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Early embryo development in V-ONESTEP, an innovative single step culture medium used for human embryo culture: a study

Karen Uhde[1§], Ann M. Mangalaraj[2§], Ralf Böhm[3], Elisabeth Schanze[3], Irena Sherameti[1], Franziska Buch[1*]
[1]VITROMED GmbH, Hans-Knöll-Str. 6, 07745 Jena, Germany; [2]Department of Reproductive medicine and Surgery, CMCH Vellore, India; [3]Cuypers & Cuypers Fertility Center Heinsberger Höfe, Hochstraße 154, 52525 Heinsberg, Germany: *correspondence to: franziska@vitromed.com

§ - these authors contributed equally to the work

Over the years the advances in the Assisted Reproduction Technologies (ART) have been rapid and inspiring. New techniques are established and integrated into clinical practice. Several factors like the age of the patients, infertility diagnosis and ovarian stimulation protocols can influence the clinical outcome. One reason for the high *in vitro* fertilization (IVF) success nowadays is the development of chemically defined media for the prolonged culture of human embryos. "Back-to-nature" and "let the embryo choose" are two approaches to define the one step or sequential protocol, which allow the cultivation of human embryos from the zygote to the blastocyst stage. IVF culture media contain many components such as salts, energy substrates, amino acids, etc., but the media formulations and the concentrations of the components vary in different media. In practice, the single step protocol compared to the sequential protocol offers several advantages: less embryo handling, a stable embryo culture environment and lower costs. V-ONESTEP (VITROMED GmbH, Germany) used in this study is a single step medium for culturing human germ cells, suitable for cultivation of all developmental stages from the fertilized oocyte to the blastocyst. The aim of this study was to observe how successful V-ONESTEP is being used in ICSI treatments and how the outcome of a treatment is, in regard to pregnancy rate or life birth rate. The fertilization rate reported here was 77.08%. Our data also demonstrate, that a day 5 transfer with a blastocyst resulted in higher implantation rates compared to a cleavage stage transfer (44.3% vs 21.4%). The good results on day 5 culture confirm that V-ONESTEP medium is very well suited for long-term culture. The outcomes confirm that V-ONESTEP fulfils all the duties of a single step embryo culture medium and can be used successfully in the IVF clinics.

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Introduction

Since the beginning of the *in vitro* fertilization (IVF) era, a lot of efforts have been undertaken to increase the success rates of assisted reproduction technologies (ART) for couples who are infertile. Many factors can influence the positive results of an *in vitro* fertilization treatment, but two of them are of particular importance: proper embryo culture conditions and medium formulation (Dieamant et al. 2017; Summers and Biggers 2003; Paternot et al. 2010). Over the past decades, improvements to the culture media for embryos have been the focus of the research worldwide (Quinn et al. 1985; Quinn 2000, 2012; Bigger 2001; Gardner et al. 2002; Pool 2005; Biggers and Summers 2008). Increasing evidence has shown that culture media can affect the number of good quality embryos, fertilization rate, implantation rate, pregnancy rate and live birth rate (Kleijkers et al. 2016; Mantikou et al. 2013). Other equally important factors, such as incubator gases and temperature, culture oils and supplements, can change the dynamics of how the embryo interacts with components of the medium, having an impact on pre- and post-implantation development and possibly on future health of the offspring (Chronopoulou and Harper 2015).

A culture medium is a complex solution that comprises a range of elements to provide the embryo with hydration, ions, and nutrients while maintaining a homeostatic and relatively non-stressful environment (Fawzy et al. 2017).

Choosing an optimal medium compound and its concentration are difficult issues that must be addressed in the design of chemically defined media (Summers and Biggers 2003). Nowadays, most commercially available culture media consist of a mixture including salts, energy sources, proteins, antibiotics, amino acids, and vitamins (Kleijkers et al. 2016).

Carbohydrates such as glucose and pyruvate are energy substrates for the embryo development (Carrasco et al. 2013). Zygotes and subsequent cleavage stages prefer pyruvate as the primary source of energy, while the eight-cell-embryo stage, uses glucose for the further development (Gruber and Klein 2011; Leese et al. 1984, 1993).

