DOI: 10.3727/096368916X693428

CT-1664 Provisionally Accepted 10/05/2016 for publication in

"Cell Transplantation"

In vitro Micro-Vibration Increases Implantation Rate after Embryonic Cell Transplantation

Vladimir Isachenko*, Karl Sterzik┼, Robert Maettner┼, Evgenia Isachenko*, Plamen Todorov[3#§], Gohar Rahimi*, Peter Mallmann*, Erwin Strehler┼, Igor Pereligin®, José Luis Alabart‡, Markus Merzenich¶

*University Maternal Hospital, Department of Obstetrics and Gynecology, Cologne University, Cologne, Germany

┼ Department of Reproductive Medicine, Christian-Lauritzen Institute, Frauenstr. 51, 89073 Ulm, Germany

§ Institute of Biology and Immunology of Reproduction, Sofia, Bulgaria

® IVF Center "Genesis Dnepr", Ukraine

‡ Servicio de Investigación Agroalimentaria (DGA), Zaragoza, Spain

¶ Praxisklinik, Schoenhauser Str. 3, 50968 Cologne, Germany

Running head: Human Embryonic Cells With Micro-Vibration

Address correspondence to Vladimir Isachenko, Dr.(SU), University Maternal Hospital, Department of Obstetrics and Gynecology, Cologne University, Kerpener Str. 34, 50931 Cologne, Germany

Tel: +49-221-4784924; Fax: +49-221-4785247; E-mail: v.isachenko@yahoo.com

Abstract:

In natural conditions the oocyte and embryo are subjected to ever changing dynamic processes. However, the routine assisted reproductive technologies today involve the use of static in vitro culture systems. Objective was to determine whether there is any difference in the viability of embryos after in vitro culture under static and mechanical micro-vibration condition. It was evaluated the viability of embryonic cells (9,624 embryos) generated from 4,436 couples after in vitro culture. For Groups ≤29 years, 30-34 years, 35-39 years and ≥40 years, the following rate of high quality embryos without fragmentation (2 to 4 blastomeres on Day 2; 6 to 8 blastomeres and compacting morula on Day 3; blastocyst, expanded and hatching blastocyst on Day 5) was detected (static vs. vibration, respectively): 65% vs. 71%, 44% vs. 69%, 67% vs. 76% (for statistic significant differences between respective rates in these three groups $P<0.05$) and 67% vs. 66% ($P>0.1$). The following baby-take-home rate was detected for Groups ≤29 years, 30-34 years, 35-39 years and ≥40 years, (static vs. vibration, respectively): 30% vs. 31% ($P>0.1$, increasing only on the level of tendency), 28% vs. 37%, 23% vs. 29% and 9% vs. 15% (differences between respective rates in these three groups with $P<0.05$). It was concluded that in vitro culture of embryos under micro-vibration (with a mimic of conditions in nature whereby oviductal fluid is mechanically agitated by the epithelial cilia) significantly increases baby-take-home rate for patients of 30 years and older.

Keywords: Embryonic cells transplantion, In vitro culture, Micro-vibration, Baby-take-home rate.

INTRODUCTION

Infertility is the inability of a person to reproduce by natural means. It is usually not the natural state of a healthy adult organism[1]. In that way infertility is a disease in essence. A reasonable question is about presence of technology of cell transplantation for the treatment of this disease. In fact, most effective method of treatment of sterility is obtaining and transplantation of embryonic cells (embryo).

Embryo transplantation refers to a step in the process of assisted reproduction in which embryos are placed into the uterus of a female with the intent to establish a pregnancy. This technique is often used in connection with in vitro fertilization (IVF). In vitro embryo culture is a component of in vitro fertilization where in resultant embryos are allowed to grow for some time in an artificial medium[1].

In vitro fertilization (IVF) is a process by which an oocyte is fertilized by sperm in vitro. The process involves monitoring and stimulating a woman's ovulation process, removing oocytes from the ovaries and letting sperm fertilize them in vitro. The fertilized oocytes are cultured for 2–6 days in a growth medium and are then transplanted in the uterus, with the intention of establishing a successful pregnancy. It is a technique of assisted reproductive technology for treatment of sterility[1].

