Day-2 serum progesterone level and IVF/ICSI outcome

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Abbreviations

AMH	anti-Müllerian hormone
ART	assisted reproductive technology
BMI	body mass index
COS	controlled ovarian stimulation
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
hCG	human chorionic hormone
hMG	human menopausal gonadotropin
hp-FSH	highly purified follicle stimulating hormone
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
IVF-ET	in vitro fertilization and embryo transfer
LH	luteinizing hormone
NICE	National Institute for Health and Care Excellence
OHSS	ovarian hyperstimulation syndrome
P	progesterone
p-FSH	purified follicle stimulating hormone
PRL	prolactin
rFSH	recombinant follicle stimulating hormone
rFSH/rLH	recombinant follicle stimulating hormone/recombinant luteinizing hormone

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Summary

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Infertility in couples is common and has become a global burden, remaining high over the years. The National Institute for Health and Care Excellence (NICE) recommends that prolonged or unresolved infertility should be treated using assisted reproduction technology. The purpose of this study was to evaluate the impact of elevated day-2 serum progesterone levels on IVF and ICSI outcome regarding pregnancy.

A one-center cohort retrospective study was conducted during the period of February 2020 to May 2020. The study includes data from 136 patients who underwent a total of 189 fresh embryo transfers in IVF/ICSI antagonist cycles from January 2014 to January 2020. Based on the levels of day-2 serum progesterone the total number of cases (n=189) were divided into two groups; group 1 (n=134) with serum progesterone level of ≤1.5 ng/mL (normal) and group 2 (n=55) with serum progesterone level of >1.5 ng/mL (high). The total dose of gonadotropins, age, BMI, AMH, FSH, LH, PRL, the number of oocytes retrieved, the number of embryos transferred and frozen, and positive pregnancy tests were compared between the two groups.

The results showed that there was no statistically significant difference between age, BMI, AMH, FSH, LH, PRL, total dose of gonadotropins, the number of oocytes retrieved, and the technique used (IVF or ICSI) between the two groups. As well there was no statistically significant difference between the two groups in regards to the number of embryos transferred and the number of frozen embryos. Although a higher percentage of pregnancies based on positive hCG was found in the normal progesterone level group (33.6%) than in the high progesterone group (23.8%), there was no statistically significant difference between the two groups (p-value 0.178).

In conclusion, this study found that an elevated day-2 serum progesterone level does not have a significant impact on the clinical outcome of IVF/ICSI antagonist cycles with fresh embryo transfers.

Key Words: In vitro fertilization, Intracytoplasmic sperm injection, Progesterone, Pregnancy

Sažetak

Naslov: Povezanost serumskih vrijednosti progesterona drugog dana menstruacijskog ciklusa sa ishodima IVF/ICSI

Autor: Ana Mičić

Neplodnost predstavlja globalni problemi uz visoku pojavnost tijekom godina. Nacionalni institut za izvrsnost u području zdravstva i skrbi (NICE) preporuča liječenje dugogodišnje ili idiopatske neplodnosti medicinski pomognutom oplodnjom. Cilj ove studije bio je istražiti utjecaj povišenih vrijednost serumskog progesterona drugog dana menstruacijskog ciklusa sa ishodima IVF/ICSI vezanim uz trudnoću.

Provedena je kohortna retrospektivna studija u razdoblju od veljače 2020. do svibnja 2020. Istraživanje je uključilo podatke od 136 pacijentica koje su od siječnja 2014. do siječnja 2020. prošle ukupno 189 antagonistički ciklusa IVF/ICSI koje su uključivale svježi embriotransfer. Ovisno o serumskoj razini progesterona 2. dana ciklusa, ukupni broj slučajeva (n=188) podijeljen je u dvije skupine; skupina 1 (n=134) s razinom progesterona u serumu od ≤1,5 ng/mL (normalna razina progesterona) i skupina 2 (n=55) s razinom progesterona u serumu >1,5 ng/mL (visoki progesteron). Uspoređivana je ukupna doza gonadotropina, dob, ITM, AMH, FSH, LH, PRL, broj aspiriranih oocita i broj transferiranih i zamrznutih embrija između obje skupine.

Nisu nađene statistički značajne razlike između dobi, ITM, AMH, FSH, LH, PRL kao niti ukupne doze korištenih gonadotropina, broja aspiriranih oocita između dviju skupina. Nije nađena niti značajna razlika između broja transferiranih embrija i broja zamrznutih embrija između istraživanih skupina. Utvrđen je veći postotak trudnoća s pozitivnim hCG-om u skupini sa normalnom razinom progesterona (33.6%) u odnosu na skupinu s visokim progesteronom (23.8%), ali ova razlika nije statistički značajna (p vrijednost 0.178).

Zaključno, rezultati ove studije pokazuju da povišena razina serumskog progesterona drugog dana ciklusa nema značajan utjecaj na ishode IVF/ICSI.