Amino acids play various roles, including the role as precursors for biosynthesis (Crosby et al. 1988), energy sources, regulators of energy metabolism (Gardner et al. 2000; Summers and Biggers 2003; Gruber and Klein 2011), osmolytes (Van Winkel et al. 1990), buffers of intracellular pH (Edwards et al. 1998) and chelators of heavy metals (Lindenbaum 1973) and therefore form an important component of culture media (Houghton et al. 2002).

Human serum albumin (HSA) is a potential source of protein (Carrasco et al. 2013). The useful role of ethylenediaminetetraacetic acid (EDTA) is based on its function as a ligand and chelating agent (Gruber and Klein 2011) that helps to reduce the glycolytic activity and protects the embryo against free radicals (Carrasco et al. 2013).

The pH is dynamic and the result of a balance between concentrations of CO_2 in the cell culture incubator and the amount of bicarbonate in the media and should range from 7.2 to 7.4. The pH depends on the association/disassociation of compounds in solution and any factors which influence this balance, such as temperature (Swain 2010).

Mineral salts provide the embryo a source of Na^{2+}, Ca^{2+}, and phosphate required for cellular function (Carrasco et al. 2013). Embryo culture media are routinely supplemented with antibiotics to prevent bacterial contamination (Lemeire et al. 2007). Nowadays, commonly used antibiotics are penicillin, streptomycin, and gentamycin.

Vitamins are organic nutrients required in small amounts by mammalian cells, however, most mammals have lost the ability to synthesize them and so vitamins must, therefore, be supplied as nutrients (McKiernan and Bavister 2000). The addition of vitamins as antioxidants to the culture media containing glucose and phosphate helped to prevent a loss in respiration and metabolic control (Lane and Gardner 1997). The following possible vitamins are components of different ART culture media: ascorbic acid, cyanocobalamin, folic acid, and tocopherol (Gruber and Klein 2011).

Over the years different strategies have been introduced to increase the rates of successful clinical pregnancies and live births and advances in cell culture media have led to a shift in IVF practice from cleavage stage embryo transfer (days 1–3) to blastocyst stage transfer (days 5–6) (Glujovsky et al. 2016; Gardner et al. 2000; Rienzi et al. 2002). Embryo culture up to day 5 – 6 allows the identification of those embryos with limited or no development potential (Wirleitner et al. 2010; Gardner 1998; Zech et al. 2006) as it improves both uterine and embryonic synchronicity and enables self-selection of viable embryos, thus resulting in better live birth rates (Glujovsky et al. 2016).

Selecting the compounds and their concentrations in a culture medium is not simple. Two approaches have been used to determine the concentrations used in a medium. "Back to nature" approach is based on sequential embryo culture medium designed to mimic *in vivo* conditions, while "let the embryo choose" approach is based on single culture medium in which the embryo is cultured in a constant medium containing all the ingredients needed for its development (Summers and Biggers 2003; Lane and Gardner 2007; Dieamant et al. 2017). In his article "Culture Systems: Sequential" published in 2012 Patrick Quinn wrote that the debate at present is which system is best, a sequential series of media to accommodate changes in physiology and metabolism of the embryo from a 1-cell zygote to the differentiated blastocyst stage or a single step culture regime using the same culture medium throughout the preimplantation period.

The medium formulation of the single step embryo culture medium V-ONESTEP (VITROMED GmbH, Germany; Table 1) used in this study was developed in cooperation with Dr. Patrick Quinn based on conventional *in vitro* culture media used in reproductive medicine. V-ONESTEP medium has been optimized to incorporate the latest changes that have been scientifically proven to be beneficial for both sequential and single step approaches from day 1 to day 5 of culture (Quinn et al. 1985; Quinn 2000, 2012). All substances necessary for early embryological development from the fertilized oocyte to the blastocyst are provided, and there is no need for a media change.

Material and Methods

Study design

From January 2015 to December 2016 a study was conducted at Department of Reproductive medicine and Surgery, CMCH Vellore, South India. ART was carried out according to the institution protocol. A written informed consent was taken from the couples undergoing ART. All procedures followed were in accordance with ethical standards laid down in the Helsinki Declaration.

