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ORIGINAL ARTICLE

Mechanical Agitation During the *in vitro* Culture of Human Pre-Implantation Embryos Drastically Increases the Pregnancy Rate

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SUMMARY

Background: **The *in vivo* developing embryo is naturally exposed to constant vibrations of around 6 Hz, increasing to 20 Hz when the oviductal fluid is mechanically agitated by the cilia. This study examines the effects on viability of subjecting human pronuclear oocytes and embryos to mechanical agitation during their *in vitro* culture before transplantation.**
Methods: **Metaphase-II oocytes were ICSI/IVF with morphologically normal spermatozoa and then divided into two groups according to whether the cells underwent mechanical agitation (20 Hz over 5 seconds once per hour) of the culture medium (Group 2, n = 23) or were cultured without mechanical agitation (Group 1, n = 23). The fertilization rate of oocytes was recorded 18 hours later. Embryo development was monitored every day during the whole period of *in vitro* culture up to the embryo transfer on day 3, 4 or 5.**
Results: **Pregnancy rates after the transfer of 3 Day embryos in Group 1 and Group 2 were 50 % and 80 %, and of 5 Day embryos in Group 1 and Group 2 were 36 % and 73 %, respectively.**
Conclusions: **The *in vitro* culture of human embryos in a medium subjected to regular short bursts of mechanical agitation drastically increases their development rate.**
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KEY WORDS

Embryo, *in vitro* culture, fertilized oocytes, mechanical vibration, pregnancy

INTRODUCTION

The *in vitro* culture of pre-implantation embryos has become a routine procedure that is widely used for *in vitro* fertilization in humans (1, 2), domestic (3, 4), wild (5) and laboratory (6) animals, and fish (7). To optimize artificial *in vitro* culture conditions by simulating conditions in the oviduct and uterus, multi-component nutrition media are used in an atmosphere of 5 to 10 % CO_2, and the whole culture system is warmed to body temperature.

Early attempts to culture cleavage stage rodent embryos in a complete *in vitro* system were made with limited success (8, 9, 10, 11).
Attempts worldwide to improve the *in vitro* culture systems used have mostly centered on modifying the components of soluble media such as salts, energy or nitrogen sources, and growth factors / hormone supplements were quickly to become the main focus of such investigations (12, 13, 14, 15).
Regardless of the type of medium and culture volume, all culture systems can be described as a standard static culture system comprising a small (a few microliters) or not so small (up to 1000 µL) volume of culture medium (16, 17, 18).
However, this type of static microfluidic culture system has not been shown to improve pregnancy rates. Subsequent investigations addressing the use of a dynamic

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microfluidic culture system with continuous media perfusion for embryo culture have reported poor embryo development rates (19) due to the removal of autocrine growth-promoting factors (20). In a recent publication (21), a new microfunnel device that mimics body motions was found to overcome the negative effects of microfluidic culture systems (20, 22). This new culture system results in significantly improved blastocyst cell numbers, developmental stage blastocysts, percentages of hatching or hatched blastocysts, and implantation and pregnancy rates (21).

However, despite such promising results, the procedure and microfunnel culture device is complex and would need to be simplified for routine use in IVF programs.

The present study was designed to assess the effects of subjecting human pronuclear oocytes and embryos to short bursts of mechanical agitation during *in vitro* culture.

MATERIALS AND METHODS

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA).

The work described here was performed at a private medical center (Endokrinologikum Ulm, Praxisklinik Frauenstrasse, Ulm, Germany). Couples were offered the choice of the *in vitro* culture of oocytes and embryos according to the standard routine or with mechanical agitation before transplantation.

Written informed consent was obtained from all the participating couples for the cryopreservation and culture of pronuclear oocytes and embryos. Patients with unexplained infertility were stimulated in the IVF-cycle (*in vitro* fertilization) or ICSI-cycle (intracytoplasmic spermatozoa injection) with triptorelin (Decapeptyl®; Ferring, Kiel, Germany) and recombinant FSH (Puregon®; Organon, Oss, The Netherlands) according to the 'long' protocol. Ovulation was induced by the administration of 10 000 IU of HCG (Pregnil®; Organon) and oocytes were retrieved 34 – 36 hours later and inseminated with the partner's sperm through conventional IVF and ICSI techniques.