Ključne riječi: in vitro oplodnja (IVF), intracitoplazmatska injekcija spermija (ICSI), progesteron, trudnoća

1. Preface

1.1. Physiology of the Menstrual Cycle

The menstrual cycle is a physiological change that occurs in females during their reproductive years and is a determinant of a woman's reproductive health. The basic biology of the menstrual cycle is a complex, coordinated sequence of events involving the hypothalamus, anterior pituitary gland, ovaries and endometrium (Hawkins and Matzuk 2008). The average cycle is 28 days long with a normal range between 25 to 35 days (Meniru 2001). The cycle depends on changes occurring after puberty within the ovaries and fluctuation in ovarian hormone levels, which are themselves controlled by the pituitary and hypothalamus within the hypothalamo-pituitary-ovarian axis (Bickerstaff and Kenny 2017). The hypothalamus secretes gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to secrete both follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Meniru 2001). The levels and timing of secretion of each gonadotropin is correlated by GnRH, feedback from sex steroid hormones and other autocrine and paracrine factors including inhibin and activin (Hawkins and Matzuk 2008). The gonadotropins stimulate the ovary to produce the steroid hormones, estrogen or progesterone. The ovarian steroid hormones in turn stimulate endometrial proliferation. Although estrogen and progesterone have some feedback at the level of the hypothalamus, the more vigorous feedback occurs at the level of the anterior pituitary (Hawkins and Matzuk 2008).

In the ovary, folliculogenesis is divided into the follicular phase, prior to ovulation, and the luteal phase, after ovulation (Bickerstaff and Kenny 2017). The follicular phase lasts an average of 14 days. During the follicular phase, FSH stimulates the growth and development of ovarian follicles (Meniru 2001). Within the follicles, there are two cell types that are involved in the processing of steroids, including estrogen and progesterone (Bickerstaff and Kenny 2017). The two cells are the theca and the granulosa cells which respond to LH and FSH, respectively. LH stimulates production of androgens from cholesterol within theca cells. These androgens are then converted into estrogens by the process of aromatization in granulosa cells, under the influence of FSH (Bickerstaff and Kenny 2017). LH stimulates cells in these follicles to produce two other hormones,

estrogen and progesterone. Estrogen is produced from the beginning of the ovarian cycle, while progesterone is produced by the corpus luteum after ovulation (Meniru 2001). LH also contributes to the development of the follicles. As the follicles grow and estrogen secretion increases, there is negative feedback on the hypothalamus and pituitary to decrease their hormonal secretions of GnRH, FSH and LH (Meniru 2001). One consequence of this is that the developing follicles are deprived of FSH. However, one follicle, the dominant follicle, is able to continue developing despite the lower FSH levels due to a higher density of FSH receptors. All the other follicles in the cohort undergo atresia (Meniru 2001). By the end of the follicular phase, which lasts an average of 14 days, the follicle matures and FSH induces LH receptors on the granulosa cells to compensate for lower FSH levels and prepare for ovulation (Bickerstaff and Kenny 2017). The high estrogen level stimulates the pituitary to suddenly produce large amounts of LH and FSH. The LH surge induces final maturational changes in the oocyte, leading to ovulation (Meniru 2001).

During ovulation, the oocyte is expulsed from the follicle with cumulus granulosa cells surrounding it. The luteal phase begins after ovulation and lasts 14 days in most women, without great variation (Bickerstaff and Kenny 2017). The remaining follicular cells in the ovary become luteinized forming the corpus luteum which then produces progesterone. Ongoing LH secretion and granulosa cell activity ensure a supply of progesterone, which stabilizes the endometrium in preparation for pregnancy (Bickerstaff and Kenny 2017). Progesterone levels are at their highest in the cycle during the luteal phase. The high level of progesterone also suppresses FSH and LH secretion to a level that will not produce further follicular growth in the ovary during that cycle (Bickerstaff and Kenny 2017). In the absence of pregnancy, the corpus luteum will regress 9 to 11 days after ovulation in a process known as luteolysis (Panaino et al. 2017). Luteolysis consists of a radical drop in circulating estradiol and progesterone levels due to apoptotic death (Vaskivuo et al. 2002). The mature corpus luteum is less sensitive to LH, produces less progesterone and will gradually disappear from the ovary (Bickerstaff and Kenny 2017). The withdrawal of the progesterone affects the uterus by causing shedding of the endometrium, known as menstruation. The reduction in the levels of progesterone, estrogen and inhibin, send a feedback signal to the pituitary to increase secretion of gonadotropic hormones and new

preantral follicles begin to be stimulated, beginning the cycle again (Bickerstaff and Kenny 2017). In the case of fertilization and implantation, the stimulation of human chorionic gonadotropin (hCG) by the placenta maintains the corpus luteum.

1.2. Infertility Treatment

Infertility, commonly defined as the inability of a couple to conceive following 12-24 months of exposure to pregnancy (Bhattacharya and Hamilton 2014), is common and has become a global burden, which has remained high over the years (Mascarenhas 2012). The National Institute for Health and Care Excellence (NICE) recommends in vitro fertilization (IVF) as the definite treatment for prolonged, unresolved infertility after other treatments have failed (NICE 2013). In vitro fertilization (IVF) means joining the egg and the sperm outside the body. The major roles of IVF therapy are (Arslan et al. 2005):

(1) to obtain multiple fertilizable oocytes of good quality that can lead to diploid fertilization and early embryo development;
(2) to establish a single, healthy (euploid) pregnancy following embryo transfer to the uterine cavity; and
(3) to cryopreserve excess embryos of good quality to optimize the total reproductive potential.

