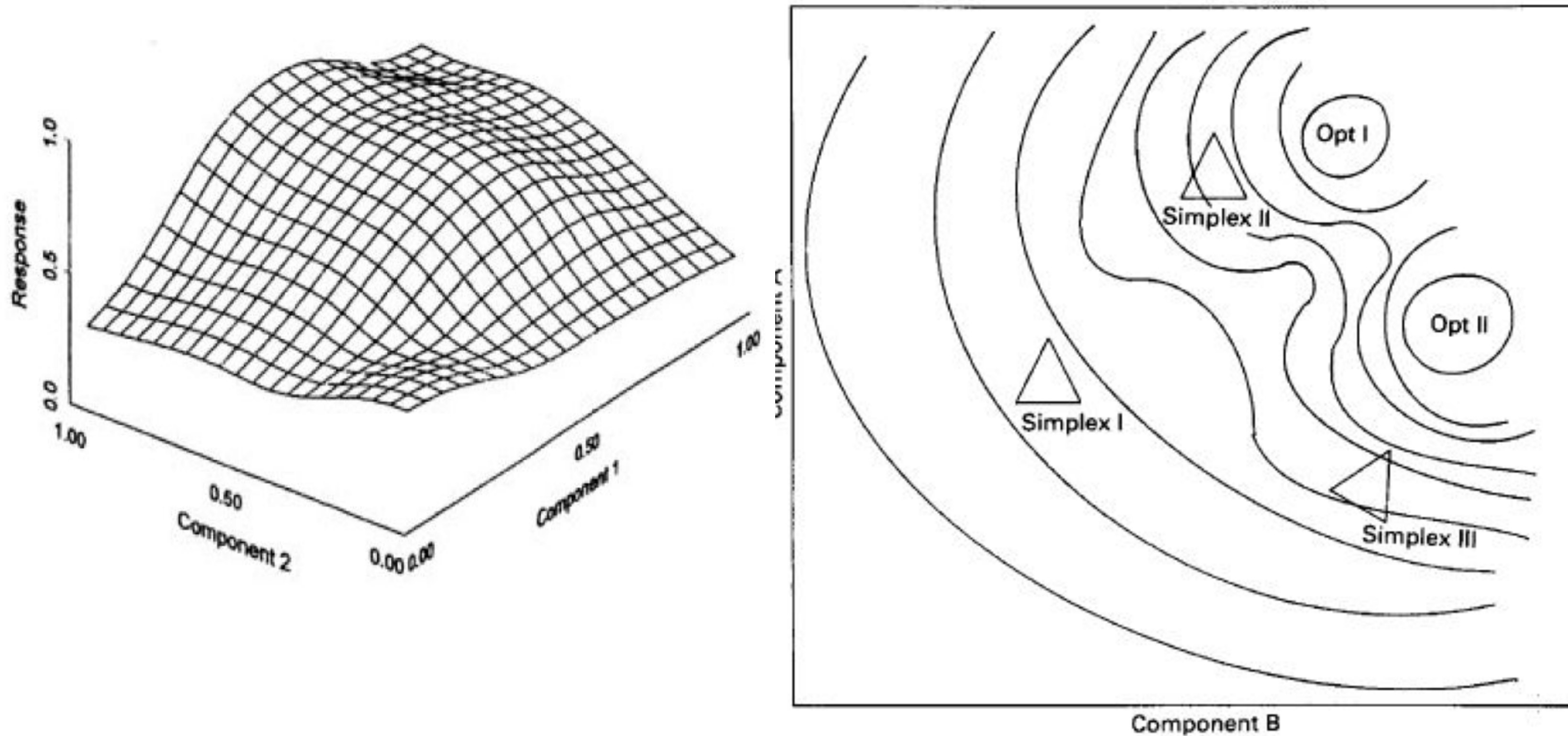


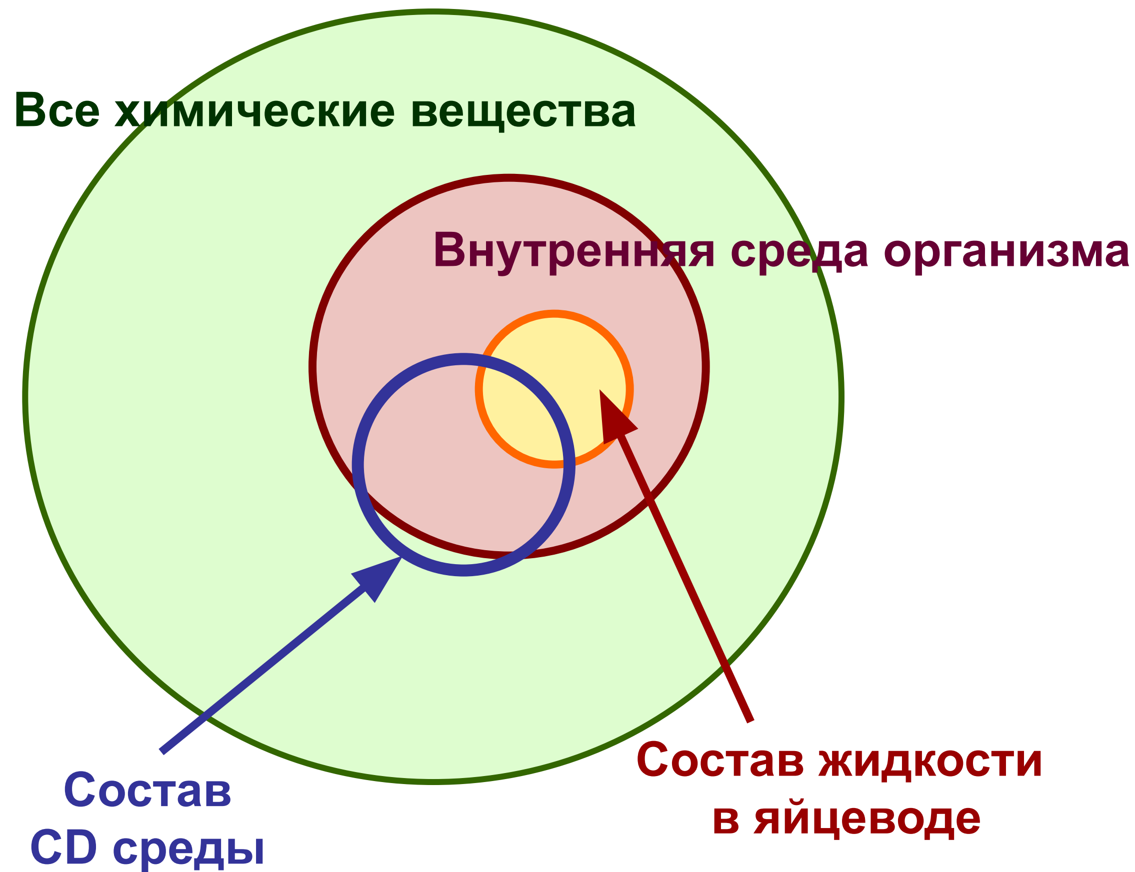
Fig. 6. Theoretical response surface with two different optima. Simplex I is started in an area of the surface where the contours of similar response are far apart. Little progress, if any, will be made. Simplex II and Simplex III are started in an area of the response surface where the contours are closer together, resulting in a rapid ascent toward the optimum, compared to Simplex I.

Simplex **(*noun*)** ***plural*** **sim·plex·es**

a spatial configuration of *n* dimensions determined by *n* + 1 points in a space of dimension equal to or greater than *n*

<a triangle together with its interior determined by its three vertices is a two-dimensional simplex in the plane or any space of higher dimension>

Состав CD (chemically defined) сред для культивирования эмбрионов



Простая среда – до 12 компонентов

Сложная среда – более 12 компонентов

Принцип "back to nature" – подбор компонентов среды для культивирования эмбрионов in vitro, в соответствии с их концентрацией в естественном для эмбрионов окружении in vivo.