Informed consent was obtained from 46 patients / couples aged 24 to 40 years (median age 35 $\pm$ 4.9) for the culture of pronuclear oocytes (two per patient) under two different conditions: Group 1 (23 patients, n = 46), without mechanical agitation of the culture medium; and Group 2 (23 patients, n = 46), with mechanical agitation (20 Hz delivered over 5 seconds once every hour). Mechanical agitation was achieved using the newly developed device NSSB-300 (Nepa Gene, Ichikawa, Chiba, Japan) (Figure 1). Embryo development rates were determined 18 hours later. The embryos were cultured in 50 µL of culture medium (Sage, Los Angeles, CA, USA) for three or five days before their transfer.

Embryo scoring was performed every day early in the morning. The embryo quality system used to grade the Day 2 and 3 embryos was that described by Steer et al. (23) as follows: Grade A, equal sized symmetrical blastomeres; Grade B, uneven blastomeres with < 10 % fragmentation; Grade C, (10 - 50 %) blastomeric fragmentation; and Grade D, (> 50 %) blastomeric fragmentation or pronucleate single cell embryos. Day 4 and 5 embryos were graded according to Veeck and Zaninovic (24).

Embryo transfer (two embryos per patient) was performed on Day 3 or Day 5 after oocytes' retrieval. In accordance with German legislation, the embryos were transferred regardless of their developmental stage, including degenerated and developmentally arrested embryos. Pregnancy was defined as an increase in serum hCG concentration (20 IU/L) determined 11 and 13 – 15 days after embryo transfer. Clinical pregnancy was recorded when the fetal sac was visualized on an ultrasound in gestational weeks seven to eight.

Statistical analysis

Results were expressed as means ($\pm$ SD) and percentages. Means for the groups were compared by analysis of variance while the χ^2-test was used to compare proportions. The level of significance was set at $p < 0.05$.

RESULTS

The mean number of oocytes retrieved per patient was 7 $\pm$ 3.4 (range 2 to 21).

The rates of pronuclei formation indicated no effects of the static (69 $\pm$ 2.5 %) and dynamic (70 $\pm$ 2.0 %) *in vitro* culture systems on the fertilization rates of the oocytes ($p > 0.05$).

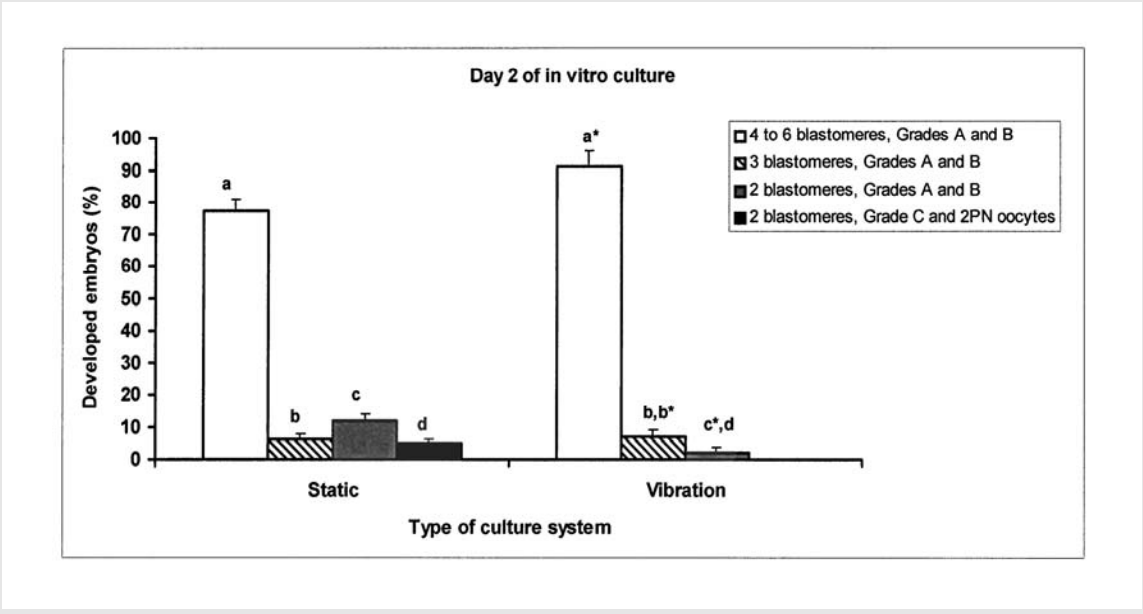
Figure 2 shows the effects of the two culture systems on embryo development recorded after 3 or 5 days of *in vitro* culture.