This study included a total of 606 patients with a patient age of 32 ± 4,4 years (women) and 37 ± 5,1 years (men). For details see Figure 1 and Results. Inclusion criteria for the study were: patients with tubal factor, male factor, unexplained infertility. Ovulatory disorders were accepted. Exclusion criteria for the study were: IVF treatment, frozen embryo culture, treatments with ET at days 2 and 4. Common demands for the study were: each patient could be included in the study only once; all subjects had to undergo Intracytoplasmic Sperm Injection (ICSI); fresh embryo transfer; constant laboratory procedures during the study

were required; culturing in reduced O_2; all patients included had to undergo ovarian stimulation.

A total number of 594 ICSI treatments were carried out (see Figure 1 for details). The embryo quality was recorded during culture period. For this study, only the outcome of fresh embryo transfers was used for analysis. The follow-up data were collected in the period 2017 – 2018 and are summarized in Figure 1.

Table 1: Key components of V-ONESTEP (VITROMED GmbH, Germany) media for the culture of preimplantation mammalian embryos

Components V-ONESTEP culture media	
Buffer	Sodium bicarbonate
Energy sources	Glucose Sodium pyruvate Calcium lactate
Physiological salts	Sodium chloride Potassium chloride Magnesium sulfate Sodium citrate
Essential/ Non-Essential Amino acids	NEAA/EAA's
pH indicator	Phenol red
Antibiotics	Gentamycin
Chelators	EDTA
Vitamins	Calcium pantothenate
Other components	Human serum albumin (additive, depending on the product variant) Sodium hyaluronate Ultra-Pure Water

V-ONESTEP culture protocol

The design of V-ONESTEP (VITROMED GmbH, Germany) media for the culture of human embryos is based on the "let the embryo choose" principle (Summers and Biggers 2003), without the need for a media change. The composition of V-ONESTEP medium and the function of its components regarding the embryo development are shown in Table 1. V-ONESTEP medium is based on IVF standard components for human cultures but consists of additional beneficial ingredients for the embryo. A schematic view of the ICSI procedure, followed by embryo development under V-ONESTEP medium is shown in Figure 2.

V-ONESTEP was used under aseptic conditions and before use it was equilibrated at 37°C, and 6% CO_2. Zygotes and embryos were cultured in an atmosphere of triple gas (6% CO_2, 5% O_2 and 89% nitrogen) in MINC-mini-incubator-COOK MEDICAL. V-ONESTEP had a pH of 7.25 to 7.4 during culture. A total number of 24 different V-ONESTEP production batches was used for this study.

Ovarian Stimulation

The standard agonist (long, ultralong, short) or antagonist protocol with 100 – 300 IU recombinant follicle-stimulating hormone (Gonal-f, follitropin alfa, Merck Serono, Inc. Rockland, USA or Recagon, follitropin beta, Schering-Plough, USA) were used. Ovulation was triggered using recombinant hCG (250 µg; Ovitrelle, Merck Serono, Inc. Rockland, USA) or GnRh agonist (Leuprolide acetate 2 mg, Lurid, Sun Pharmaceuticals Industries, Ltd, India) subcutaneously when at least three follicles achieved a diameter of 17 mm. Transvaginal oocyte retrieval was performed under conscious sedation 35 – 36 h following trigger.

Luteal support

Luteal support was administered using micronized progesterone vaginally, 400 mg (Naturogest, Zydus Healthcare, Ltd, India) twice daily and intramuscular progesterone 100 mg (Gestone, Ferring Pharmaceuticals, India) twice weekly.

Pregnancy was diagnosed by a positive serum beta hCG test and confirmed by transvaginal ultrasound conducted two weeks later. All women with an ongoing pregnancy were referred at 10 weeks to obstetric units for follow-up till delivery.

Information regarding clinical and laboratory variables such as age, indication, oocyte numbers, embryo quality, number of embryos transferred, day of transfer (cleavage vs

blastocyst), and outcomes was obtained from the departmental ART database. Data regarding live birth were obtained through electronic media or phone interviews.

Intracytoplasmic Sperm Injection (ICSI) treatment

All media and microtool products used were from VITROMED GmbH, Germany.