HTF human tubal fluid (Quinn et al., 1985).

Сравнение состава среды HTF с опубликованными данными о составе жидкости яйцеводов (по Biggers, 2001)

Compound (mmol/l)	Lippes *et al.* (1976)[a]	Lopata *et al.* (1976)[b]	Medium HTF[c] (1985)	David *et al.* (1973)[d]	Borland *et al.* (1980)[e]	Gardner *et al.* (1996)[f]
Na^+	139–140	149.2	148.3	142–148	130	–
K^+	7.7–9.9	4.5	5.06	6.7	21.2	–
Cl^-	117–120	–	108.3	112–127	132	–
Ca^{2+}	3.8–4.8	1.38	2.04	–	1.13	–
Mg^{2+}	–	0.19	0.2	–	1.42	–
Glucose	2.39–3.04	–	2.78	–	–	2.32
Pyruvate	–	0.18	0.33	–	–	0.16
Lactate	–	2.52	21.4	–	–	6.19

[a]Samples collected over 24 h by cannulation ($n = 16$).
[b]Samples collected at laparoscopy ($n = 2$).
[c]Concentrations of ions calculated from the concentrations of constituents in Table 7 from Quinn *et al.* (1985).
[d]Samples collected at laparotomy ($n = 33$).
[e]Microsamples collected by microsampling ($n = 7$).
[f]Microsamples collected during the luteal phase at laparoscopy ($n = 9$).