IVF may be used to overcome female or male infertility problems (NICE 2013). IVF is the best form of treatment for women with damaged fallopian tubes, where a blockage prevents the egg and sperm from meeting (Davies et al. 2009). IVF is also recommended if scarring or ‘adhesions’ around the tubes and ovaries prevents the egg from getting down the tubes, for example, after a burst appendix or in cases of severe endometriosis (Davies et al. 2009). Men with very low sperm counts or poorly moving sperm may require a special technique of IVF called intracytoplasmic sperm injection (ICSI). ICSI is a micromanipulation technique for in vitro insemination, aimed at improving the fertilization rate in couples with severe male factor infertility or those with previous failed fertilization during IVF attempts (Coughlan et al. 2011). ICSI can be used for men with profound oligospermia, asthenoteratozoospermia and obstructive azoospermia following microsurgical or direct aspiration of sperm from either the epididymis or the testis

(Coughlan et al. 2011). ICSI involves the injection of a single spermatozoon directly into the cytoplasm of the oocyte. The spermatozoon is immobilized before ICSI, usually by breaking the tail, as flagellation within the ooplasm is undesirable and only the genetic material of the sperm head is required (Coughlan et al. 2011). ICSI is used in up to 60% of reported assisted reproductive technology (ART) cycles (Bhattacharya and Hamilton 2014). It has revolutionized the management of male infertility and has provided the possibility of a pregnancy for men who previously would have required their partners to undergo donor insemination (Coughlan et al. 2011). Additionally, IVF is recommended for couples with unexplained infertility if they have been trying for a long time (more than 5 years) or the woman is older than 35 (Davies et al. 2009).

1.3. Controlled Ovarian Stimulation

The success of IVF depends in part on obtaining a sufficient number of eggs to create high-quality embryos for uterine transfer, without exposing the patient to the risks of excessive ovarian stimulation (Sunkara 2011). Complex endocrine changes happen while a woman undergoes ovarian stimulation as part of IVF treatment (Gallos et al. 2017). During IVF treatment, the ovaries are stimulated with relatively large doses of FSH, which are administered as daily injections. This approach is known as controlled ovarian stimulation (COS). COS is a term used to describe the use of fertility drugs with the aim of growing and releasing more than one egg (Davies et al. 2009). When they are given to a woman who is already ovulating naturally, these drugs boost her fertility and increase the chance of pregnancy. The two main aims of controlled ovarian stimulation are to create a cohort of developing follicles and to prevent premature spontaneous ovulation (Gallos et al. 2017). Controlled ovarian stimulation (COS) includes three basic elements (Gallos et al. 2017):

(1) exogenous gonadotropins to stimulate multi-follicular development;
(2) co-treatment with either gonadotropin releasing hormone (GnRH) agonist or antagonist to suppress pituitary function and prevent premature ovulation; and
(3) triggering of final oocyte maturation 36 to 38 hours prior to oocyte retrieval.

Gonadotropins are the fundamental agents used in ovulation stimulation. The combination of GnRH agonist pituitary suppression and exogenous gonadotropins in ART protocols has resulted in significant beneficial effects (Arslan et al. 2005). According to Arslan et al. (2005), these effects include improvement in the stimulation of follicular development and in the quality of developing oocytes, prevention of a premature LH release, decrease in cancellation rates and an overall improvement in the total reproductive potential. There are several brands available; they all contain FSH, which acts directly on the ovaries (Davies et al. 2009). Gonadotropin preparations available for use include human menopausal gonadotropin (hMG), a urinary product with FSH and LH activity, purified FHS (p-FSH), highly purified FSH (hp-FSH) and various recombinant FSH (rFSH) and LH (rFSH/rLH) preparations. GnRH agonists or antagonists are used in different protocols. According to Gallos et al. (2017), in the 'long-protocol', the GnRH agonist is started at least 2 weeks before stimulation and continued up until oocyte maturation is achieved. Alternatively, in the 'short-protocol' a GnRH agonist is started simultaneously with stimulation and continued up until the day of oocyte maturation trigger (Gallos et al. 2017). GnRH agonists are administered intramuscularly, subcutaneously or intranasally. In the 'long-protocol', the initial flare effect of the GnRH agonists is followed by desensitisation and down-regulation of the pituitary gland with an internalisation of the GnRH receptors (Gallos et al. 2017). This protocol is associated with a higher oocyte number and clinical pregnancy rates, however, there is evidence of an increase in the requirement of gonadotropins compared to a 'short-protocol' (Siristatidis 2015).

Another option for COS is the use of GnRH antagonists which require a shorter duration of use compared with the agonist GnRH 'long-protocol' and are mostly started on day 6 after initiation of stimulation (termed fixed antagonist protocol), continuing up until administration of a drug to trigger oocyte maturation (Gallos et al. 2017). GnRH antagonists act by binding to the GnRH receptors and preventing endogenous release of GnRH from the pituitary gland (Gallos et al. 2017). The GnRH antagonist protocols are associated with immediate LH suppression and decreased gonadotropin use. As a result, the antagonist protocols are associated with a significant reduction in ovarian hyperstimulation

syndrome (OHSS) without significantly decreasing the live birth rate (Al-Inany 2016).