Immediately after puncture, oocytes were identified in follicular fluid and hold at 37°C in HEPES buffer. Before ICSI procedure, oocytes were enzymatically denuded by pipetting them in hyaluronidase. In parallel to puncturing, semen was prepared by density gradient centrifugation, according to manufacturer instruction with little modifications. In short, 1.5 ml of gradient 40% were laid on 1.5 ml gradient 80%, followed by 20 min centrifugation at 300 *xg*. After centrifugation the pellet was washed twice with sperm washing media and hold until ICSI in sperm wash media at 37°C. A small amount of sperm suspension was placed into PVP. In the same dish metaphase II oocytes were placed in small V-ONESTEP droplets for ICSI. A sperm was immobilized in PVP and taken into an injection pipette. An oocyte was gently aspirated by a holding pipette, with the polar body in 6 or 12 o'clock position. Injection of a sperm into the oocyte was done by gently aspirating ooplasm into the injection needle to confirm the oolemma breakage. Then the ooplasm and the sperm were gently released into the oocyte.

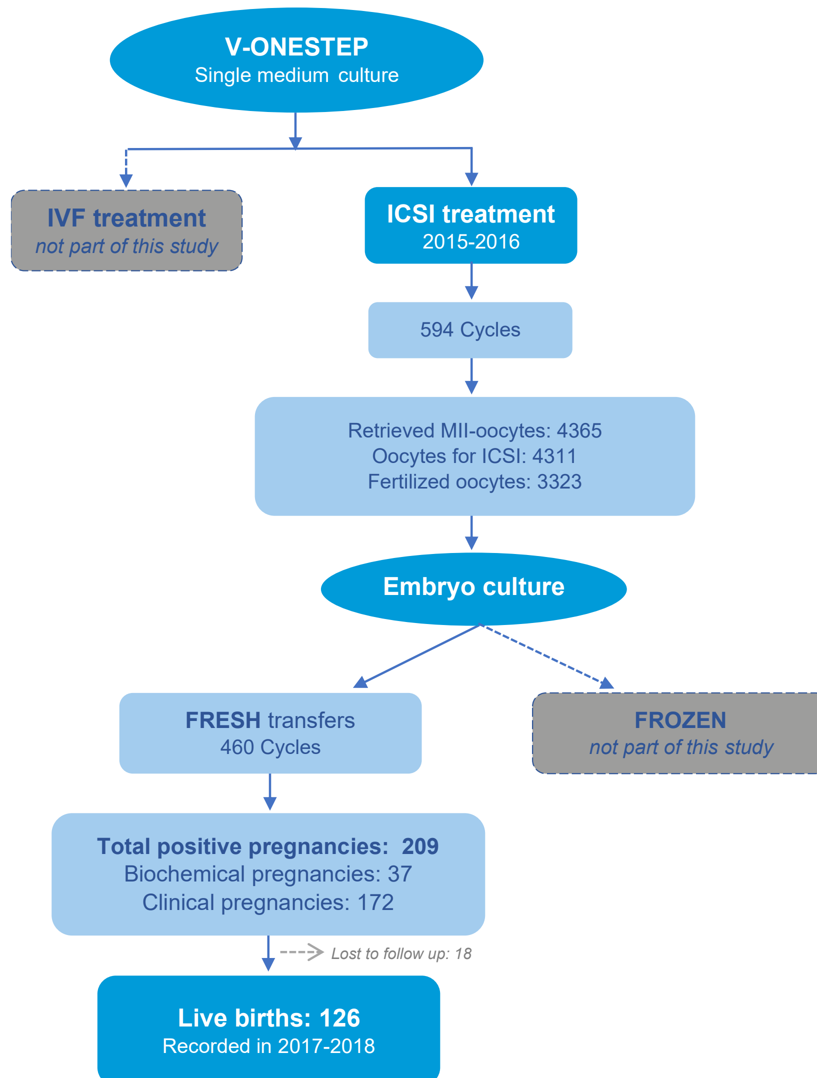


Figure 1. Flow diagram of the conducted study using V-ONESTEP – a single step culture medium for human embryo culture