Table II. Compositions of media HTF (Quinn *et al.*, 1985), modified HTF (Quinn *et al.*, 1995), P1 (Gardner *et al.*, 2000) and KSOM (Lawitts and Biggers, 1993)

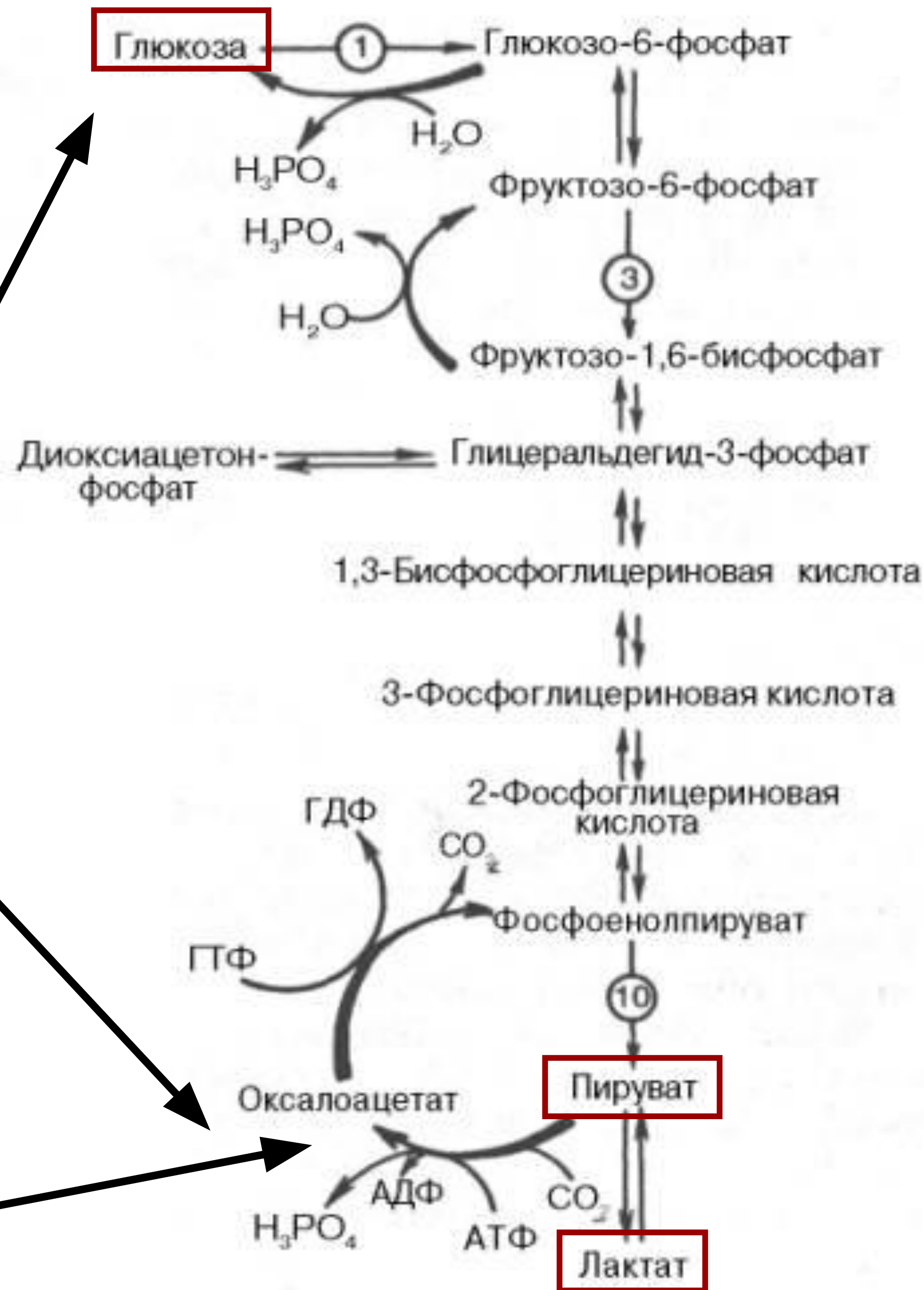
Compound (mmol/l)	HTF	Modified HTF	P1	KSOM
NaCl	101.6	101.6	101.6	95.0
KCl	4.69	4.69	4.69	2.50
KH_2PO_4	0.37	–	–	0.35
$CaCl_2$	2.04	2.04	2.04	1.71
$MgSO_4$	0.20	0.20	0.20	0.20
$NaHCO_3$	25.0	25.0	25.0	25.0
Glucose	2.78	–	–	0.20
Na pyruvate	0.33	0.33	0.33	0.20
Na lactate	21.4	21.4	21.4	10.0
Citrate	–	–	0.5	–
Glutamine	–	1.0	-	1.0
Taurine	–	–	0.05	-
EDTA	–	0.1	–	0.01
Penicillin (U/ml)	100	100	–	–
Streptomycin SO_4 (50 µg/ml)	100	100	–	–
Phenol red (µg/ml)	10	10	5	–

Источники энергии

пируват
лактат
глюкоза

Полная функциональность возвращается только на стадии 8-ми бластомеров!