Gonadotropins can also be used in ovulation induction protocols. A small dose of the gonadotropin is given daily or on alternate days, starting early in the menstrual cycle, usually by injection under the skin. The main risk of this treatment is a multiple birth and it can cause ovarian hyperstimulation. Due to the risk of multiple pregnancy, it is important to monitor the cycle with ultrasound to check the number of eggs developing (Davies et al. 2009). Most cycles lead to the release of 2 or 3 eggs, which leads to higher success rates, however, couples should be warned of the risk of multiple pregnancy. If too many eggs develop, the treatment may be cancelled and resumed the following month (Davies et al. 2009). Alternatively some couples may be offered 'follicle reduction' which removes excess eggs with ultrasound guidance or the treatment cycle may be converted to IVF (Davies et al. 2009).

At the end of the stimulation phase of an IVF cycle, a drug is used to trigger the final oocyte maturation, which is used to mimic the natural endogenous LH surge and initiate the process of ovulation before the mature eggs are collected from the woman and fertilized with sperm in the laboratory (Gallos et al. 2017). Two drugs are currently used: human chorionic gonadotropin (hCG), which is the most common drug, or GnRH agonist in an antagonist protocol. The advantage of using a GnRH agonist to trigger an oocyte maturation in an antagonist protocol is the further reduced incidence of OHSS compared to hCG because of it's sustained luteotrophic effect (Gallos et al. 2017). However, in the GnRH agonist trigger protocol, live birth and ongoing pregnancy rates are lower, which is thought to be a consequence of the luteal phase defect. Modifying the luteal phase support protocols to include small dosages of hCG can increase pregnancy rates, but it also increases the risk of OHSS (Humaidan 2011).

Controlled ovarian hyperstimulation is, therefore, a principle step of IVF therapy. Further, identifying a patient's ovarian response to gonadotropin stimulation as high, intermediate or low is important for prospectively optimizing COS protocols and for decreasing the risk for complications such as cycle cancellation due to inadequate response, or conversely, development of OHSS (Arslan et al. 2005). The ovarian response and the potential for conception of an

IVF cycle can be determined with high accuracy by the assessment of the ovarian reserve (Arslan et al. 2005).

IVF therapy has become increasingly simplified in recent years. The use of gonadotropin releasing hormone agonists with gonadotropins has resulted in greater ease of planning the superovulation stimulation than was possible with the earlier use of clomiphene citrate with gonadotropins (Saadat et al. 2004). That regimen had to be monitored carefully in order to predict and prevent the occurrence of endogenous preovulatory LH surge. In the absence of GnRH analog controlled cycles there is a cancellation rate of 15-20 percent because oocyte retrieval has to be performed 26-28 hours after the detection of the endogenous surge and this often meant that oocyte collections were performed at night and during weekends (Balen and Jacobs 2003). When GnRH agonists are used the oocyte retrieval can be precisely timed to occur 34-38 hours after the administration of hCG. Human chorionic gonadotropin acts as a surrogate for the normal mid-cycle LH surge and causes resumption of meiosis within the oocytes and their preparation for fertilization (Balen and Jacobs 2003). The use of GnRH antagonists prevents premature growth of LH, allows maximum mobilization of oocytes by minimizing the suppressive effect of GnRH agonists on ovarian receptors and eliminating ovarian suppression at the level of follicle mobilization (Lambalk et al. 2017). Transient gonadotropin suppression occurs a few hours after their administration, in contrast to GnRH agonists. Premature luteinization is also reduced in the GnRH-antagonist protocol, and this protocol has a number of different benefits for patients, including shorter stimulation, lower gonadotropin doses, and a lower risk of cyst formation (Lambalk et al. 2017).

1.4. Elevated Follicular Phase Progesterone and COS

Regardless of the stimulation protocols used in IVF cycles, a premature elevation in progesterone during the follicular phase of ovarian stimulation may occur in up to 38 percent of cycles (Bosch et al. 2003). In recent years, the importance of this serum progesterone elevation during IVF treatment has become a matter of intense debate (Lawrenz et al. 2016). Elevated progesterone levels before administration of hCG have been reported to adversely affect the outcome of IVF and embryo transfer (IVF-ET) in some studies (Urman et al.

1999). Different cut-offs have been used to define elevated progesterone during stimulated cycles ranging from 0.8 to 2.0 ng/mL (Givens et al. 1994; Ubaldi et al. 1995). The decision to administer the ovulatory dose of hCG is commonly based on the serum estradiol levels and follicle size (Urman et al. 1999). However, a high progesterone level during the follicular phase is often considered to be an indication for a freeze-all protocol because of the assumption that high progesterone levels may have a negative impact on endometrial receptivity (Urman et al. 1999).