Figure 2A provides the data recorded on the second day of *in vitro* culture. These data reveal that under the dynamic culture conditions, a significantly higher percentage of excellent (Grade A) and good (Grade B) quality embryos at the 4 to 6 blastomere stage was observed compared to the static culture system (91 $\pm$ 4.9 % vs 77 $\pm$ 3.8 %, $p < 0.05$). Also noted was a non-statistical increase (10 %) in the number of 2-blastomere Grade A and Grade B embryos under conditions of static compared to dynamic culture (12 $\pm$ 1.9 % vs 2 $\pm$ 1.6 %, $p > 0.05$). Additionally, fertilized oocytes rendering two-cell Grade C embryos arrested at the 2 PN-stage were only observed in the static culture system though, once again, this difference lacked significance ($p > 0.05$).

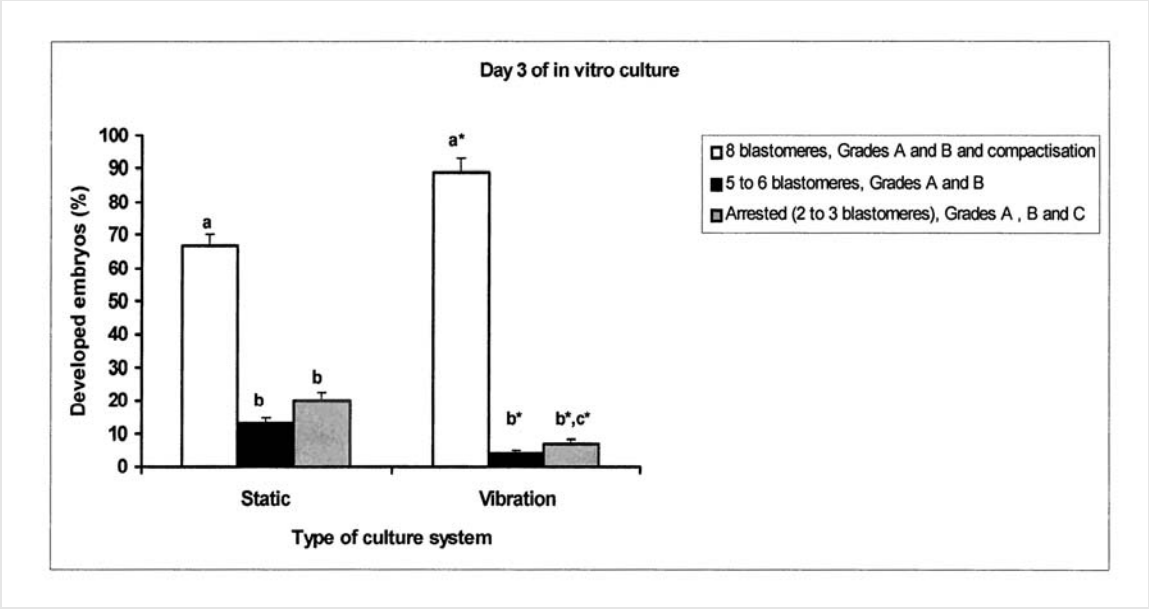
The data recorded for the *in vitro* cultured Day 3 embryos are provided in Figure 2B. These data reveal the positive influence of the dynamic *in vitro* culture system on the developmental competence of these embryos. Thus, the percentage of Grade A and Grade B 8-cell embryos obtained under dynamic compared to static

Figure 1. The NSSB-300 device (Nepa Gene, Ichikawa, Chiba, Japan) used for the mechanical agitation of the *in vitro* culture system. Petri dishes contain the fertilized oocytes, and embryos are placed in the machine's CO_2 incubator. Scale bar = 5 cm.