Ооциты и зиготы способны усваивать только пируват !



Глюкоза: отрицательное воздействие на эмбрионы на стадиях дробления?

BIOLOGY OF REPRODUCTION 39, 1183–1192 (1988)

Two-Cell Block to Development of Cultured Hamster Embryos Is Caused by Phosphate and Glucose[1]

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ABSTRACT

The failure of hamster 2-cell embryos to develop in vitro (2-cell block) was examined with experiments in which concentrations of glucose and phosphate in the culture medium were varied. Embryos were cultured in a protein-free modified Tyrode's solution that normally contains 5.0 mM glucose and 0.35 mM sodium dihydrogen phosphate. In the presence of 0.35 mM phosphate but without glucose, 23% of 2-cell embryos reached the 4-cell stage or further after culture for 1 day and 27% after 2 days. Glucose inhibited embryo development even at 0.1 mM (4% development to ≥4-cells after culture for 2 days); there was no dose-related inhibition above this glucose concentration. In a second experiment, phosphate levels were varied in the absence of glucose. Phosphate was highly inhibitory to development, with 97% of 2-cell embryos reaching the 4-cell stage or further after culture for 1 day in the absence of phosphate compared to 9–21% in the presence of 0.1–1.05 mM phosphate. After culture for 2 days, 26% of embryos reached the 8-cell stage or further when phosphate was absent compared to 0% development to 8-cells with 0.1 mM phosphate or higher. In a factorial experiment, phosphate blocked development when glucose was present or absent, whereas glucose did not block embryo development in the absence of phosphate. However, 2-deoxyglucose (a non-metabolizable analogue of glucose) inhibited embryo development in the absence of phosphate. These data show that the in vitro block to development of hamster 2-cell embryos is caused at least in part by glucose and/or phosphate. Deletion of these compounds from the culture medium eliminates the 2-cell block to development in virtually all embryos, and approximately 25–75% of embryos develop to the 8-cell or morula stages in vitro. The observations provide a possible explanation for the 2-cell and 4-cell blocks that occur in conventional culture media: stimulation of glycolysis by glucose and/or phosphate may result in inefficient adenosine triphosphate (ATP) production. The data indicate marked dissimilarities in the regulation of in vitro development of early cleavage stage hamster embryos compared with embryos of inbred mice, since the latter have an inactive glycolytic pathway prior to the 8-cell stage of development and will grow from 1-cell to blastocyst with both phosphate and glucose in the culture medium.

TABLE 1. Composition of modified culture medium TLP-PVA[a] used for culture of 2-cell hamster embryos.

Component	Concentration (mM)
NaCl	98.0
KCl	3.2
$CaCl_2$	2.0
$MgCl_2$	0.5
$NaHCO_3$	25.0
Na lactate	10.0
Na pyruvate	0.5
L-Alanine	0.28
L-Arginine	0.30
L-Asparagine	0.50
L-Aspartic Acid	5.00
L-Cysteine	0.10
L-Glutamic Acid	5.00
L-Glutamine	1.00
Glycine	7.00
L-Histidine	0.10
L-Isoleucine	0.15
L-Leucine	0.20
L-Lysine	0.75
L-Methionine	0.70
L-Phenylalanine	0.30
L-Proline	0.17
L-Serine	0.24
Taurine	7.00
L-Threonine	0.50
L-Tryptophan	0.10
L-Valine	0.20
Polyvinylalcohol	1.0 mg/ml

[a]The medium was supplemented in some experimental treatments with sodium dihydrogen phosphate, glucose, and/or 2-deoxyglucose.

TABLE 2. Effect of glucose concentration on in vitro development of hamster 2-cell embryos (Experiment 1).[a]

		Day 1[c]	Day 2[d]
Glucose conc. (mM)	No. of 2-cell embryos[b]	No. of ≥4-cells (% ± SEM)	No. of ≥4-cells (% ± SEM)
0.0	147	34 (23.1 ± 5.5)[e]	40 (27.2 ± 5.6)[e]
0.1	148	2 (1.4 ± 2.1)	6 (4.0 ± 2.5)
0.5	149	9 (6.0 ± 3.4)	7 (4.7 ± 2.9)
5.0	147	1 (0.7 ± 1.5)	4 (2.7 ± 2.2)

Спор:

Bavister:

Глюкоза и фосфат тормозят развитие дробящихся эмбрионов

Biggers:

Глюкоза не тормозит развитие эмбрионов (в концентрации, присутствующей в плазме крови – 5,56ммоль), фосфат оказывает небольшое тормозящее действие.

Результат:

Специальные среды для эмбрионов на стадиях дробления (до 3 сут.) и для более поздних (3-5 сут.)