All fertilization steps in nature (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. The tubal mucosa is arranged as longitudinal folds and consists of a single layer of cuboidal or columnar epithelium. The major cell types of this epithelium are secretory and ciliated cells[2], whose cilia have a vibrating, or beating[3]. Some investigations have shown an increase in ciliary beat frequency to 5.8 ±

0.3 Hz in the fimbrial section of the tube during the secretory phase[2] compared with the proliferative phase (4.9 ± 0.2 Hz).

What is known about micro-vibration in general? Vibration is a natural phenomenon that refers to mechanical oscillations about an equilibrium point. Since life began, the Earth has subjected all living things to a natural pulsation frequency. This natural phenomenon was predicted in 1952[(4)] and named the global electromagnetic resonance phenomenon or Schumann resonances. Schumann resonances are quasi-standing electromagnetic waves that exist in the Earth's 'electromagnetic' cavity (the space between the surface of the Earth and the ionosphere). Schumann resonances are the principal background in the electromagnetic spectrum between 3 and 69 Hz and appear as distinct peaks at extremely low frequencies of around 7.83 (strongest), 14, 21, 27, 39 and 45 (weakest) Hz. In daily life, this vibration could be 'desirable' (for example musical instruments), but more often is undesirable (wasting energy and creating unwanted sound – noise).

The stimulating effect of vibration on living systems is well known and plays a relevant role in mechanical transduction, which is essential for the survival of both cells and higher organisms.

A retrospective analysis of a cohort of ICSI patients attending a German private fertility center during about 2 years was done[5]. In vitro culture was performed either in a static environment with single oocyte/ embryo culture (n=291 patients) or under micro-vibration and group culture (n=244 patients). In the static group, oocytes/ embryos were cultured individually, while in the micro-vibration all the oocytes were cultured together and up to 4 embryos were cultured in the drop with a three dimensional vibration of 56 Hz for 5 s/60 min. Authors have observed a significant increase in fertilization rate in oocytes cultured in

groups and under micro-vibration conditions compared to oocytes cultured individually in a static culture (82% vs. 78%), as well as in the implantation rate (42% vs. 35%). in the same time, authors have noted that the clinical pregnancy rate showed a tendency to be higher in the micro-vibration group but it did not reach significance (47% vs. 43%)[5].

The aim of our investigations was to determine whether there is any difference in the viability of embryos after in vitro culture under static and mechanical micro-vibration condition.

MATERIALS AND METHODS

The authors confirm that all ongoing and related trials for this drug/intervention are registered. Clinical trial registration: ISRCTN13773904 "In vitro culture and transfer of human embryos "**.**

The current work was performed from January 2011 to December 2015 at a private medical center (Endokrinologikum Ulm, Praxisklinik Frauenstraße, Ulm, Germany, www.kinderwunsch-ulm.de). Permission was granted by the Ethical Commissions of Medical Faculties of university Ulm, Germany (permission 321/10-UBB/bal. from 12.11.2011) and university Cologne, Germany (permission 13-147 from 20.11.2013) for the in-vitro culture of embryos under mechanical micro-vibration. In vitro culture of embryos of each odd couple was performed in accordance with routine methodology in static system, without micro-vibration. Each even couple were offered the choice of the in-vitro culture of oocytes and embryos according to the standard routine or with mechanical agitation (micro-vibration) until transplantation. Written informed consent was obtained from all the participating couples. Eight participants were not agreed to perform the in vitro

culture of their embryos with micro vibration and that culture was performed in static "traditional" system.

All Patients with infertility were stimulated for in vitro fertilization-cycle (IVF) or intracytoplasmic spermatozoa injection-cycle (ICSI) with triptorelin (Decapeptyl®, Ferring, Kiel, Germany) and recombinant FSH (Puregon®, MSD Sharp & Dohme GmbH, Haar, Germany or Menogon®, MerckSerono GmbH, Darmstadt, Germany or Gonal-f®, MerckSerono GmbH, Darmstadt, Germany) according to the "short" protocol. Ovulation was induced by the administration of 5000 IU of HCG (Brevactid®, Ferring GmbH, Kiel, Germany) and oocytes were retrieved 34 – 36 hours later and inseminated with the partner's sperm through conventional IVF and ICSI techniques.

