THE EFFECT OF CHANGES IN CULTURE MEDIA, pH AND TEMPERATURE ON IN VITRO FERTILIZATION AND PRE-IMPLANTATION EMBRYONIC DEVELOPMENT OF MICE

Mohammed-Baqir M-R. Fakhrildin Ayad M.A. Fadhil M. A. Ibrahim
Institute for Embryo Research and Infertility Biotechnology Dept/College of Science
Treatment/University of Baghdad Al-Nahrain University/Baghdad

May R. Jafar Ban S. Al-Temimie
Biotechnology Research Center/Al-Nahrain University/Baghdad

Received 12/5/2004 Accepted 2/4/2006

ABSTRACT

The present study was conducted to investigate the effect of different types of culture media, degrees of temperature of culture medium and wide range of pH within culture medium on the percentages of in vitro fertilization of mouse oocytes and embryonic development. Nine hundred and twenty four oocytes were recovered from eighty one superovulated mice. Groups of mature oocytes were incubated with active spermatozoa within different culture media (Ham’s F-10, Medi-Cult. IVF, TCM-199, Earl’s + TCM-199 and RPMI-1640) at different degrees of temperature (36, 37, 38 and 39), as well as, the pH of culture medium was changed (range: 6.9-8.1) in 5% CO₂ for 18-20 hour. Percentages of in vitro fertilization and abnormal embryonic development were assessed.

The best results for IVF and normal embryonic development were achieved with RPMI-1640 medium at temperature 37 °C and acidity (7.3-7.4) of medium. However, best results for in vitro development of mouse embryos were obtained by using TCM-199 medium and Ham’s F-10 medium at temperature 36 °C and acidity (7.3-7.4). In conclusion, the mouse spermatozoa and oocytes are highly sensitive to changes in the environmental conditions. In addition, conditions of culture medium and its selection are may be the main limited factor for improvement in vitro fertilization and embryonic development in mice. Further biochemical and molecular studies are recommended to assess the effect of culture medium on content of RNA and protein synthesis of different stages of mouse embryo.

Key words: Superovulation, in vitro fertilization and embryonic development.
تأثير التغييرات في الأوساط الزراعية والباهاء ودرجة الحرارة على الأخصاب الخارجي
وتطور الأجنحة قبل الانغراس في الفنر

محمد باقر محمد رشاد فخر الدين
أياد محمد علي فاضل
محمد عبد القادر إبراهيم
معهد أبحاث الأجنحة وعلاج العقم/ جامعة بغداد
قسم التقنيات الاحياتية/ كلية العلوم/ جامعة النهرين
ميه روسي جعفر
مركز أبحاث التقنيات الاحياتية/ جامعة النهرين/ بغداد

استلم بتاريخ 12/5/2006
قبل تاريخ 2/4/2006

الخلاصة

أجريت هذه الدراسة لمعرفة تأثير أنواع مختلفة من الأوساط الزراعية (Culture medium) على النسب المئوية للأخصاب الخارجي (pH) ودرجات الحرارة (Temperature) وتطور الأجنحة (In vitro fertilization) وتطور الاجنحة (Embryonic development) في الفنر. فقد أُستُرِدَت
تسعينات وأربع وعشرين بويضة من أربع وثمانين لث فاز مختبر للإباضة فاز.V. تغيُّرات أنواع الأدوات الباهائية إشعاعية لجسم (Superovulation) وتُستغل (10) و (TCM-199) و (Ham’s F-12) و (Medi-Cult. IVF) و (RPMI-1640) و (إمغاسة دورة 18-20 حيث) تقييم الت מספר المئوية لأشخاص الوجبات وكثافة النتائج في
الفنر.