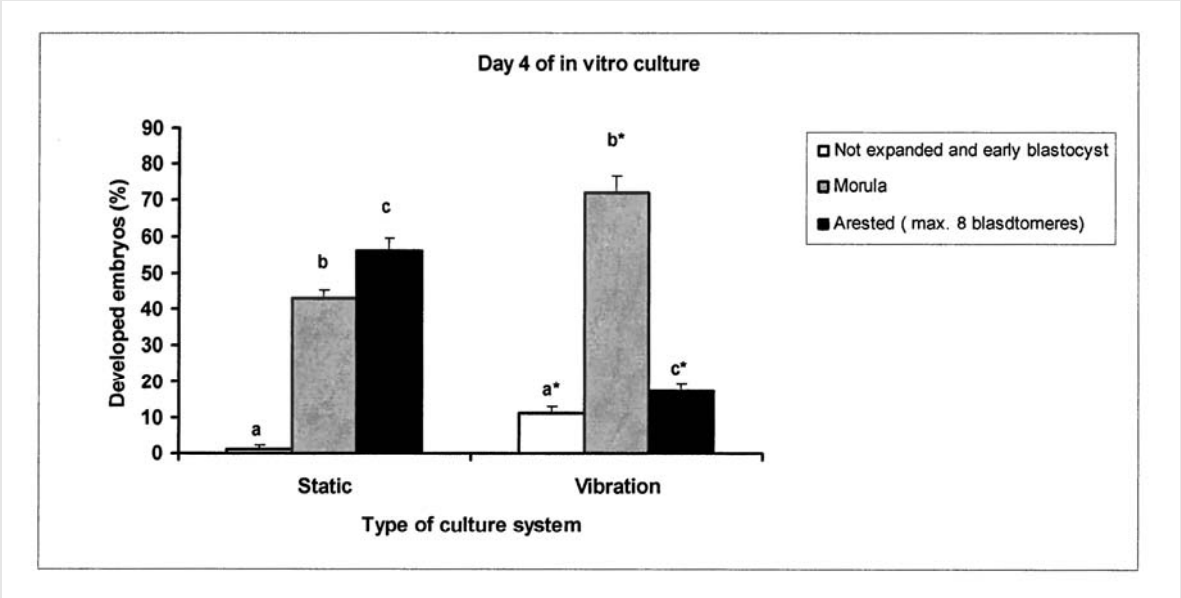
2A



2B


Day 3 of in vitro culture
Developed embryos (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
a*
b*
b*,c*
Static
Vibration
Type of culture system
8 blastomeres, Grades A and B and compactisation
5 to 6 blastomeres, Grades A and B
Arrested (2 to 3 blastomeres), Grades A , B and C

2C


Day 4 of in vitro culture
Developed embryos (%)
90
80
70
60
50
40
30
20
10
0
a
b
c
a*
b*
c*
Static
Vibration
Type of culture system
Not expanded and early blastocyst
Morula
Arested (max. 8 blasdtomeres)