Progesterone is usually measured during the luteal phase because it reflects the quality of the corpus luteum coming from lutenization of granulosa cells after the LH surge (Sonigo et al. 2014). As noted above, progesterone is essential before and during pregnancy as it plays a critical role in supporting the endometrium and hence survival of the conceptus (Spencer et al. 2004). Endometrial receptivity results from several molecular events triggered by progesterone after estrogen priming (Rosario et al. 2003). Moreover, a short exposure to physiological concentration of progesterone (in the range of follicular phase values) after estrogen priming has a stimulatory effect on LH secretion, by acting directly at the pituitary level (Couzinet et al. 1992).

In contrast to modest progesterone levels that characterize the first part of the menstrual cycle, a progressive and significant increase in the serum concentrations of progesterone takes place throughout the follicular phase in COS (Fanchin et al. 1996). In pituitary-functioning COS cycles, an acceleration of this phenomenon is observed toward the last days of the follicular phase while plasma LH levels start to increase (Urbancsek et al. 1990). The substantial circulating progesterone levels present during the late follicular phase of COS may be attributed to an amplified response of the granulosa cells of multiple follicles to endogenous LH (Trounson and Calabrese 1984). The follicular production of progesterone resulting from endogenous LH surges or premature luteinisation, is virtually eliminated by GnRH agonist administration, as these drugs prevent the LH surge, however, the progressive increase in plasma progesterone during the follicular phase of COS is surprisingly not prohibited by GnRH analogues (Adda-Herzbog 2018). Due to the presumably different nature of these two phenomena, Adda-Herzog et al. (2018) proposed to name the persistent increase in circulating progesterone levels observed during COS, even more with pituitary control by

GnRH analogues, as premature progesterone elevation rather than premature luteinization. A study done by Sonigo et al. (2014) supports this idea stating that the progesterone elevation does not reflect 'premature luteinization' because progesterone elevation, which occurs as the risk of endogenous LH surge, is usually controlled by simultaneous administration of GnRH analogues. Adda-Herzbog et al. (2018) further stated that the mechanisms responsible for the unexpected lack of influence of the pituitary suppression by GnRH analogues on the progesterone profile during the follicular phase of COS have been debated. However, one proposed hypothesis is the sensitivity of hyperstimulated ovaries to residual endogenous LH levels. Another possibility that could account for the occurrence of premature progesterone elevation is that granulosa cells are stimulated, not by low residual LH levels, but rather by the considerable amount of exogenous LH activity administered to foster the maturation of multiple follicles during COS (Adda-Herzbog 2018).

Late follicular phase progesterone elevation, commonly defined as progesterone levels of 1.5 ng/mL (4.77 nmol/L) or greater at the day of hCG trigger, has been reported in 6-30 percent of controlled ovarian stimulation cycles (Bosch et al. 2010; Venetis et al. 2015). The observation of worse pregnancy outcomes in fresh embryo transfer in IVF/ICSI cycles among patients with progesterone elevation compared to non-progesterone elevation has prompted clinicians to monitor progesterone levels during the late follicular phase or at the day of hCG trigger (Esteves et al. 2018).

The presence of elevated progesterone levels on day 2 of the cycle might be elicited by advanced or disrupted endometrial receptivity (Kolibianakis et al. 2004). Furthermore, the presence of a still functioning corpus luteum may provide a suboptimal endocrine milieu for new follicular growth and subsequently affect pregnancy rates (Mersereau et al. 2008). Little information is available concerning the association of elevated progesterone levels at initiation of ovarian stimulation with IVF outcome (Hamidine et al. 2014). In long GnRH agonist cycles, suppression of gonadotropins results in basal levels of steroid hormones at initiation of stimulation, and thus, consistently normal progesterone levels (Huang et al. 1996). However, elevated baseline progesterone levels have been reported in short GnRH agonist cycles and GnRH antagonist cycles (Kolibianakis et al. 2004). The study done by Sonigo et al. (2014) supports this by stating that serum

progesterone at the time of hCG is significantly higher in women treated with GnRH agonist as compared with GnRH antagonist. This difference can be explained by the stronger ovarian response to FSH as attested by the average difference of about 2 oocytes in favour of GnRH agonist. In addition, it has been shown that a higher endogenous LH concentration is observed during the last few days of stimulation in women treated with GnRH agonist as compared with those who received GnRH antagonist (Hugues et al. 2011).

Several publications have demonstrated that the risk of progesterone elevation is mainly dependent on the degree of ovarian response to FSH (Sonigo et al. 2014). Indeed, the serum progesterone rise at the time of hCG is strongly correlated to the serum estradiol concentration, to the number of retrieved oocytes and to the total dose of FSH required for ovarian stimulation (Bosch et al. 2010; Xu et al. 2012; Fatemi et al. 2013). Sonigo et al. (2014) stated that this is probably explained by the increase in 'granulosa cell mass' which produces progesterone under the control of FSH and that therefore, the incidence of progesterone elevation depends on the intensity of the ovarian response to FSH. Several studies have observed a linear relationship between the percentage of patients with progesterone elevation and the number of retrieved oocytes (Fatemi et al. 2013).

In order to prevent progesterone elevation during COS, delaying the administration of gonadotropins in GnRH antagonist cycles could result in normalization of progesterone values (Kolibianakis et al. 2004). Recently it has been suggested that pretreatment with a GnHR antagonist during 3 consecutive days before ovarian stimulation leads to normalization of progesterone levels, resulting in adequate ovarian stimulation and acceptable pregnancy rates (Blockeel et al. 2011).