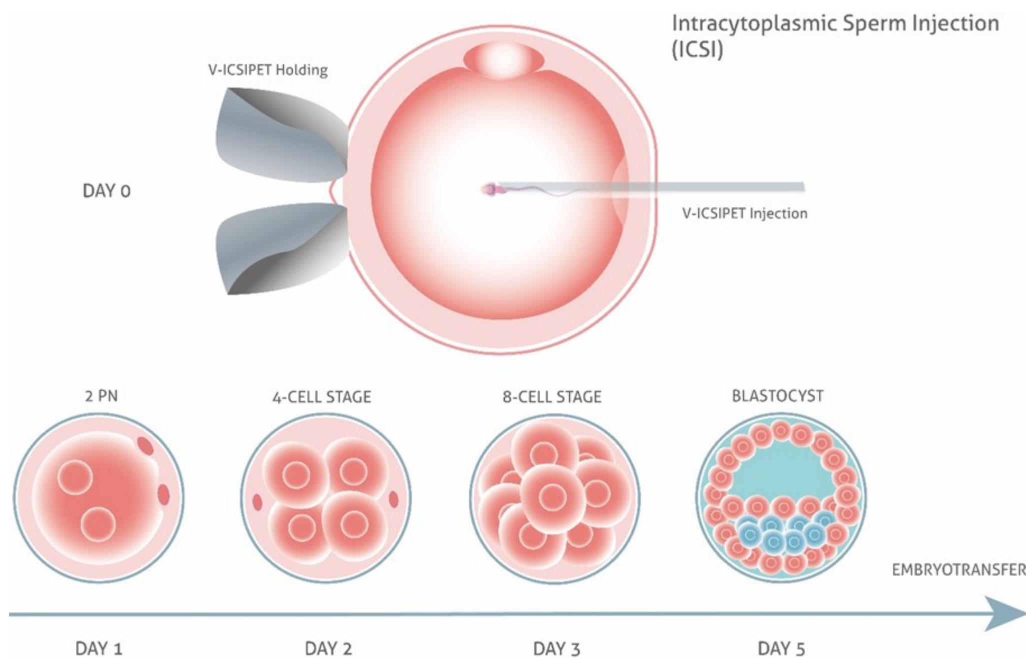


Figure 2: Schematic view of ICSI procedure, followed by embryo development under V-ONESTEP culture from day 0 to day 5. Blastocysts with good morphology were used for embryo transfer.

After ICSI the fertilized oocytes were transferred into V-ONESTEP for cultivation. Approximately 16 h after insemination, fertilization was checked by the presence of two pronuclei. Only oocytes with two pronuclei were checked until day 5 for blastocysts formation. Embryos were graded according to the Istanbul consensus, 2011. Average of 2 cleavage stage embryos or blastocysts were transferred.

Statistical analysis

The primary outcome of this study was the clinical pregnancy rate, as well as the live birth rate. In addition, live birth rates were calculated relating to the BMI. Also, infertility cause and pregnancy rates were considered. For graph creation SigmaPlot was used.

Results

The age of the women was between 20 and 44, which results in an average age of 32 ± 4.4 years. For male patients the mean age was 37 ± 5.1 years. In total 606 patients were included in this analysis (Figure 1; Table 2). In 594 punctures, 4311 oocytes which were used for ICSI, were retrieved (Table 2). Of these oocytes 3323 were fertilized, which results in a fertilization rate of 77.08% (Table 2).

Table 2: Overview of treated patients, retrieved MII oocytes and fertilization rate.

	Number
Punctures	594
Oocytes for ICSI	4311
Fertilized	3323
Fertilization rate	77.08%

Following Vienna Consensus (ESHRE 2017) the implantation rate (defined as the number of gestational sacs observed divided by the number of embryos transferred) was calculated (ESHRE Special Interest Group, 2017). Within 460 embryo transfers, 917 embryos were given back. In total 223 (24.3%) embryos implanted (Table 3). If this is distinguished between embryos transferred in the cleavage stage (in total 802 transferred). and the blastocyst stage (in total 115 transferred), it results in an implantation rate of 21.4% and 44.3%, respectively (Table 3).