Patients were alternately assigned to the two embryo culture groups. Only two or three embryos per patient were cultured, as according to German law no more than three pronuclear oocytes/embryos from one patient (usually two) can be cultured in vitro and all cultured oocytes/embryos must be later transferred to the patient independently of the developmental rate of these embryos.

Oocytes for the culture of pronuclear embryos were obtained from 4436 informed patients aged n 26–44 years (median age 32.8). Pronuclear embryos (two or three per patient) were cultured in vitro under two different conditions: Group 1 (n = 4821), without mechanical agitation of the culture medium (standard routine conditions); and Group 2 (n = 4803), with mechanical agitation (44 Hz delivered over 5 s once every hour and acceleration (660 mV/g at 3.3 V: X = ±1.0g, Y = ±0.7g, Z = ±0.15g).

Mechanical agitation was achieved using the developed device Viboviduct 1500 (SimSoTec GmbH, Cologne, Germany, www.vibration-oviduct.com). This device before using was calibrated by measurement of vibration with special device PCE-VT 2700 (PCE Instruments UK Ltd., Southampton, U.K.). Viboviduct 1500 generates micro-vibrations on basis of special electric motor with low electromagnetic noise. The generated vibrations is forwarding directly to the plate with Petri dishes. Harmful high frequencies damping and smoothing by the intelligent control software developed on the microprocessor. The control software monitors the motor movements. Petri dishes with embryos are fixed on the plate. The device is designed and developed for use in CO_2-incubator.

Embryo development rates were determined on the day of transfer (Day 2, Day 3 or Day 5). The embryos were cultured in 50 µl of culture medium (Sage, Los Angeles, CA, USA) under mineral oil (Sigma, St. Louis, MO, USA) for their transfer.

The embryo on Day 2 and 3 quality system used to grade of the embryos was described by Steer et al.[6] 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. Day 5 embryos were graded according to Veeck and Zaninovic[7].

Embryo transfer (usually two and in some cases three embryos per patient) was performed on Day 2, Day 3, or Day 5 after retrieval of oocytes. Pregnancy was defined as an increase in serum hCG concentration (20 IU/L) determined on 11 and 13 – 15 days after embryo transfer. Clinical pregnancy was recorded when the fetal sac was visualized on an ultrasound on gestational weeks seven to eight.

Statistical analysis

Quality of embryos, amount of transferred embryos, amount of morphologically ideal developed embryos (Grade A and B), amount of sacs, pregnancy outcome (ongoing pregnancy, abort, abrasion, biochemical pregnancy) and baby-take-home rate (the number of life births per number of IVF/ICSI treatments (cycles) in percent) were evaluated by ANOVA. Various characteristics were summarized by mean and SD within groups. The level of statistical significance was set at $P<0.05$. Clinical pregnancy rates were analysed by ANOVA for categorical variables using the CATMOD Procedure of SAS Institute Inc.[8]. Comparisons between age groups were performed by pairwise contrasts and Bonferroni-Holm adjustment for multiple comparisons using the MULTTEST procedure of SAS.

RESULTS

The mean number of transferred embryos per patient for static group was 2.17 $\pm$ 0.32. The mean number of transferred embryos per patient for micro-vibration group was 2.17 $\pm$ 0.36.

For Groups $\leq$29 years, 30-34 years, 35-39 years and $\geq$40 years, the following rate of high quality embryos without fragmentation (2 to 4 blastomeres on Day 2; 6 to 8 blastomeres and compacting morula on Day 3; blastocyst, expanded and hatching blastocyst on Day 5) was detected (static vs. vibration, respectively): 65.2% vs. 70.8%, 44.3% vs. 69.3%, 67.% vs. 76.4% (for statistic significant differences between respective rates in these three groups $P<0.05$) and 67% vs. 66% ($P>0.1$) (Tables 1, 2).

The following baby-take-home rate was detected for Groups $\leq$29 years, 30-34 years, 35-39 years and $\geq$40 years, respectively (static vs. vibration): 30% vs. 31% ($P>0.1$, increasing

only on the level of tendency), 28% vs. 37%, 23% vs. 29% and 9% vs. 15% (differences between respective rates in these three groups with P<0.05) (Table 3).