As discussed, some studies have reported no connection between progesterone elevation in early follicular phase and IVF/ICSI outcomes, while other studies have showed that progesterone elevation is associated with lower pregnancy rates. Since a clear conclusion has not been reached, it remains important for clinicians to understand how ovarian stimulation might affect IVF/ICSI outcomes, therefore further research is required.

2. Hypothesis

Elevated serum progesterone level (>1.5 ng/mL) on day-2 of the menstrual cycle is associated with a lower pregnancy rate in IVF/ICSI antagonist cycles with fresh embryo transfer.

3. Objectives

The purpose of this study was to evaluate the effects of a normal and elevated day-2 serum progesterone level on IVF and ICSI outcomes regarding pregnancy rates. Further, the aim was to determine whether or not it is important to measure progesterone level in the early follicular phase. Currently, many clinicians are questioning the clinical validity of measuring progesterone level during the early follicular phase of stimulated cycles. One of the concerns is the ambiguity of which patients might benefit from progesterone monitoring during stimulated cycles and what would be the practical consequences of progesterone elevation to pregnancy success in an IVF/ICSI procedure (Esteves et al. 2018).

4. Patients and Methods

This was a one-center cohort retrospective study conducted at the Division of Human Reproduction, Department of Obstetrics and Gynecology, University Hospital Center Zagreb, Zagreb, Croatia during the period of February 2020 to May 2020. Data from 626 IVF/ICSI antagonist cycles were initially retrieved from the hospital charts. However, only the cases that had a measured day-2 serum progesterone level, and those in which the patient underwent fresh embryo transfers, were selected for this study. The study includes data from 136 patients who underwent a total of 189 fresh embryo transfers in IVF/ICSI antagonist cycles from January 2014 to January 2020. Serum levels of day-2 progesterone, anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH,) luteinzing hormone (LH), prolactin (PRL), total dose of stimulation, the number of oocytes retrieved, and the number of embryos transferred and frozen were compared among participants. The treating physician decided the protocol and type of gonadotropins before starting the stimulation, on a case-by-case basis per patient characteristics (age, BMI and AMH). Ovarian stimulation was carried out using recombinant FSH (rFSH, Gonal F®, MerckSerono, Germany; Puregon®, Organon, Netherlands; Ovaleap®, Theramex, Ireland or Bemfola®, GedeonRichter, Plc) or highly purified human menopausal gonadotropin (Menopur®, Ferring Pharmaceuticals, Switzerland). Follicle growth was assessed by transvaginal ultrasound. A daily dose of 0.25 mg of GnRH antagonist cetrorelix acetate (Cetrotide®, MerckSerono Germany) or garnirelix (Orgalutran®, Merck Sharp & Dohme, Netherlands) administration was initiated on stimulation day 6 and continued until the day of human chorionic gonadotropin (hCG) administration. Final oocyte maturation was triggered by subcutaneous administration of 7500 IU of hCG (Ovitrelle®, MerckSerono, Germany) when at least one follicle had reached 18 mm in diameter and two had reached 16 mm measured by transvaginal ultrasonography (Voluson S8, General Electric). Transvaginal oocyte retrieval was scheduled 34-36 hours later. Embryo transfer of a maximum of 2 embryos was done on day 3 or 5 after retrieval. All other good quality embryos were frozen on day 5.

To test our hypothesis that elevated serum progesterone levels during COS decreases pregnancy rates, we selected a cut-off value of >1.5 ng/mL. This

threshold was based on previous studies (Hamdine et al. 2014; Panaino et al. 2017; Mahapatro and Radhakrishan 2017). According to the day-2 serum progesterone level the total number of cases (n=189) were divided into two groups, normal progesterone level ($\leq$ 1.5 ng/mL) group (n=134) and high progesterone level (>1.5 ng/mL) group (n=55), respectively.

Category variables are described as percentages, and the continuous ones are described with the arithmetic mean ± standard deviation. To compare groups (independence testing) with categorical variables, we used Pearson's χ^2 test, while we used parametric Student's t-test to compare continuous variables. All statistical calculations were performed using the computer program for statistical analysis SPSS for Windows, version 22.0 (Statistical Package for the Social Sciences Inc., Illinois, USA). For all tests, the difference was considered statistically significant when $p<0.05$.

5. Results

A total of 189 cases were included in the study and according to the day-2 serum progesterone level they were divided into two groups. A high progesterone level was defined as a value of >1.5 ng/mL.

Out of the 189 cases, 134 cases had a normal progesterone level (≤1.5 ng/mL), while 55 cases had a high progesterone level (>1.5 ng/mL). Table 1 shows the demographic distribution of age, and body mass index (BMI).

Table 1. Demographic characteristics of each group

Parameters	Normal P group	n	High P group	n	p-value*
Age (years)	35.1±4.3	134	34.6±3.5	55	0.408
BMI (kg/m^2)	23.2±3.6	72	22.1±2.3	33	0.068

*Student's t-test

There was no statistically significant difference in these parameters between the two groups (p=0.408 and p=0.068, respectively). The mean age of the normal progesterone group was 35.1±4.3 years and of the high progesterone group was 34.6±3.5 years. Meanwhile, the mean BMI for the normal progesterone group and the high progesterone group was 23.2±3.6 kg/m^2 and 22.1±2.3 kg/m^2, respectively.