The correlations between the patients age and the clinical pregnancy rate, as well as the live birth outcome were analysed (Figure 3). Within the group of ≤ 29 years old women, 45.9% of the cases resulted in a clinical pregnancy, from which 41% resulted in a live birth. The outcome was lower for the two other groups. In the group of 30 – 34 years

old women, 32.8% of the treatments resulted in a clinical pregnancy and 26.5% of them had a live birth, while the older group of patients (≥ 35 years) resulted in a clinical pregnancy rate of 24.16%, from which 17.4% had a live birth (Figure 3).

Table 3: Implantation rates after fresh transfer

	Total implanted	Total transferred	Percentage
Implantation rate	223	917	24.3%
Implantation rate (cleavage stage)	172	802	21.4%
Implantation rate (blastocyst stage)	51	115	44.3%

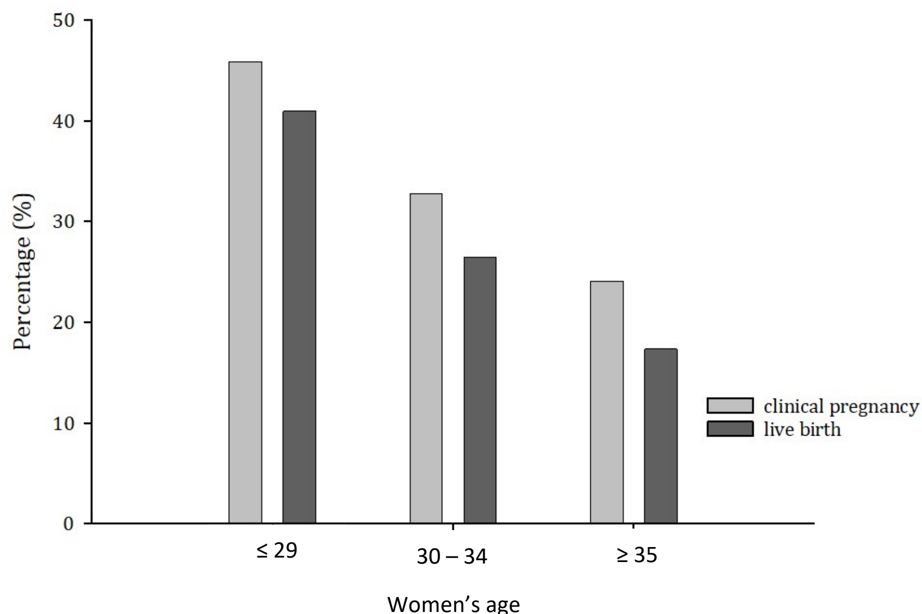
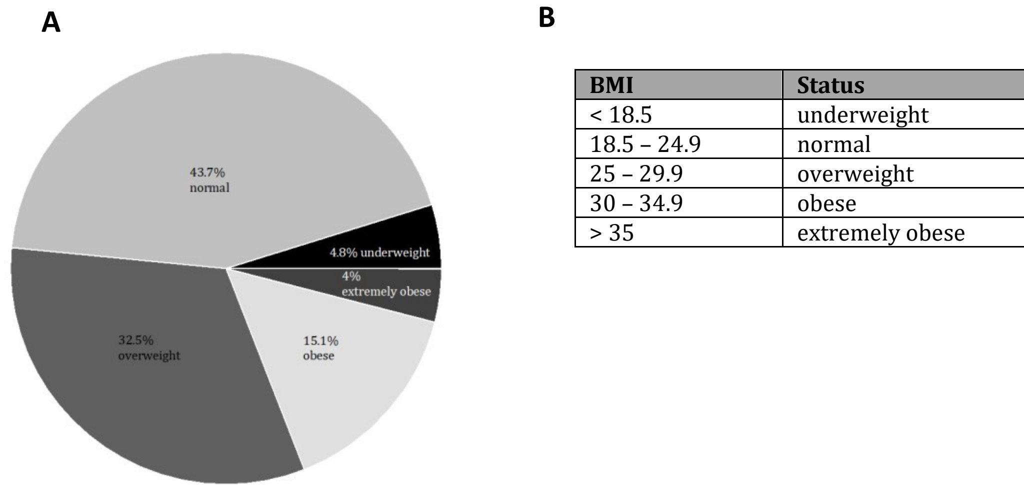


Figure 3: Outcome of ICSI treatment in percentage (%) related to female age. The patients were divided in 3 groups: ≤ 29 years, 30 – 34 years, ≥ 35 years. The outcome is divided into clinical pregnancy (light gray) and live birth rate (dark gray).