2D

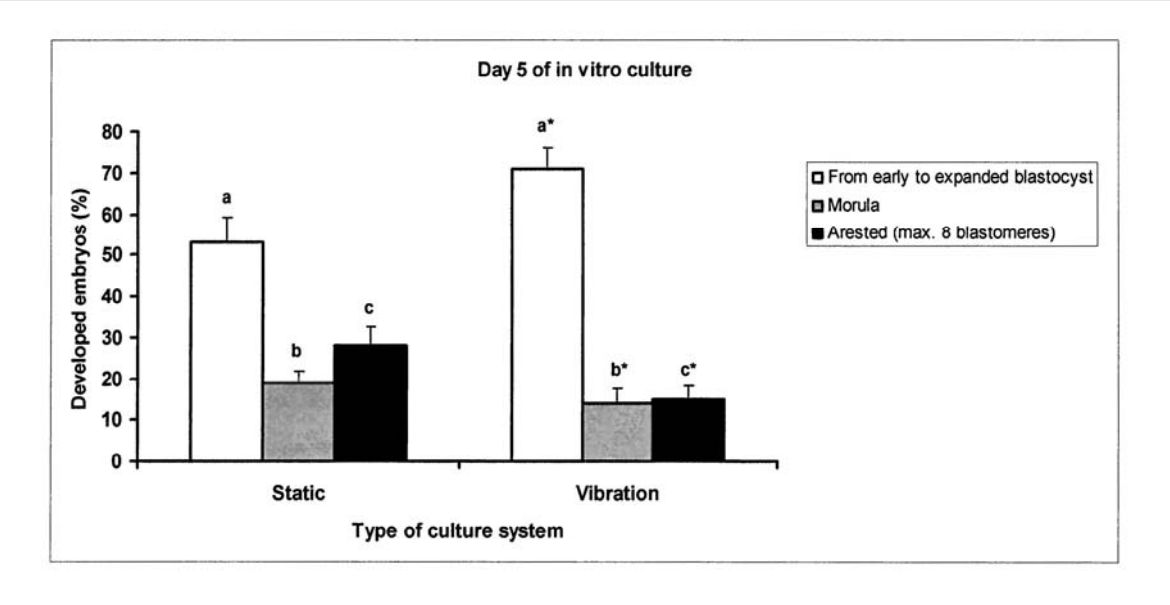


Figure 2. Effects of mechanical agitation on embryo development rates. (A) Day 2, (B) Day 3, (C) Day 4, and (C) Day 5 of *in vitro* culture. Values with different subscripts differ significantly ($p < 0.05$).

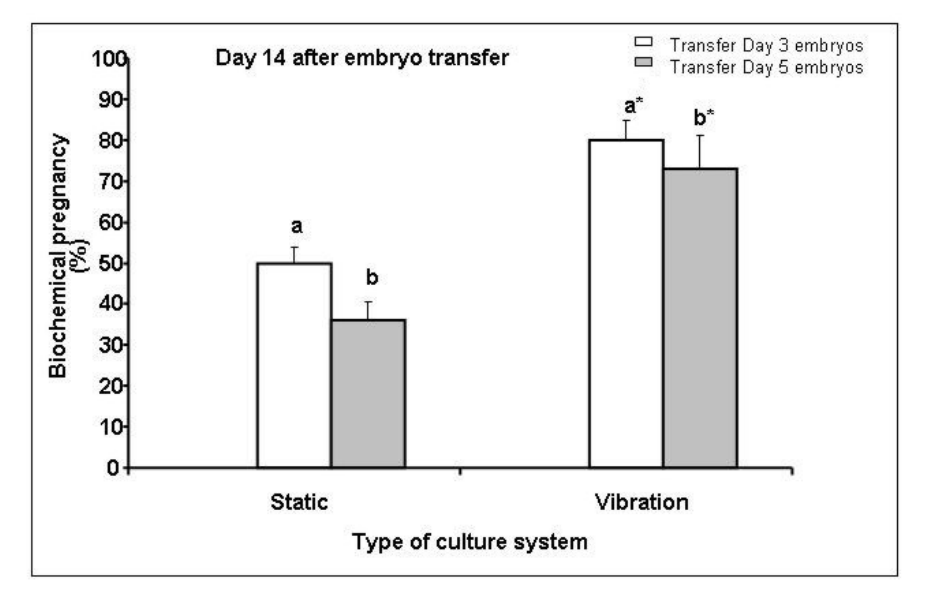


Figure 3. Biochemical and clinical pregnancy rates depending on the type of culture system. Values with different subscripts differ significantly ($p < 0.05$).

culture conditions was significantly higher by 22 % (67 ± 3.1 % vs 89 ± 4.2 %, $p > 0.01$). This observation was accompanied by a non-significant 9 % increase in the number of Grade A and B 5-cell and 6-cell embryos in the static cultures (13 ± 1.7 % vs 4 ± 0.9 %, $p > 0.05$). The proportion of developmentally arrested embryos (2 and 3 blastomere Grade A, B and C embryos) was significantly higher for the static culture system (20 ± 2.6 % vs 7 ± 1.3 %, $p < 0.05$).