تم تحقيق أفضل النتائج للأخصاب الخارجي وتطور الأجنحة الطبيعية باستخدام الوسط الزراعي (RPMI-1640) ودرجة حرارة (37) độ C ودرجة حرارة (7.3-7.4) تأثير (RCM) ودرجة حرارة (37) °C

حال تحقق أفضل النتائج بالنسبة لنمو الأجنحة خلال الجسم باستخدام الوسطين الزراعيين (RCM-199) و (Ham’s F-10) و (Ham’s F-12) و (TCM-199) و (Ham’s F-10) و (RCM-199) و (Ham’s F-12) و (TCM-199) و (Ham’s F-10) و (RCM-199). 

نستنتج أن

الوظائف والبيولوجيا ذات حساسية عالية للتغييرات في هذه الظروف المحيطة. كذلك تعتبر ظروف تفاصيل الزراعي اختيارها هو عامل حاسم رئيس لتحسين الأخصاب الخارجي وتطور الأجنحة خلال الجسم في الفنر.

تم iarvi4 دراسات جزيئية وميكروهية إضافية لتقييم تأثير الوسط الزراعي على حمضية الرنا (RNA) لوصف مراحل أجنحة الفنر.

80
INTRODUCTION

During the past two decades, a great experience and large number of information was collected to optimize conditions of culture medium and techniques for \textit{In vitro} Fertilization and embryonic development (1,3). It was reported there were many conditions affect either sperm, or oocyte or both to achieve fertilization included biological, chemical and physical factors (4,5).

It was reported that many improvements were introduced on culture medium including chemical, physical and biological conditions. Chemical factors involving enrichment of culture medium with different hormones, synthetic human serum albumin, antibiotics, antioxidants, ions, organic and inorganic materials (6,11). However, physical improvements involving temperature, pH and osmolarity of culture medium (12,16). While, biological additives was considered an important factor to increase the rate of fertilization \textit{in vitro} involving addition of growth factors, fetal calf serum (FCS), bovine serum albumin (BSA), amino acids, follicular fluid and oviductal fluid (3,5,17,19).

In general, successful fertilization \textit{in vitro} is dependent on maintenance of suitable culture conditions for male and female gametes and pre-implanted embryos (18). Therefore, this study was designed to investigate, in mice, the effects of different environmental conditions of culture medium including different types of culture media, changes in the degrees of temperature and various acidity of culture medium on the percentages of IVF and abnormal embryonic development.

MATERIALS AND METHODS

1- Animals and superovulation regimen:

Eighty one healthy, female, mothered Swiss white mice (age: 15-16 weeks) were kept in an air-conditioned room at a temperature of 26 ± 2 °C and exposed to 14 hour day light program (20). Females were intraperitoneally (IP) injected with 15 IU of human Postmenopausal Gonadotrophin (hMG; Pergonal 500, Serono, Italy). After 72 hour of first injection, same females were IP injected with 15 IU of human Chorionic gonadotrophin (hCG; Profasi 5000 IU, Serono, Italy). After 16-18 hour of last injection, oocytes were flushed from oviducts, and treated with hyaluronidase (Medi-Cult, Denmark) to remove cumulus cells. Then, recovered oocytes were pipetted and washed two times and classified into immature, mature and atretic depending on the presence of 1\textsuperscript{st} polar body and other morphological features. For \textit{in vitro} maturation, immature oocytes were grouped (8-10 oocytes) and incubated at 37 °C in 5% CO\textsubscript{2} for 4-6 hours, and presence of 1\textsuperscript{st} polar body was determined and considered the best indicator for oocyte maturation (21).
2- Experimental design:

The present study was designed to examine the effects of changes in various environmental factors on the percentages of IVF, in vitro development of embryos and abnormal development of pre-implanted embryos. According to the nature of this study, three experiments were applied involving:

a- Culture media experiment: Several types of culture media including Ham’s F-10, TCM-199, Earl’s + TCM-199, Medi-Cult. IVF and RPMI-1640 were used.
b- Temperature experiment: Various temperature degrees including: 36, 37, 38 and 39 °C were applied.
c- Acidity (pH) experiment: A wide rang of acidity (pH range: 6.9 - 8.1) of culture medium was tested.