The comparison of hormonal parameters between the groups is presented in Table 2. The mean serum levels of AMH, FSH, LH and PRL for the normal progesterone group versus the high progesterone group were not significantly different as presented in Table 2.

Table 2. The comparison of hormonal parameters between groups

Parameters	Normal P group	n	High P group	n	p-value*
AMH (pmol/L)	18.9±16.1	101	16.0±17.1	38	0.361
FSH (IU/L)	7.2±2.4	115	6.9±2.4	51	0.387

Parameters	Normal P group	n	High P group	n	p-value*
LH (IU/L)	5.6±6.6	111	5.6±2.3	50	0.975
PRL (μg/L)	15.4±10.2	82	14.5±7.6	28	0.603

*Student's t-test

The total dose of stimulation was similar in both groups (p=0.837, Table 3). The average number of oocytes retrieved was also similar between the two groups (7.6±4.8 in the normal progesterone group vs. 7.1±5.1 in the high progesterone group). The frequency between IVF and ICSI was comparable between groups. Table 3 also shows the number of embryos transferred and the number of embryos that were frozen. No significant difference was found between the two groups for these two characteristics either (p-value for the number of embryos transferred was 0.743, while the p-value for the number of embryos frozen was 0.765).

Table 3. Stimulation and embryological parameters for each group

Parameter	Normal P group n = 134	High P group n = 55	p- value*
Total dose of stimulation (IU/L)	2259.5±633.1	2285.9±854.1	0.837
Number of oocytes retrieved	7.6±4.8	7.1±5.1	0.515
IVF (%)	43.3	41.8	0.873
ICSI (%)	56.7	58.2	
Number of embryos transferred	1.5±0.5	1.6±0.5	0.743
Number of frozen embryos (%)			0.765
0	83.5	78.2	
1	3.0	7.3	
2	8.3	7.3	
3	2.3	1.8	
4	2.3	3.6	
5	0.8	1.8	

*Student's t-test continuous variables, Pearson's χ^2 test categorical variables

Although a higher percentage of pregnancies based on positive hCG was found in the normal progesterone level group (33.6%) than in the high progesterone group (23.8%), there was no statistically significant difference between the two groups (p=0.178) as shown in Table 4 and Figure 1.

Table 4. Clinical outcome between the two groups

Parameters	Normal P group n = 134	High P group n = 55	p-value*
Positive hCG (%)	33.6	23.8	0.178

*Pearson's χ^2 test

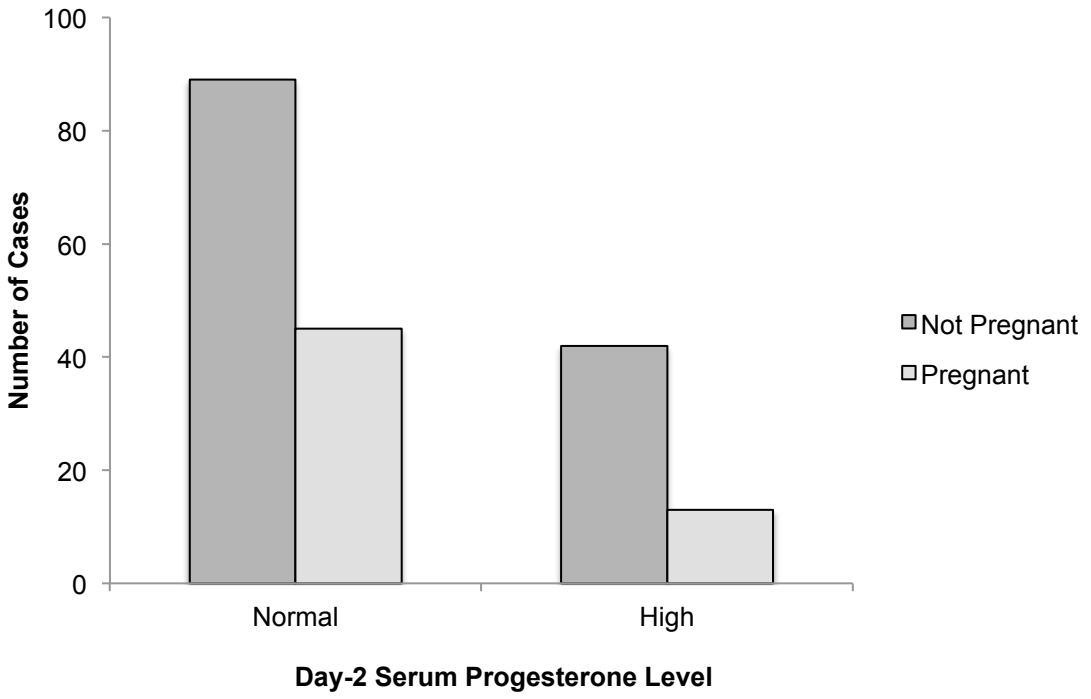

Figure 1. IVF/ICSI outcome based on day-2 serum progesterone level between the two groups