DISCUSSION

The potential question can agitate the following fact: there is no significant difference between quality of embryos from some groups (for example, in group ≥40 years), but the baby-take-home rate is significantly different. We can explain this fact by subjectivity of evaluation of embryos using official classification. In the same time, the baby birth is absolutely objective rate that is free from subjectivity. Just with orientation on this rate we have done our conclusions.

The process of embryo development is the complex involving ciliary beating just from the moment of ovulation, fertilization and during embryo transport via the oviduct to the uterus[9].

'Ciliary' refers to the cilia, which is Latin for vibrating hairs. Baseline cilia beating frequencies have been reported to vary widely between individuals in the range of 5–20 Hz[10,11]. At the higher frequencies, mechanical contact between oocyte/embryo during the secretory phase also increases significantly. The ciliary beat has the following characteristics: (i) its rate is remarkably uniform[1]; and (ii) the beat of a particular cilium and its adjacent cilium appears to be well co-ordinated and a definite metachronal wave is established[12]. This metachronism is defined as co-ordinated oscillation including a definite phasing of micro-vibration between the cilia of a single cell and a definite phasing of this vibration between the cilia of adjacent cells. The fluid that surrounds the cilia and forms a blanket above the tips of the cilia is a suspension of mucus[13].

The positive effect of pulsative mechanical micro-vibration for the cytoplasmic maturation of in-vitro matured pig oocytes was published[14]. These authors subjected cumulus–

oocyte–complexes cultured in micro-drops to pulsatile mechanical vibration at a frequency of 20 Hz with acceleration (660 mV/g at 3.3 V: X = ±1.0g, Y = ±0.7g, Z = ±0.15g; instruction of manufacturer). During in-vitro maturation, vibration did not affect the proportion of oocytes reaching the Metaphase-II stage. However, blastocyst formation rates after the activation of oocytes exposed to vibration were significantly higher than those obtained for oocytes matured without mechanical vibration (27% vs. 12% and 26 vs. 15%, respectively, for the 5 s and 10 s pulses).

In medicine, the embryonic development rates and clinical results were compared between a static culture group (n = 159 cycles) and a micro-vibration culture group (n = 166 cycles) in poor responders. A micro-vibrator was set at a frequency of 42 Hz, 5 s /60 min duration of embryo development (15). In poor responders, the embryo development rate was improved to a limited extent under the micro-vibration culture conditions, but the clinical results were significantly improved[15].

It should be noted that the vibration at a frequency 44 Hz and the acceleration described above are the parameters of movement of the plate on which Petri dishes with culture medium and embryos are located. The study laboratory's observations on bovine oocytes have shown that the amplitude of vibration of embryos as well as acceleration of cells are lower than these rates with which the plate is vibrating (data not shown). This fact is due to the inertness of oocytes suspended in a liquid environment: vibration is drastically suppressed by the culture medium and is dependent on the composition and volume of this medium. Parameters of the 'real' vibration of embryos is calculated mathematically and make up 33 Hz. It is necessary to underline that the observing in vivo and published rate of vibration of cilia is only the vibration of cilia, not real vibration of embryos because of inertness of these "swimming" embryos. In fact, we need to know the rate vibration of

apparatus plate in order to change it if it will be necessary. Only developmental rate of embryos is criterion for optimization of parameters of vibration of apparatus.

The acceleration accompanying any movement (**$a = r\omega^2$** and **$\omega = 2\pi f$**, (**a**) acceleration, (**r)** radius of movement, (**f)** frequency) is crucially important for biological objects. The maximal rate of acceleration is achieved at a maximal frequency 50 Hz. That is why it would be not correct to use the "optimal" rate of frequency established for vibration apparatus of certain construction to an apparatus of different construction: with the same frequency the acceleration rate can be different.

In conclusion, in vitro culture of embryos under micro-vibration (with a mimic of conditions in nature whereby oviductal fluid is mechanically agitated by the epithelial cilia) significantly increases baby-take-home rate for patients of 30 years and older.

Disclosure: The authors declare no conflicts of interest.

REFERENCES

1. Infertility. Embryo transplantation. In vitro fertilisation. Available from: https://en.wikipedia.org/wiki/Main_Page.

2. Lyons R.A, Djahanbakhch O, Mahmood T, Saridogan E, Sattar S, Sheaff M.T, Naftalin AA; Chenoy R. Fallopian tube ciliary beat frequency in relation to the stage of menstrual cycle and anatomical site. Hum. Reprod. 2002;17:584–588.