3- Sperm collection:

Spermatozoa were flushed from both vas deferens of male mouse (No.: 29 males) within 1ml of sperm preparation medium (Medi-Cult, Denmark). Sperm function tests were examined pre- and post-incubation for at least 1 hour. Active vasal spermatozoa were adjusted to 1 X 10^6 / 0.75 ml within selected culture medium was prepared for experiment previously, and then placed under paraffin oil (Medi-Cult, Denmark) within four well IVF Petri dish (Nunclon, Denmark).

4- In vitro fertilization technique:

Each 5-6 mature oocytes were introduced inside a drop of vassal spermatozoa-containing medium at 37 °C in 5% CO₂ for 18-20 hour to assess the percentages of fertilization (21).

5- Statistics:

Data were statistically analyzed to obtain mean and standard error of mean (SEM). Also, MANOVA test was used to compare level of significance among different means of each experiment (22).

RESULTS AND DISCUSSION

In the present study, results showed that RPMI-1640 medium has the best result for the percentages of IVF and lowest abnormal embryonic morphology as compared to other culture media used. On the other hand, insignificant differences are reported for the percentages of IVF between RPMI-1640 and Ham’s F-10 media Figure (1).
Figure (1): Effect of different culture media on the percentages of \textit{in vitro} fertilization and embryonic abnormality in mice

*: Significantly (P<0.005) different as compared to other corresponding groups.
#: Significantly (P<0.01) different as compared to groups of Earl’s+TCM-199 and Medi-Cult. IVF media.

These results are certified that the constituents of culture medium have direct and specific influence on the consecutive steps, which leads to fertilization and embryo formation later (23). It is reported that the successful IVF has been highly dependent on the pre-fertilization events, which is affected by the composition of culture medium (24). The basic chemical composition of RPMI-1640 medium is characterized by the presence of high concentration of glucose, in addition to various additives like hormones, vitamins and amino acids (25). In spite of Medi-Cult medium contains specific additives including human serum albumin and synthetic serum replacement (11), Medi-Cult medium has occupied the second degree for success IVF after RPMI-1640 and Ham’s F-10 culture media Figure (1). It is certified that the absence of natural serum like fetal calf serum, bovine serum albumin and fetal bovine serum causes the zona pellucida to be more solid, and subsequent fertilization is reduced (26,27).
Significant (P<0.005) and least result for abnormal embryonic development was detected when RPMI-1640 is used as compared to other culture media. In contrast, largest percentage for abnormal embryonic development was observed where (Earl’s+TCM-199) medium is used Figure (1). An increased percentage of abnormal embryonic development mirror the micro-environmental conditions of the grown embryos. Al-Katanani and Hansen (16) reported that the heightened sensitivity of the early embryo to stress may be the fact that embryonic genome is largely suppressed during early cleavage stages. In addition, it is known that mouse spermatozoa, oocytes and embryos are highly sensitive to any shift in chemical or physical conditions, therefore, mouse embryos are used for control and safety materials used for human embryos (28). Furthermore, the differences in the percentages of abnormal embryonic development within different culture media were used may be as a result of differences in osmotic pressure among culture media. It is reported that the oocytes of each species has specific range of osmotic pressure, which play main role for normal embryonic development, in addition to oocyte in vitro maturation and fertilization (29-31).