Figure 2C provides the data recorded for the Day 4 embryos. Under the dynamic culture conditions, the percentage of embryos at different blastocyst stages was higher than that recorded for the static culture system (11 ± 1.9 % vs 1 ± 1.1 %, $p > 0.05$), including a small proportion (3 %) of non fully-expanded blastocysts. The difference between the percentages of embryos at the morula stage observed under the dynamic and static culture conditions was highly significant (72 ± 4.8 % vs 43 ± 2.4 %, respectively, $p > 0.01$). The percentage of embryos with signs of developmental arrest (8-cell or fewer-cell embryos) was significantly higher for the static culture system compared to the dynamic culture system (56 + 3.6 % vs 17 ± 2.1 %, respectively, $p < 0.05$).

The data recorded on the fifth day of *in vitro* culture are shown in Figure 2D.

It may be observed that the percentage of embryos at the blastocyst stage (from hatched to early blastocyst) was significantly higher for the dynamic than the static system (71 ± 4.9 % vs 53 ± 6.1 %, $p < 0.01$). Under static culture conditions, significantly higher rates of developmentally arrested 2- to 8-blastomere embryos were obtained (28 ± 4.6 % vs 15 ± 3.2 %, $p < 0.05$).

Figure 3 provides the biochemical and clinical pregnancy rates recorded after embryo transfer. Thus, the *in vitro* culture under dynamic conditions resulted in a signifycantly higher pregnancy rate independent from day of embryo transfer. So, on the third day of embryo transfer the dynamic embryo culture resulted in pregnancy rates 30 % higher (80 % vs 50 %, respectively) and on the fifth day 37 % higher (73 ± 5.8 % vs 36 ± 4.9 %, respectively, $p < 0.01$) compared to static embryo culture.

DISCUSSION

Conventional assisted reproductive technologies involve the use of static culture systems. Notwithstanding, in natural conditions (*in vivo*) the egg, sperm, and embryo are subjected to continuously changing dynamic processes. In the popular system whereby embryos are cultured in small droplets, for example, toxic substances such as oxygen-derived radicals (25) and ammonia (26), with known detrimental effects on embryos (27), are likely to build up and concentrate. The main principle of such a culture system is refreshment of the medium. However, although periodic media changes may prevent toxins from accumulating, this manipulation leads to an imbalance with the elimination of beneficial auto- and paracrine factors (28).

In natural dynamic conditions, the development of the embryo as it passes through the oviduct is accompanied by the constant removal of metabolites, gas exchange, and exposure to numerous factors, absent *in vitro*, that mediate maternal-embryonic communication (20, 29). This difference in conditions could be responsible, at least in part, for the impaired *in vitro* development and viability of *in vitro* cultured preimplantation embryos (30).

In a static culture system, the only thing we can do to prevent the deleterious effects of metabolites on the embryo is to replace the culture medium with fresh medium. The mammalian preimplantation embryo is a relatively autonomous system. Such a self-controlling system can regulate its own cell division and differentiation without being in contact with the maternal reproductive tract (31). Thus, *in vitro* this self-controlling system produces diffusible embryotrophic paracrine / autocrine factors, such as PAF (platelet-activating factor), which are in part responsible for the regulation of early embryo development (32). The data from this study shows that the distance between individual bovine embryos in culture affects preimplantation development, especially blastocyst formation, cell number, and metabolism. During *in vitro* culture, when refreshing the culture medium, besides unwanted embryo-metabolites, we also remove embryotrophic paracrine / autocrine factors.

In nature, all fertilization steps (receiving the ovulated oocytes, providing a suitable environment for fertilization) and embryo development with subsequent transport to the uterus take place inside the fallopian tube. This multifunctional organ is mechanically active and undergoes two types of movement: muscular and cellular. The movement of the ovum changes to a complex pattern of rapid forward and backward motion associated with segmental contractions of the oviduct wall. The purpose of these muscular (peristaltic) and segmental muscular contractions may be to stir the tube's contents and ensure the mixing of gametes and embryos with tubal secretions (33).