Гликолитический путь деградации глюкозы до пирувата:

Голубой цвет: участие в процессе АТФ и АДФ;

Желтый цвет: NAD и NADH;

Все промежуточные (между глюкозой и пируватом) компоненты фосфорилированы

Human Reproduction Vol.20, No.12 pp. 3376–3384, 2005
doi:10.1093/humrep/dei228
Advance Access publication August 25, 2005.

One-step versus two-step culture of mouse preimplantation embryos: is there a difference?

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[3]To whom correspondence should be addressed. E-mail: john_biggers@hms.harvard.edu

BACKGROUND: A comparison has been made of the development of mouse zygotes in either one-step or two-step culture systems. METHODS: Em
significant differences were observ
cell mass (ICM) and trophectoder
optimized medium supplemented w
and two-step culture in G1.2/G2.2
in the one-step protocol using KS
groups of fetuses were not signific
differences were observed in the p
that developed in protocols: one-s
culture in DM2/DM1. EDTA is not
l in $KSOM_g^{AA}$. CONCLUSIONS:
development *in vitro* but they are

Human Reproduction Vol.21, No.7 pp. 1935–1944, 2006

Letters to the Editor

One-step versus two-step culture of mouse preimplantation embryos

Sir,

We would like to offer a different perspective on the study reported by Biggers *et al.* (2005) whose findings are contradictory to our own previous studies on these embryo culture media systems.

