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Use of a medium buffered with n-hydroxyethylpiperazine-n-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization

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Objective: This study was conducted to determine whether N-hydroxyethylpiperazine-N-ethanesulfonate (HEPES)—buffered medium used for the microinjection of sperm into oocytes may be detrimental for the embryo.

Design: Controlled randomized study.

Setting: Private IVF center.

Patient(s): Women (n = 708) undergoing ICSI.

Intervention(s): The women were randomized into two study groups: 2,204 oocytes from 357 women were treated using a medium buffered with bicarbonate without HEPES during the ICSI procedure, and 2,168 oocytes from 351 women were treated using a medium buffered with HEPES during the ICSI procedure.

Main Outcome Measure(s): Fertilization rate, degeneration rate, triploid rate, cleavage rate, embryo quality, pregnancy rate, implantation rate, and abortion rate.

Result(s): Oocytes treated with a HEPES-buffered medium showed a statistically significant higher rate of triploid and degenerated oocytes after fertilization with ICSI compared with oocytes treated with a medium without HEPES. The embryos obtained from oocytes microinjected with a HEPES-buffered medium showed a statistically significant higher rate of highly fragmented embryos compared with the controls. Pregnancy rate and implantation rate were statistically significantly lower in the patient group with oocytes treated with the HEPES-buffered medium. The other parameters evaluated did not show any statistically significant differences.

Conclusion(s): Our study showed that the use of media buffered with HEPES, during the microinjection of sperm into the oocytes, is detrimental for IVF outcome and should be avoided. (Fertil Steril® 2006;xx:xxx. ©2006 by American Society for Reproductive Medicine.)

Key Words: HEPES, intracytoplasmic sperm injection, IVF outcome, embryo quality

The widespread use of assisted reproductive techniques has highlighted the role of the culture media, which have to allow human embryos to arrive at the blastocyst stage (1–4). Furthermore, it has been shown that HEPES may promote cell fragmentation in the early cleavage stage embryo (5). For these reasons the media generally used in IVF for human embryos are manufactured without HEPES and phosphate buffers (3, 4, 15).

In the microinjection of sperm into the oocyte during intracytoplasmic sperm injection (ICSI), sperm selection is generally performed using a medium buffered with HEPES (16). Long standing time of gametes under the microscope for the procedure may change the pH of the medium, and the presence of an organic pH buffer may help to maintain the pH in a stable state.

However, the use of these substances may be detrimental to embryo development after fertilization, especially with the direct injection of an organic buffer into the cytoplasm of oocytes as shown in an early report (17). In that study the authors reported that the HEPES injected into the oocyte determined a rise in intracellular pH with a reversible meiotic arrest.

For these reasons we hypothesized that, because the ICSI procedure can be performed in a few minutes by experienced
embryologists, avoiding HEPES in the medium used for microinjection may improve the IVF outcome.

In this study we tested with a controlled trial whether avoiding the use of a medium buffered with HEPES during ICSI may be useful for embryo development after fertilization.

**MATERIALS AND METHODS**

All patients referred to Biroma, Rome, Italy, undergoing their first ICSI cycle from January 2002 to December 2003 were eligible for the study. The study was reviewed and approved by the institutional review board.

The patients were randomly assigned by a computer-generated number sequence to the two arms of the study: one in which a medium buffered only with bicarbonate, not containing HEPES (IVF; Vitrolife, Kungsbacka, Sweden), was used for the ICSI procedure and other procedures, including sperm washing and oocyte collection, and the other in which the medium with HEPES (Gamete; Vitrolife) was used during ICSI.

A total of 357 cycles were included in the group treated without HEPES, and 351 in the control group with HEPES. For patients attending more than one cycle of IVF, only the first cycle was included in the study. Each patient was included in only one arm of the study. Azoospermic patients were excluded from the study.

Controlled ovarian hyperstimulation was performed according to the long protocol in all patients. Subcutaneous buserelin, 0.4 mg daily, on days 22 to 24 of their previous cycle was administered to the patients. When suppression was confirmed by E2 and ultrasound examinations, FSH was commenced at 300 IU daily (Gonal-f; Serono, Rome, Italy) on the second day of the menstrual cycle.

From the seventh day of stimulation in both groups, daily monitoring of follicle size by ultrasound was performed, and plasma levels of E2 were measured. From this stage the dose of FSH was adjusted depending on the individual response of each patient.

The criteria used for triggering ovulation with 10,000 IU hCG (Gonasi HP 5000, AMSA, Rome, Italy) IM were plasma E2 between 1,000 and 4,500 pg/mL and at least four follicles >16 mm diameter. The cycle was canceled in case of poor ovarian response, when less than three follicles were observed on the ninth day, or in case of ovarian hyperstimulation syndrome, E2 >4,500 pg/mL.