After a meticulous search of published data on improving *in vitro* embryo culture, our goal was to develop a system that could offer the combined benefits of the microfunnel pulsatile and tilting embryo culture systems.

The first article reporting the benefits of pulsatile mechanical vibration for the cytoplasmic maturation of *in vitro*-matured pig oocytes was published by Miyoshi's group (34). These authors subjected oocyte cumulus complexes cultured in micro-drops to pulsatile mechanical vibration (PMV) at a frequency of 20 Hz for 5 seconds at intervals of 30 - 60 minutes, or for 10 seconds at intervals of 60 minutes. During *in vitro* maturation, PMV did not affect the proportion of oocytes reaching the metaphase-II stage. However, blastocyst formation rates after the activation of oocytes exposed to PMV were significantly higher ($p < 0.05$) than those

obtained for oocytes matured without mechanical vibration (26 - 28 % vs 12 % and 26 vs 16 %, respectively for the 5 seconds and 10 seconds pulses). The question we then asked ourselves was why these authors chose a vibration frequency of 20 Hz. The reply to this question is based on: (i) the significant increase in ciliary beating frequency of the human isthmus and ampoule produced after ovulation, that is, in the secretory phase (35, 36); (ii) the baseline frequency of ciliary beating varies among individuals from 5 to 20 Hz (37, 38).

The results of our study directly or indirectly support the findings of all these investigations. However, they are mostly consistent with the recent results of Heo (21) and Matsuura (39). Thus, we here report that the mechanical stimulation of embryos during *in vitro* culture with 100 Hz / 5seconds (20Hz / second) per hour significantly increased the developmental competence and quality of human embryos, compared to the standard static culture system, but did not influence the fertilization rate of the injected oocytes.

Under dynamic culture conditions, significant increases of 14 % among 4- to 6-cell embryos (Grades A and B); 22 % among 8-cell embryos (Grades A and B); 29 % among morula stage embryos; and 18 % among blastocyst stage embryos (hatched to early blastocysts) were obtained. If we compare the two culture systems (dynamic versus static) in terms of rates of arrested embryos, these were significantly lower overall in the dynamic system. On Day 2 of embryo culture, fertilized oocytes arrested at the 2 PN-stage were only present in the static system; on Day 3, a significantly higher rate (13 %) of arrested 2- and 3-cell embryos (Grades A, B and C) was recorded in the static system; on Days 3 and 4, significantly higher percentages (39 %) of embryos arrested at the 8- or fewer cell stages (Grades A, B and C) were recorded in the static system; and on Day 5, the percentage of arrested 8-cell or fewer-cell less embryos (Grades A, B and C) was significantly higher by 13 % again for the static culture system. Consistent with these results, pregnancy rates (biochemical) were significantly increased by 30 % and 37 % respectively after the transfer of Day 4 and Day 5 embryos cultured under dynamic conditions.

Thus, the main characteristic shared by the three perspective methods of *in vitro* culture discussed here (pulsatile dynamic microfunnel culture; tilting embryo culture, and mechanical vibration) is mechanical stimulation, which apparently induces cell to cell communication. We believe that further beneficial effects on embryo development of all these techniques are similar and consist from: refreshing of the medium surrounding the embryo, achieved by perfusion (21), tilting (39) or mechanical vibration (34), resulting not only in mixing of the embryo-surrounding medium and reducing concentrations of toxic embryo metabolites, but apparently have a multi-factorial influence of mechanical stimulation due to activation of intracellular cell to cell communication.

In conclusion, the *in vitro* culture of human embryos in a medium subjected to regular short intervals of mechanical agitation leads to drastically increased development rates. This type of treatment tries to mimic conditions in nature whereby oviductal fluid is mechanically agitated by the epithelial cilia.

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Declaration of Interest:

I certify that this material is original that no actual or potential conflict of interest in relation to this article exists.

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