In our previous publication comparing KSOMAA to sequential media (Gardner and Lane, 2002, 2003; Reed *et al.*, 2003), we were careful to ensure that all conditions used were exactly the same for the medium being tested, e.g. the same volume of medium. This was done because in our extensive experience, the volume of medium has a significant impact on culture outcome if there are problems with the oil source used. Specifically, when suboptimal oil is used, embryo development increases with increasing volumes of medium. In the article by Biggers *et al.* (2005), two different volumes were used, 50 µl for the KSOMAA and 20 µl for the sequential media. The source of oil used was one not sold for use in human IVF. One therefore

Furthermore, the addition of ammonium chloride to the culture medium can induce the same birth defect (Lane and Gardner, 1994; Sinawat *et al.*, 2003). Therefore, three independent laboratories, including that of Biggers, have demonstrated that ammonium can induce exencephaly in mouse fetuses, following the exposure of the preimplantation embryo. What has been debated is the frequency at which the birth defect is induced. What is not for discussion is that the birth defect is induced by ammonium. As a result, we are obligated to renew media which contain amino acids at 48 h, irrespective of the formulation, to ensure that ammonium levels cannot build up sufficiently to induce the neural tube birth defect. So, irrespective of the article by Biggers *et al.* (2005), his own previous

David K.Gardner[1,3] and Michelle Lane[2]
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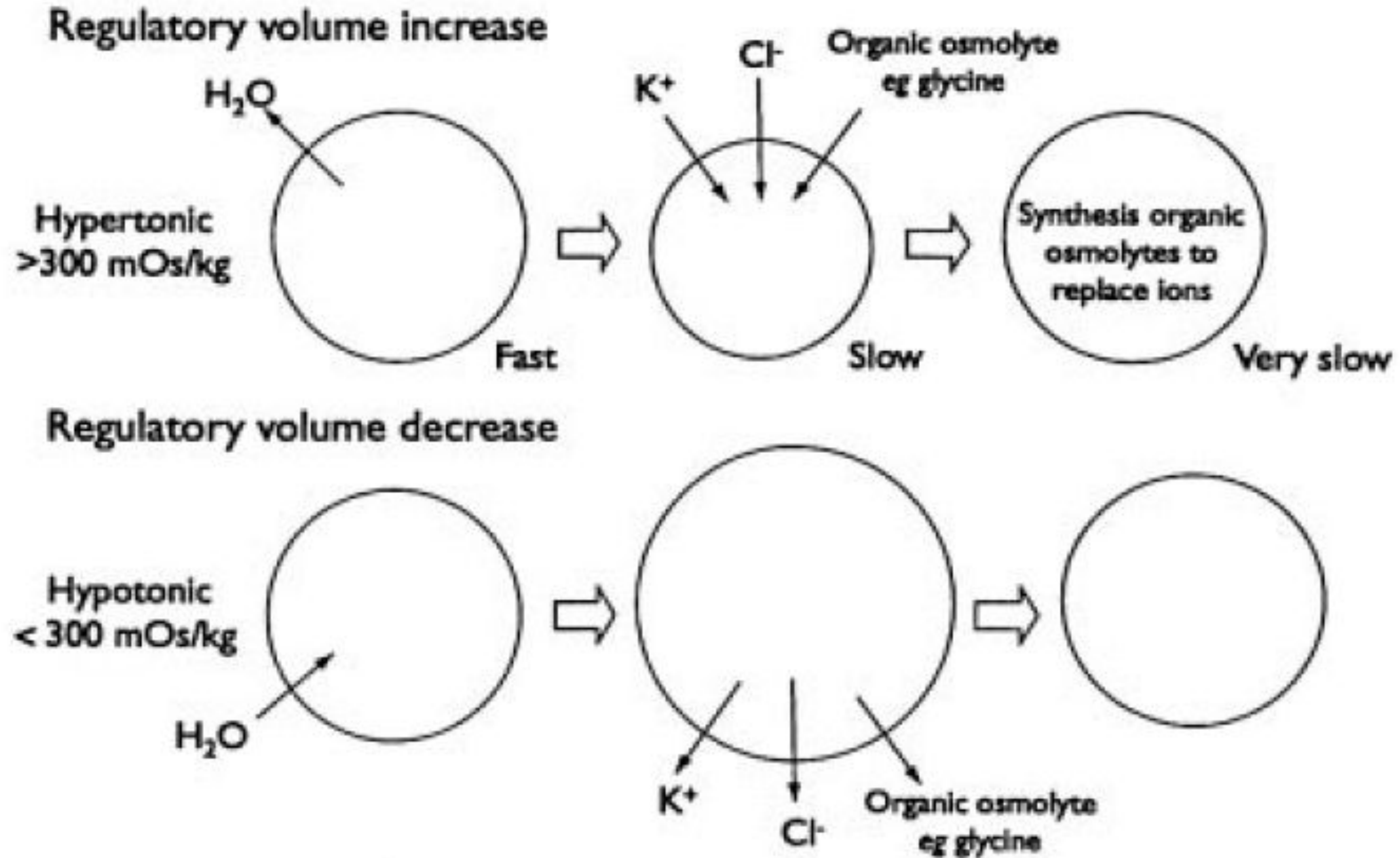
[2]*Obstetrics and Gynaecology Department, The Queen Elizabeth Hospital, University of Adelaide, Adelaide, South Australia, Australia*