Although TCM-199 medium has the lowest percentage of IVF, the TCM-199 medium and Ham’s F-10 medium was the best culture media for in vitro embryonic growth. In the next degree, was RPMI-1640 medium was appeared to support the growth of most stages of embryonic growth Table (1).
Table (1): Effect of different culture media, temperatures and pH of culture medium on the percentages of in vitro embryonic development in mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number of oocytes</th>
<th>IVF (%)</th>
<th>1-cell embryo</th>
<th>2-cells embryo</th>
<th>4-cells embryo</th>
<th>8-cells embryo</th>
<th>16-cells embryo</th>
<th>32-cells embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Types of culture media</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCM – 199</td>
<td>96</td>
<td>12.40</td>
<td>0</td>
<td>20</td>
<td>33.33</td>
<td>26.66</td>
<td>13.33</td>
<td>6.66</td>
</tr>
<tr>
<td>Earl’s + TCM-199</td>
<td>99</td>
<td>14.88</td>
<td>46.66</td>
<td>20</td>
<td>20</td>
<td>13.33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medi – Cult. IVF</td>
<td>80</td>
<td>16.36</td>
<td>23.07</td>
<td>15.38</td>
<td>23.07</td>
<td>23.07</td>
<td>15.38</td>
<td>0</td>
</tr>
<tr>
<td>Ham’s F-10</td>
<td>88</td>
<td>56.79</td>
<td>8.00</td>
<td>18.00</td>
<td>16.00</td>
<td>34.00</td>
<td>20.00</td>
<td>4.00</td>
</tr>
<tr>
<td>RPMI - 1640</td>
<td>82</td>
<td>63.62</td>
<td>12.06</td>
<td>25.86</td>
<td>25.86</td>
<td>20.68</td>
<td>13.79</td>
<td>1.75</td>
</tr>
<tr>
<td><strong>Degrees of temperature (°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>82</td>
<td>63.62</td>
<td>12.06</td>
<td>25.86</td>
<td>25.86</td>
<td>20.68</td>
<td>13.79</td>
<td>1.75</td>
</tr>
<tr>
<td>38</td>
<td>63</td>
<td>12.71</td>
<td>62.50</td>
<td>25.0</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>92</td>
<td>7.14</td>
<td>85.72</td>
<td>14.28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Acidity (pH) of culture medium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.9 – 7.0</td>
<td>80</td>
<td>2.54</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.3 – 7.4</td>
<td>82</td>
<td>63.62</td>
<td>12.06</td>
<td>25.86</td>
<td>25.86</td>
<td>20.68</td>
<td>13.79</td>
<td>1.75</td>
</tr>
<tr>
<td>7.6 – 7.7</td>
<td>80</td>
<td>14.76</td>
<td>16.66</td>
<td>33.33</td>
<td>16.66</td>
<td>33.33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.0 – 8.1</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

These results may mean that the constituents of two culture media conserve and regulates the microenvironment with balanced and non-static state, and then support normal embryonic development. Trounson and Caro (32) remarked that the successful human and animal IVF is dependent on maintenance of suitable culture conditions for gametes and early developed embryos. Certainly, the presence of adequate number of normal embryos is very important for embryo transfer and successful implantation and gestation (33).

Best and significant (P<0.005) results for IVF and normal embryonic development were achieved within culture medium at temperature degree 37 °C as compared to other degrees of temperature were used. In contrast, least percentage for IVF was observed with culture medium at temperature degree 39 °C Figure (2).
Figure (2): Effect of different temperatures (°C) on the percentages of *in vitro* fertilization and embryonic abnormality in mice

* : Significantly (P<0.005) different as compared to other corresponding groups.
# : Significantly (P<0.05) different as compared to groups of temperature (36 and 38) °C.