Live birth rates were analyzed according to Body Mass Index BMI (Figure 4A), calculated by dividing the body weight in kilogram by the square of height in meter. Following only the group of women with a treatment that resulted in a live birth is considered. For the group of the treated women with a normal BMI (18.5 – 24.9; Figure 4B) ICSI treatment resulted in 43.7% live births (Figure 4A). In the group of overweight women (BMI 25 – 29.9) and obese women (BMI between 30 and 34.9) the live birth rates were 32.5% and 15.1% respectively (Figure 4A). The lowest rates with 4% and 4.8% were calculated for extremely obese (BMI ≥ 35) and underweight (BMI ≤ 18.5) women (Figure 4A).

The reasons for infertility of the treated couples were of different origins and were divided into female-cause (for example endometriosis), male-cause (for example necrozoospermia), or both (female and male). If the reason for an IVF treatment was due to a female cause, the clinical pregnancy rate resulted in 33.9% (Figure 5). In 26.4% of these cases the pregnancy resulted in a live birth. In the case of male infertility, the clinical pregnancy (33.65%) and birth rates (26.9%) were comparable to the rates in the case of female infertility. If both, male and female, were affected the clinical pregnancy and live birth rate were 32.97% and 28.57%, respectively (Figure 5). Considering the overall outcome, 126 deliveries were recorded in total. In 102 of the cases, the outcome resulted in a singleton birth, and 24 cases resulted in twins (Table 5). Of 172 clinical pregnancies, 28 resulted in a miscarriage (Table 5).



BMI	Status
< 18.5	underweight
18.5 – 24.9	normal
25 – 29.9	overweight
30 – 34.9	obese
> 35	extremely obese

Figure 4: (A) Live birth rates related to the BMI of the women. Within each section the percentages and the BMI classification is described. (B) BMI values and their corresponding status

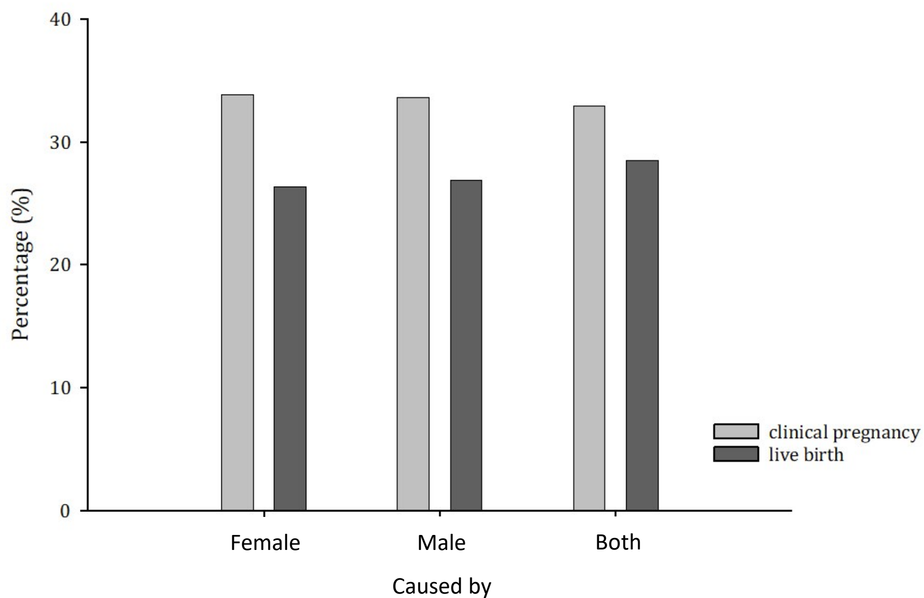


Figure 5: Outcome of clinical pregnancy (light gray) and live birth (dark grey) related to the reason for the fertility treatment, that were attributed to causes in female, male, or both genders.