Oocyte retrieval was performed under ultrasound control by the transvaginal route on day 0, 36 h after the injection of hCG. ICSI was performed in all cases according to published procedures (16). Oocytes were observed 18 h after ICSI for their pronuclei. The embryologists performing ICSI were blind to the solution used.

The embryos obtained were categorized on day 3 into three categories, depending on their morphologic appearance. Grade A had 6–8 or more equal and regular blastomeres without the presence of cytoplasmic fragments; grade B had less than 6–8 unequal blastomeres with or without cytoplasmatic fragments; and grade C were fragmented (more than 50%) embryos (18).

The embryos were transferred 72 h after insemination using the Wallace embryo transfer catheter (H. G. Wallace, UK). All transfer procedures were performed by the same physician to avoid inter-operator variability.

All pregnancies were confirmed by a rising titer of serum β-hCG from 12 days after embryo transfer and ultrasound demonstration of the gestation sac 4 weeks after the transfer. Biochemical pregnancies alone have not been included.

The same luteal phase support was used in both groups: 50 mg daily progesterone (Prontogest; AMSA) intramuscularly from the day of replacement.

### Statistical Analysis

All statistical analyses were performed using the SPSS statistical package (SPSS, Chicago, IL). Pregnancy rate and implantation rate were the primary outcomes, and secondary outcomes were days of stimulation, E2 at the day of hCG, number of oocytes collected, fertilization rate, degeneration rate, number of triploid embryos, number of embryos obtained, quality of embryos, number of embryos transferred, and abortion rate.

Mann-Whitney U test and Student t test were used for continuous variables (days of stimulation, E2 at the day of hCG, amount of FSH administered, number of oocytes collected, number of embryos obtained, and number of embryos transferred), and Fisher exact test was used for dichotomous variables (clinical pregnancy rate, implantation rate, fertilization rate, quality of embryos, degeneration rate, number of triploid embryos, and abortion rate).

A sample size analysis a priori was done for a difference of 20% between the two groups in pregnancy rate. The statistical results of P obtained were corrected for the number of comparisons performed.

### RESULTS

In Table 1 the epidemiologic data of the two patient groups are reported. No statistically significant differences were found between the two study groups. The two groups were homogeneous for female age, male age, time of infertility, and frequency of infertility causes.

In Table 2 the clinical data of controlled ovarian hyperstimulation are reported. No statistically significant differences were found between the two study groups for days of stimulation, E2 levels at the hCG day, FSH amount administered to the women, number of oocytes recovered, metaphase two rate, or immature oocytes rate.
In the group of 357 patients without HEPES, ICSI was performed on 2,204 oocytes and normal fertilization was observed in 69.9%, and in the control group of 351 patients ICSI was performed on 2,168 oocytes with a normal fertilization rate of 69.2%; no statistically significant differences were found. The degenerated oocytes rate after ICSI was 6.5% in the group without HEPES and 8.8% in the control group (P < .0001). Triploid embryo rate was 3.3% in the group without HEPES and 6.1% in the control group (P < .0001). No differences were observed for embryo cleavage rate between the two groups. The frequency of bad-quality embryos with high fragmentation was statistically significantly lower in the group without HEPES, 30.2%, compared to the control group, 37.5% (P < .0001). The embryos graded B were statistically significantly higher in the group without HEPES, 46.3%, compared to controls, 39.4% (P < .0001). The rate of top-quality embryos obtained and replaced in uterus were similar in both groups.

The pregnancy rate and implantation rate in the group without HEPES were statistically significantly higher than in the control group (37.8% vs. 28.2%; P < .01; 18.3% vs. 12.3%; P < .01). No differences were observed for the number of embryos transferred, top-quality embryo rate transferred, or abortion rate between the two groups. The data are reported in Table 3.

**DISCUSSION**

In recent years the culture media for IVF have been improved to respond to the needs of human embryos during the first stages of development. With the introduction of sequential media, each step from fecundation to the blastocyst embryo stage development has a specifically designed medium without a phosphate buffer and with only bicarbonate buffer and different concentrations of pyruvate lactate and glucose (1–4).

In the ICSI procedure, to avoid an excessive pH change during micromanipulation under the microscope, a medium buffered with HEPES, an organic pH buffer, is generally used. However, it is well known that this organic pH buffer may be detrimental to the zygote and during early embryo cleavage stages and may be toxic to embryo development (5–14). An early report showed that the injection of HEPES into starfish eggs inhibited meiosis reinitiation (17).

It has been shown that a concentration of 25 mmol/L HEPES in the culture medium is detrimental to embryo development in bovines (19). Several authors showed the detrimental role of HEPES in the media used for fertilization (8, 9, 11). Furthermore, the role of HEPES in promoting embryo fragmentation and retarded development has also been reported (5, 20–22).