Mahi and Yanagimachi (12) reported that the high percentage of sperm hyperactivation and acrosomal reaction were occur at 37 °C, while, sperm require long period to perform sperm hyperactivation and acrosomal reaction within low temperature of culture medium (34). However, the degree of temperature is highly affect the molecular state of the membrane during sperm-oocyte fusion (13), as well as, pre-, peri- and post-events of sperm-oocyte binding (15). Moreover, there is evidence that high temperature can change the composition of phospholipids and reduced protein synthesis within cytoplasm of mouse and cattle oocytes (35).
Elevation of temperature of culture medium negatively affected the fluidity of the plasma membrane of sperm and oocyte, alterations in levels of CO$_2$ solubility and acidity within culture medium for *in vitro* fertilization and embryonic development and lastly mitotic divisions of early embryonic stages (15). Baumgartner and Chrisman (36) observed that the raise in the temperature of culture medium during *in vitro* incubation has direct impact on activities and functions of oocytes and embryos through reduction of enzymatic activity, and subsequently reduction of protein synthesis requisite for embryo growth and development to advanced stages (37). Furthermore, an increased temperature causes reduction in the level of CO$_2$ solubility within culture medium, which leads to raise acidity of culture medium and suppression of enzymatic activity and anabolism within cytoplasm of oocytes and embryos (35).

It was noticed that the best progressiveness for *in vitro* embryonic development within culture medium at temperature degrees (36 and 37 °C). Meanwhile, at degrees of temperature (38 and 39 °C) were negatively affects the growth of mouse embryos Table (1). These results are in agreement with two reports (37,38). They reported that the culture of embryos at high temperatures reduce embryonic development. It is known that the several proteins produced within cytoplasm of embryonic blastomeres have a defense role against slight elevation of temperature. However, high degrees of temperature may be over the defense role of these proteins. Early embryonic development is highly affected by the wide fluctuation of temperature of culture medium, especially with high elevation of temperature. An increased temperature of culture medium causes abnormal division of embryonic blastomeres during mitotic division. In addition to sensitivity of embryonic genome, formation of mitotic spindle is highly sensitive to high temperature (16).

In general, great percentages for IVF and normal embryonic development were achieved within culture medium at narrow range of pH: 7.3-7.4. While in pH: 8.0-8.1 of culture medium, embryonic development obtained in IVF were zero Figure (3).
Figure (3): Effect of different pH of culture medium on the percentages of *in vitro* fertilization and embryonic abnormality in mice

* : Significantly (P<0.01) different as compared to other corresponding groups.
# : Significantly (P<0.01) different as compared to groups of pH (6.9-7.0 and 8.0-8.1).

The percentage of IVF within culture medium at pH: 7.6-7.7 better than pH: 6.9-7.0, but the percentage of abnormal embryonic development within culture medium at acidity (pH: 6.9-7.0) better than the acidity (pH: 7.6-7.7) Figure (3). In the present study, optimum growth of mouse embryos was documented in culture medium with pH: 7.3-7.4. In contrast, bad or no results for *in vitro* embryonic development were observed in culture medium with pH: 6.9-7.0 and (pH: 8.0-8.1); respectively Table (1). It is mentioned that the optimum acidity for oocyte maturation and fertilization between (pH: 7.3-7.5) (39). However, Jainudeen *et al.* (31) certified that the best results for IVF and embryonic development are achieved with acidity of culture medium between (pH: 7.2-7.6). Altitude acidity of culture medium yielded increased rate of polysperm fertilization, and subsequent an increased percentage of abnormal embryonic development (40).
Alterations in pH of culture medium used for IVF may be negatively affect the final maturation of oocyte, sperm capacitation and acrosomal reaction, formation and fusion of male and female pronuclei and lastly spindle formation during early mitotic divisions (12,40). All these processes considered the essential requirements for successful sperm-oocyte binding and IVF (41). With the other words, acidity (pH) of culture medium is highly affects the regulation of macromolecules of plasma membrane and metabolism of sperm, as well as motility and viability of sperm. Further, technique of IVF was achieved under standard micro-environmental conditions, which mimics in vivo fertilization (42,43).

In conclusion, the mouse spermatozoa and oocytes are sensitive to changes in the environmental conditions. In addition, conditions of culture medium and its selection are may be the main limited factor for improvement in vitro fertilization and embryonic development in mice.
REFERENCES

3. History of In-Vitro Cell Culture (2002). History of In-Vitro Cell Culture (What is in-vitro cell culture all about?) Internet file.