Discussion

Several studies have suggested that culture media have an impact on the quality of embryos generated in IVF/ICSI cycles thereby influencing implantation and pregnancy rates (Cooke et al. 2002; Friedler et al. 2007). Despite the large number of publications, it is still unclear which culture medium leads to the best IVF/ICSI success rates (Mantikou et al. 2013) and whether sequential or continuous media should be favored (Stimpfel et al. 2020). Although there are pros and cons for single step culture systems, this system shows advantages especially for the continuous culturing

system, which usually is used in time lapse systems. With a single step medium, the embryo can develop without any disturbance during the early stages until the blastocyst stage (Biggers and Summer 2008; Gardner and Lane 1996; Machtinger and Racowsky 2012; Hardarson et al. 2015; Greenblatt et al. 2005; Angus et al. 2006; Matsubara et al. 2006; Zech et al. 2006). Another advantage is the reduction of costs when compared to the sequential protocol.

The aim of this study was to observe how successful V-ONESTEP culture medium is being used in ICSI treatments and how the outcome of a treatment is, in regard to pregnancy rate or life birth rate. This study was conducted with the single step V-ONESTEP medium, which is developed for the early human embryo growth. The main outcome was the fertilization rate of 77.08% for the treated group of women with an age of 32 ± 4.4 years. The pregnancy rate per aspiration achieved was 25.9%, while the delivery rate per aspiration was 21.2%. These results were comparable to those outcomes reported in ESHRE Annual Report (2017) where a pregnancy rate per aspiration of 27.8% and the delivery rate per aspiration of 20.1% with ICSI were reported. Other literature data also confirm the outcomes of this study. In Wirleitner et al. (2010), the fertilization rate after using a single medium was reported to be 76.5%, which is comparable to our V-ONESTEP study (77.08%). Our data also demonstrate that a day 5 transfer with a blastocyst resulted in higher implantation rates compared to a cleavage stage transfer (44.3% vs 21.4%). The good results on day 5 culture confirm that the single step V-ONESTEP medium is very well suited for long-term culture, which is also the mission that a single step medium should have.

Table 5: Overview of clinical pregnancies which resulted in a miscarriage, a singleton or twin birth

<table>
<tr><td>Clinical Pregnancies</td><td>172</td><td></td><td></td><td></td></tr>
<tr><td>Miscarriage</td><td>28</td><td></td><td>16.3%</td><td></td></tr>
<tr><td>Singelton</td><td>102</td><td rowspan="2">total =126</td><td>59.3%</td><td rowspan="2">total = 73.3%</td></tr>
<tr><td>Twins</td><td>24</td><td>14%</td></tr>
<tr><td>Lost to follow up</td><td>18</td><td></td><td>10.4%</td><td></td></tr>
</table>

According to the report of WHO, in 2016 about 40% of the women were overweight. During the period 1975 to 2016, the worldwide prevalence of obesity nearly tripled (WHO). The tendency of an increasing BMI worldwide also affects the fertility of men and women. Women with an elevated BMI, have a higher chance of infertility or related disorders compared to women with a normal BMI (Brewer et al. 2010). It is not yet clear, if obesity also affects IVF treatments. In our study, we have found that normal and overweight women had a higher chance for a live birth (43.7% and 32.5%) compared to obese or extremely obese women (15.1% and 4%). In the study of Banker et al. (2017) no correlation between BMI and ART success could be shown. But this will need further investigation, since it is known that elevated BMI levels also lead to general physical impairments. In contrast to the findings of Banker et al. (2017), the study of Kudesia et al. (2018) showed a poorer outcome of the IVF treatment if the patient was overweight or obese. In our study a slight decrease of live birth was seen in the overweight group, compared to the normal weight women. A clear decrease was seen in the obese women compared to the normal weight women (Figure 4A). Also, this contrary study situation shows that the BMI and its relation to ART outcome need further investigations.

In this study, we did not only include patients with a good prognosis, like patients between 25 and 35 years and with a normal to overweight BMI. Patients with an age over 40 years or obese patients were also considered, which of course influences the results. If only the treated women with a normal BMI (18.5 – 24.9; Figure 4B) were considered, the ICSI treatment resulted in 43.7% live birth (Figure 4A) which is comparable to the other studies. As a conclusion, the results of this study confirm that V-ONESTEP medium fulfils all the duties of a single step embryo culture medium and can be used successfully in IVF clinics.

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