3. Chauveau A, Arloing A, Fleming G. 1973. The comparative anatomy of the domestic animals; Ed. By G. Fleming. New York; D. Appleton and Company; pp. 957.

4. Schumann WO. Über die Dämpfung der elektromagnetischen Eigenschwingnugen des Systems Erde – Luft – Ionosphäre. Zeitschrift und Naturfirschung. 1952;7a: 250–252. German.

5. El Danasouri I, Sandi-Monroy N, Winkle T, Reeka N, Gagsteiger F. Micro-vibration culture and group culture increase fertilization and implantation rates in human embryos. Abstr. Ann. Meet. ESHRE; Munich; Germany i147;2014.

6. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum. Reprod. 1992;7:117-119.

7. Veeck LL, Zaninovic N. An atlas of human Blastocysts. The Parthenon publishing group; New York; 2003.

8. SAS Institute Inc. 2011. SAS OnlineDoc® 9.3. Cary; NC: SAS Institute Inc.

9. Fauci LJ, Dillon R. Biofluidmechanics of reproduction. Ann. Rev. Fluid. Mech. 2006;38:371–394.

10. Paltieli Y, Weichselbaum A, Hoffman N, Eibschitz I, Kam Z. Laser scattering instrument for real time in-vivo measurement of ciliary activity in human fallopian tubes. Hum. Reprod. 1995;10:1638-1641.

11. Weström L, Mårdh PA, Mecklenburg CV, Håkansson CH. Studies on ciliated epithelia of the human genital tract. II. The mucociliary wave pattern of fallopian tube epithelium. Fertil. Steril. 1997;28:955-96.

12. Holwill M.E.J. Hydrodynamics aspects of ciliary and flagellar movements. in : M. A. Sleigh (Ed.); Cilia and Flagella; Academic Press; 143-75;1974.

13. Miller CE. The kinematics and dynamics of ciliary fluid systems. J. Exp. Biol. 1968;49: 617-629.

14. Mizobe Y, Yoshida M, Miyoshi K. Enhancement of cytoplasmic maturation of in vitro-matured pig oocytes by mechanical vibration. J. Reprod. Dev. 2010;56:287-290.

15. Hur YS, Park JH, Ryu EK, Park SJ, Lee JH, Lee SH, Yoon J, Yoon SH, Hur CY, Lee WD, Lim JH. Effect of micro-vibration culture system on embryo development. J. Assist. Reprod. Genet. 2013;30:835-841.

LEGENDS

Table 1. High-quality embryos in different age groups just before transplantation: in vitro culture in static system.

(2A) 2 blastomeres, no fragmentation; (4A) 4 blastomeres, no fragmentation; (6A) 6 blastomeres, no fragmentation; (8A) 8 blastomeres, no fragmentation; (8A compacting) 8 blastomeres, beginning of compacting; (EB) early blastocyst; (B3) blastocyst with small blastocoel; (B2) blastocyst; (B1) expanded blastocyst, (B1h) fully expanded or hatching blastocyst.
Different superscripts indicate significant difference (P<0.05) between the respective rates in "Static" and "Vibration" groups (see also Table 2 for comparison).

Table 2. High-quality embryos in different age groups just before transplantation: in vitro culture with micro vibration.

(2A) 2 blastomeres, no fragmentation; (4A) 4 blastomeres, no fragmentation; (6A) 6 blastomeres, no fragmentation; (8A) 8 blastomeres, no fragmentation; (8A compacting) 8 blastomeres, beginning of compacting; (EB) early blastocyst; (B3) blastocyst with small blastocoel; (B2) blastocyst; (B1) expanded blastocyst, (B1h) fully expanded or hatching blastocyst.

Different superscripts indicate significant difference (P<0.05) between the respective rates in "Vibration" and "Static" groups (see also Table 1 for comparison).

Table 3. Number of formed sacs and "baby-take-home" rate after transplantation of embryos in different age groups: in vitro culture in static system and with micro vibration.

Different superscripts indicate significant difference (P<0.05) between the respective rates in "Static" and "Vibration" groups.