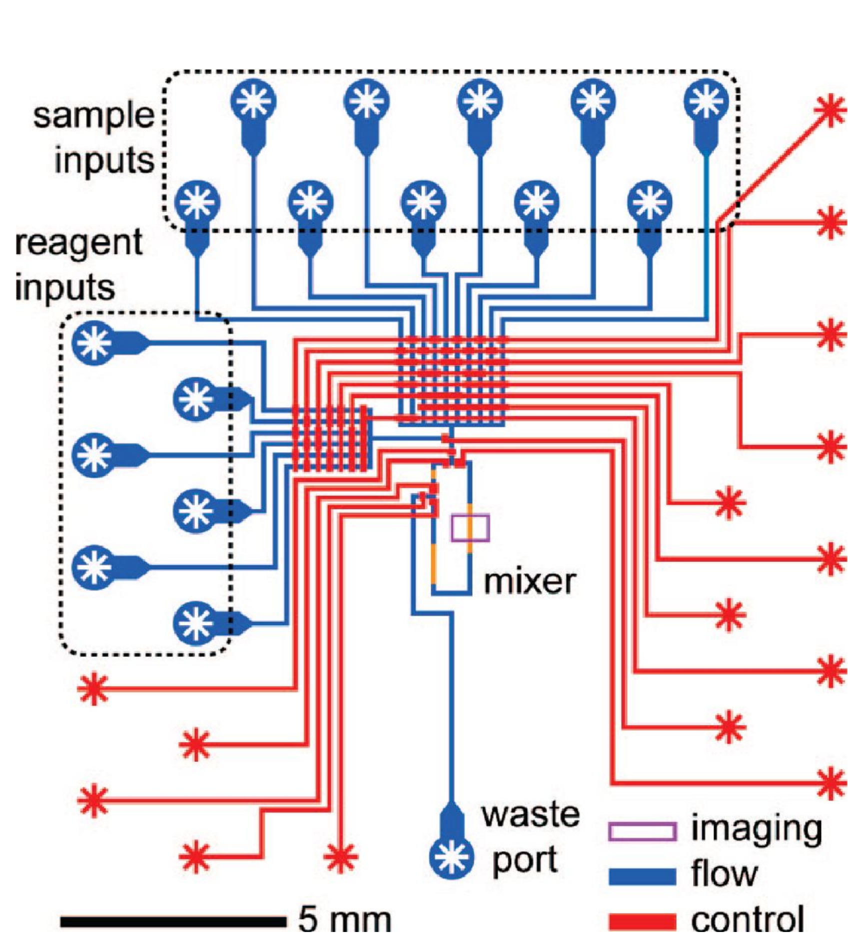
Осмотические свойства среды

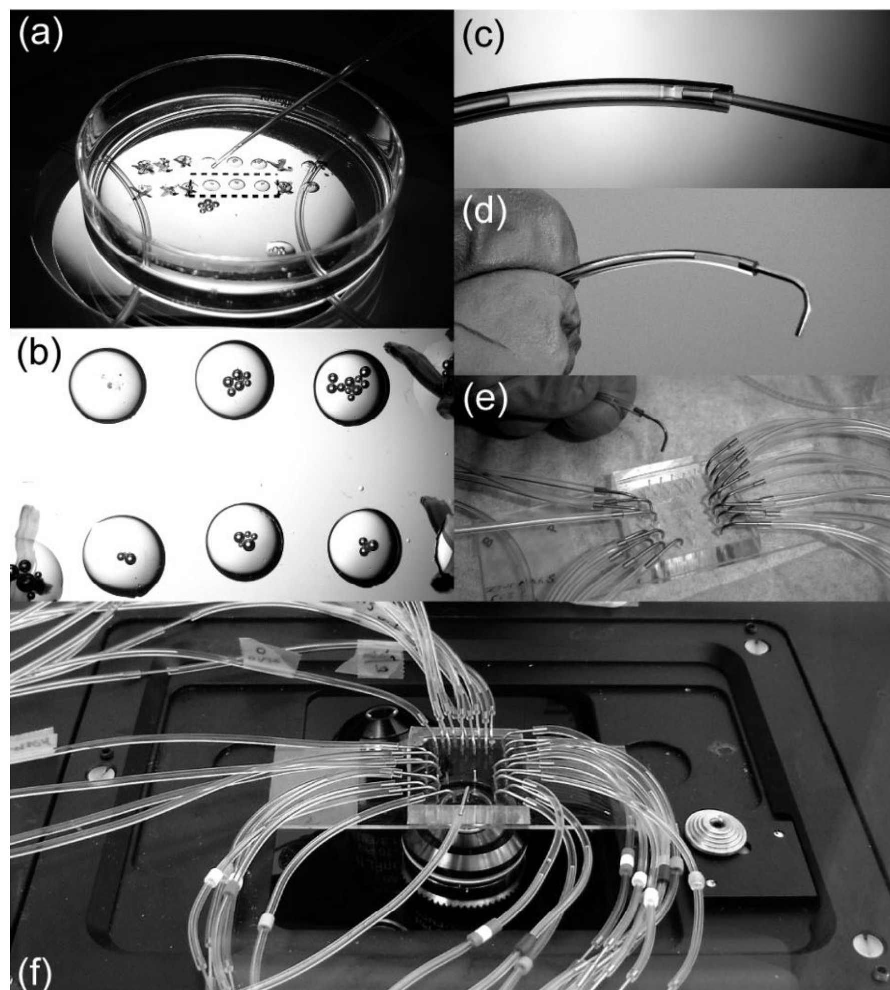


Biggers JD (2002), Reproductive BioMedicine Online

Noninvasive Metabolic Profiling Using Microfluidics for Analysis of Single Preimplantation Embryos