The ICSI procedure was introduced in 1991 (16), and now experienced embryologists can perform it in a reduced time, avoiding the oocytes’ prolonged exposure to light and a different environment from the incubator. For these reasons we have evaluated the possibility of using a medium without HEPES during the ICSI procedure.

The data of our study showed that the use of a medium without HEPES during the ICSI procedure permits a statis-
A well trained embryologist proficient in microinjection can perform ICSI in a few (3–5) minutes, therefore avoiding a long standing time of the gametes under the microscope outside the incubator. This permits the use of a medium buffered with bicarbonate, without HEPES, for all the steps of ICSI, from fertilization throughout the embryo culture, without the stress of changing the medium.

In some cases after microinjection we measured the pH of the medium used for the micromanipulation, and we did not observe significant differences between the HEPES-buffered medium and the bicarbonate only–buffered medium.

Furthermore, the policy in our center is to microinject no more than ten eggs per patient, and in this study we excluded patients undergoing testicular sperm extraction, because in these cases finding sperm in the testicular tissue may require that eggs stay for a longer time under the microscope. In these cases we still use a HEPES-buffered medium, even though for all other cases we do not routinely use this medium.

Even though more studies are needed to elucidate the effects of the HEPES buffer on embryo development, our data suggest that the use of HEPES-buffered media should be reconsidered, especially in established IVF programs with

### Table 3

Results of comparison between ICSI cycles using during microinjection medium with HEPES and without.

<table>
<thead>
<tr>
<th></th>
<th>Without HEPES</th>
<th>Control</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes treated</td>
<td>2,204</td>
<td>2,168</td>
<td>—</td>
</tr>
<tr>
<td>No. of oocytes fertilized</td>
<td>69.9%</td>
<td>69.2%</td>
<td>NS</td>
</tr>
<tr>
<td>Triploid</td>
<td>3.3%</td>
<td>6.1%</td>
<td>.0001</td>
</tr>
<tr>
<td>Degenerated</td>
<td>6.5%</td>
<td>8.8%</td>
<td>.007</td>
</tr>
<tr>
<td>No. of embryos cleaving (%)</td>
<td>68.1%</td>
<td>65.1%</td>
<td>NS</td>
</tr>
<tr>
<td>Embryos obtained</td>
<td>4.2 ± 2.7</td>
<td>4.0 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Embryo grading:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade A (%)</td>
<td>348 (23.4)</td>
<td>323 (23.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Grade B (%)</td>
<td>688 (46.3)</td>
<td>553 (39.4)</td>
<td>.001</td>
</tr>
<tr>
<td>Grade C (%)</td>
<td>450 (30.2)</td>
<td>525 (37.5)</td>
<td>.0001</td>
</tr>
<tr>
<td>Embryos transfered</td>
<td>2.5 ± 0.8</td>
<td>2.6 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Top-quality embryo</td>
<td>36.0%</td>
<td>33.8%</td>
<td>NS</td>
</tr>
<tr>
<td>Pregnancy rate</td>
<td>37.8%</td>
<td>28.2%</td>
<td>.01</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>18.3%</td>
<td>12.3%</td>
<td>.01</td>
</tr>
<tr>
<td>Abortion rate</td>
<td>14.8%</td>
<td>15.1%</td>
<td>NS</td>
</tr>
</tbody>
</table>

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The detrimental role of HEPES in embryo development and IVF outcomes observed in our study may be due to the increase of intracellular pH caused by this organic buffer when injected into the oocytes. Several authors reported that HEPES significantly increases intracellular pH (14, 20, 21), which is associated with the inhibition of meiosis reinitiation (17). The alteration of meiosis phases may determine damage in the spindle functions, with abnormal chromosome segregation during the second meiotic division after fertilization, and in the first embryo mitotic division, with the increase of embryos with chromosomal aberration.

The significant increase of triploid embryos when a medium with HEPES was used with ICSI is intriguing. Triploid embryos in ICSI are mostly due to a nondisjunction and extrusion of the second polar body. This may be due in our case to an altered function of the cytoskeleton and meiotic spindle after oocyte activation and fertilization. We can speculate that the injection of HEPES inside the oocyte may modify its function, increasing cellular pH and in some cases enhancing triploid embryo.

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experienced embryologists, in order to obtain a better embryo development after ICSI procedure.

REFERENCES


Use of a medium buffered with n-hydroxyethylpiperazine-n-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization


Rome, Italy

The use of a medium buffered with N-hydroxyethylpiperazine-N-ethanesulfonate for microinjection of sperm into oocytes is detrimental to the outcome of in vitro fertilization when compared with a medium buffered only with bicarbonate.
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