Baby-take-home rate notes the number of life births per number of IVF/ICSI treatments (cycles) in percent.

Quality of embryos	Age of patients			
Day 2	**≤29 years**	**30 – 34 years**	**35 – 39 years**	**≥40 years**
2A	26%	13%	12%	6%
4A	35%	25%	49%	59%
Total high-quality embryos, n (%)	18 (61%)[a]	23 (38%)[d]	110 (61%)[g]	71 (65%)[j]
Day 3	**≤29 years**	**30 – 34 years**	**35 – 39 years**	**≥40 years**
6A	17%	5%	13%	15%
8A	46%	29%	48%	48%
8A compacting	9%	6%	9%	9%
Total high-quality embryos, n (%)	55 (64%)[b]	98 (40%)[e]	524 (70%)[h]	393 (72%)[k]
Day 5	**≤29 years**	**30 – 34 years**	**35 – 39 years**	**≥40 years**
EB	14%	14%	17%	24%
B3	1%	1%	1%	2%
B2	18%	1%	19%	11%
B1	27%	24%	24%	20%
B1h	9%	15%	8%	6%
Total high-quality embryos, n (%)	264 (69%)[c]	633 (55%)[f]	568 (69%)[i]	292 (63%)[l]

Table 1

Quality of embryos	**Age of patient**			
Day 2	**≤29 years**	**30 – 34 years**	**35 – 39 years**	**≥40 years**
2A	22%	16%	20%	20%
4A	44%	48%	51%	38%
Total high-quality embryos, n (%)	22 (66%)[m]	72 (64%)[p]	222 (71%)[t]	97 (58%)[w]
Day 3	**≤29 years**	**30 - 34 years**	**35 - 39 years**	**≥40 years**
6A	9%	11%	12%	11%
8A	55%	48%	54%	52%
8A compacting	6%	7%	12%	14%
Total high-quality embryos, n (%)	106 (70%)[n]	335 (66%)[r]	448 (78%)[u]	263 (77%)[x]
Day 5	**≤29 years**	**30 – 34 years**	**35 - 39 years**	**≥40 years**
EB	17%	17%	19%	20%
B3	1%	4%	0%	0%
B2	14%	9%	17%	13%
B1	28%	26%	33%	22%
B1h	17%	21%	9%	7%
Total high-quality embryos, n (%)	263 (77%)[o]	653 (77%)[s]	804 (78%)[v]	238 (62%)[l]

Table 2.

CT-1664 Cell Transplantation early e-pub; provisional acceptance 10/05/2016

	In viro cultute in static system				In viro cultute with micro vibration			
Age of patients (embryos developed to respective stage before transplantation)	**≤29 years** (496 embryos from 250 patients)	**30 – 34 years** (1457 embryos from 715 patients)	**35 – 39 years** (1751 embryos from 804 patients)	**>40 years** (1117 embryos from 457 patients)	**≤29 years** (527 embryos from 277 patients)	**30 – 34 years** (1469 embryos from 690 patients)	**35 – 39 years** (1911 embryos from 850 patients)	**≥40 years** (893 embryos from 393 patients)
Number of sacs, n (%)	149 (30%)[1]	525 (36%)[6]	595 (34%)[10]	68 (16%)[14]	268 (51%)[18]	646 (44%)[21]	671 (35%)[10]	286 (32%)[28]
Singleton, n (%)	88 (59%)[2]	315 (60%)[7]	327 (55%)[11]	178 (76%)[15]	145 (54%)[19]	323 (50%)[22]	463 (69%)[25]	254 (89%)[29]
Twins, n (%)	61 (41%)[3]	204 (39%)[8]	268 (45%)[12]	43 (24%)[16]	123 (46%)[20]	200 (48%)[23]	201(30%)[26]	32 (11%)[30]
Triplet, n (%)	0[4]	6 (1%)[4]	0[4]	0[4]	0[4]	13 (2%)[4]	7 (1%)[4]	0[4]
" Baby-take home" rate, n (%)	149 (30%)[5]	408 (28%)[9]	403 (23%)[13]	100 (9%)[17]	163 (31%)[5]	543 (37%)[24]	556 (29%)[27]	134 (15%)[31]

Table 3