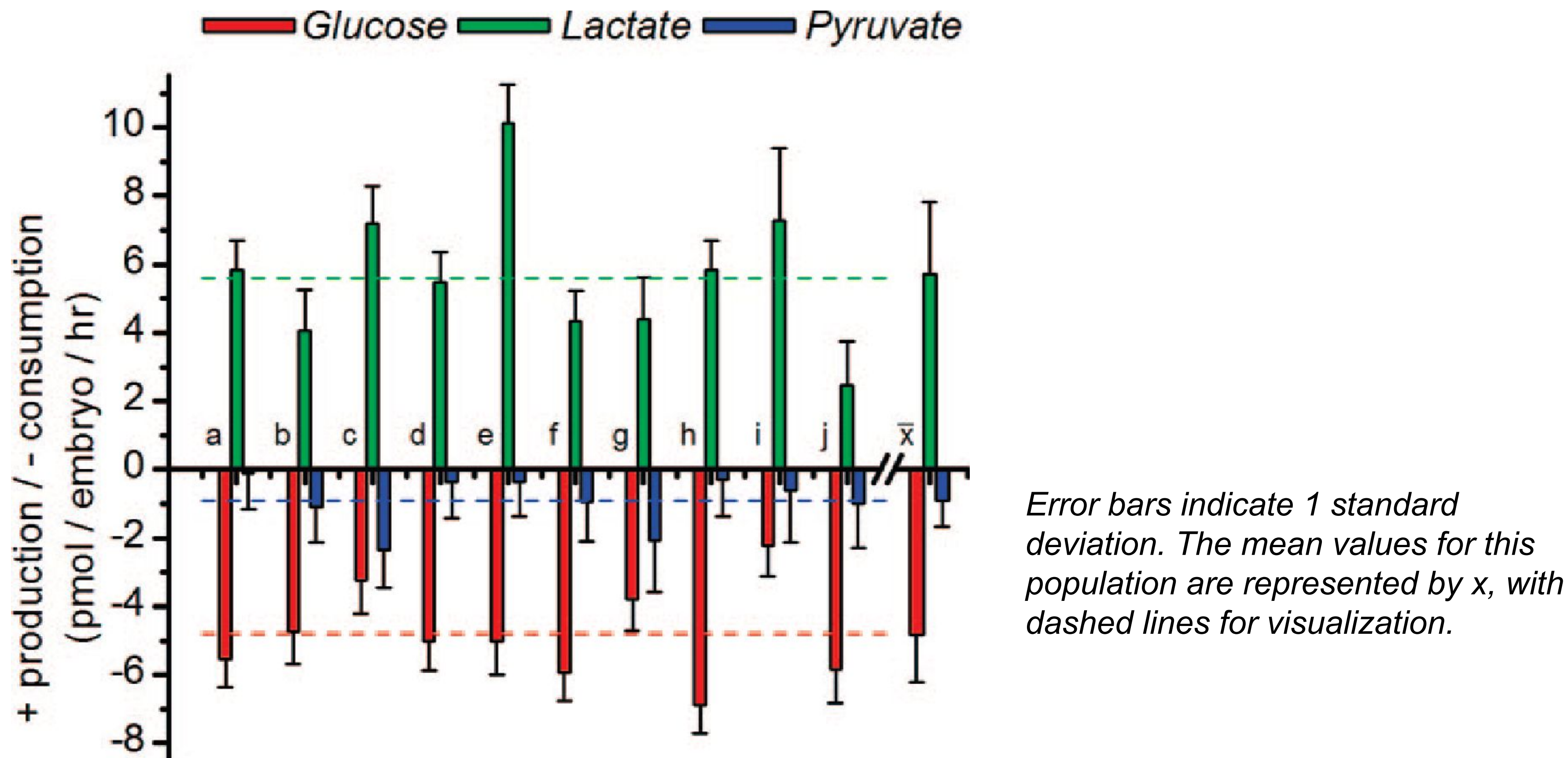
John Paul Urbanski, Mark T. Johnson, David D. Craig, David L. Potter, David K. Gardner,|
and Todd Thorsen
Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge,
Massachusetts Fertility Laboratories of Colorado, Englewood, Colorado 80110





Anal. Chem. **2008,** *80,* 6500–6507

Анализ метаболической активности единичных эмбрионов



Error bars indicate 1 standard deviation. The mean values for this population are represented by x, with dashed lines for visualization.

Metabolic profiles obtained from 10 murine embryos, labeled a-j. Results are from day 4 of the culture and are presented relative to original G2 media (analyzed in parallel with culture samples) to ascertain the changes in metabolite levels due to embryo activity.

Individual embryos were cultured in 1 *μ*L of medium G2 for 48 h prior to analysis. Heterogeneity in metabolism becomes apparent among morphologically similar embryos.

Anal. Chem. **2008,** *80,* 6500–6507

РОССИЙСКАЯ ФЕДЕРАЦИЯ

ПАТЕНТ

НА ИЗОБРЕТЕНИЕ

№ 2281777

СПОСОБ УПРАВЛЕНИЯ КАЧЕСТВОМ ООЦИТОВ И КОМПОЗИЦИЯ ДЛЯ ДОБАВЛЕНИЯ В СРЕДУ КУЛЬТИВИРОВАНИЯ ООЦИТОВ

Патентообладатель(ли): ***Семенова Мария Львовна (RU), Захарова Елена Евгеньевна (RU), Залетов Сергей Юрьевич (RU), Заева Виктория Викторовна (RU)***

Автор(ы): ***см. на обороте***

Заявка № **2005111862**

Приоритет изобретения **21 апреля 2005 г.**

Зарегистрировано в Государственном реестре изобретений Российской Федерации ***20 августа 2006 г.***

Срок действия патента истекает **21 апреля 2025 г.**

Руководитель Федеральной службы по интеллектуальной собственности, патентам и товарным знакам

Б.П. Симонов

РОССИЙСКАЯ ФЕДЕРАЦИЯ

ПАТЕНТ

НА ИЗОБРЕТЕНИЕ

№ 2281778

СПОСОБ КУЛЬТИВИРОВАНИЯ ЗИГОТЫ И/ИЛИ ЭМБРИОНА И КОМПОЗИЦИЯ ДЛЯ ДОБАВЛЕНИЯ В СРЕДУ КУЛЬТИВИРОВАНИЯ ЗИГОТ И/ИЛИ ЭМБРИОНОВ

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Заявка № **2005111859**

Приоритет изобретения **21 апреля 2005 г.**

Зарегистрировано в Государственном реестре изобретений Российской Федерации ***20 августа 2006 г.***

Срок действия патента истекает **21 апреля 2025 г.**

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