6. Discussion

To date, little information is available on the incidence or outcomes of elevated progesterone levels in early follicular phase IVF patients (Tang et al. 2007). Further, the origin and regulation of progesterone secretion throughout the follicular phase of the natural menstrual cycle remain poorly understood (Hamdine et al. 2014). Synthesis of progesterone involves the cytochrome P450 side-chain cleavage enzyme, which is abundant in the corpus luteum, the theca interna of the growing follicle, the theca externa, and the cortex of the adrenal glands (Tang et al. 2007). Therefore, all of these tissues are potential sources of elevated serum follicular phase progesterone (Tang et al. 2007). During the early follicular phase, it is even postulated that the adrenal glands might be the main source of circulating progesterone, while during the late follicular phase ovaries mainly contribute the progesterone (De Geyter et al. 2002). However, there is no established direct link between the gonadal axis and the adrenal axis and as a result the question of why certain women have elevated baseline progesterone levels still remains (Hamdine et al. 2014). As well, what is reported as the beginning on menstruation, may actually be breakthrough bleeding before the menstruation actually begins so the high progesterone level could be considered as normal late luteal phase progesterone levels (Hamdine et al. 2014). Regardless of the origin, it is important to know what the effects of elevated progesterone levels in the early follicular phase are, however according to Hamdine et al. (2013 Sept) there is an obvious lack of information in the literature on this topic. This study showed that there was no statistically significant difference between pregnancy rates of normal and elevated day-2 serum progesterone levels. These findings were supported by the study performed by Mahapatro and Radhakrishan (2017). In that study there was no statistically significant difference in terms of age, total dose of gonadotropins, the total number of oocytes retrieved or the number of oocytes transferred between the two groups. Mahapatro and Radhakrishan (2017) also did not find a statistically significant difference in those same parameters, however, they did find a statistically significant difference in antral follicle count, with the cycles in which the progesterone level was normal, experiencing an increased number of antral follicles.

Hamdine et al. (2014) carried out a prospective study in combination with a systemic review and meta-analysis, to assess the effect of day-2 progesterone level on pregnancy outcomes. They found that among 158 patients, 13.3% had an elevated day-2 serum progesterone level. In this study among 189 cases (136 patients), 29.1% had an elevated day-2 serum progesterone level. Similar to our results, in the prospective intervention study done by Hamdine et al. in 2014, it was found that elevated progesterone levels on day 2 may affect ongoing pregnancy rates in GnRH antagonist cycles, however the observed differences did not reach statistical significance. In this study we also found a lower pregnancy rate in the elevated progesterone group (23.8% vs. 33.6%, p-value 0.178), but it was also not statistically significant. From the meta-analysis that was conducted on this topic in that same paper, Hamdine et al. (2014) concluded that early or late GnRH antagonist initiation had no statistically significant differential impact on the effect of high or normal progesterone on ongoing pregnancy rates. This conclusion suggests that management of patients with elevated progesterone levels at the beginning of stimulation cannot be achieved by early initiation of GnRH antagonist treatment. Overall, the Hamdine et al. (2014) paper concluded that routine screening for progesterone is not recommended due to the relatively low incidence of elevated day-2 progesterone level affecting ongoing pregnancy rates as well as the absence of a proven effective treatment strategy.

Similarly the study, done by Sims et al. (1994), supports the findings in the current study. Sims et al. (1994) found that in patients with a risk of poor response to stimulation after treatment with a GnRH-agonist down-regulation short protocol and high-dose FSH, 85% of the cycles showed elevated serum progesterone (>1 ng/mL) on cycle days 2-6. Those cycles were linked to a decreased ovarian response, however, no significant difference in pregnancy outcomes were found. Additionally, the initial assumption that a high progesterone level is detrimental for implantation was not supported by the study done by Urman et al. (1999); instead they observed a higher implantation rate in the high progesterone group, although it was not statistically significant.

Another study done in 2015 by Listijono et al. found a low incidence of raised early follicular progesterone levels in their monitored group, leading them to question it as a useable measurement. Further they found that in cases where the early follicular progesterone measurement was high, there was an association

with a significantly lower incidence of elevated pre-trigger progesterone levels. However, the clinical pregnancy rate was the same in both groups, indicating little value in measuring early follicular progesterone levels (Listijono et al. 2015). Several reports have suggested an association between elevated serum progesterone levels on day of hCG administration and unfavourable cycle outcomes in patients undergoing assisted reproduction (Urman et al. 1999). The Listijono et al. (2015) study also confirmed that elevation in pre-trigger progesterone level is associated with decreased clinical pregnancy rate, demonstrating that the most important progesterone measurements are those in the late follicular/pre-trigger phase with the potential to improve outcomes by undertaking a freeze all and transfer in a future cycle protocol.

In the Blockeel et al. (2011) prospective study, in the case of the elevated progesterone level group, GnRH antagonist was administered for three consecutive days in order to normalize the baseline progesterone levels. With this approach, a non-statistically significant difference in pregnancy rates between the normal and the high progesterone group was subsequently reported. However, in the meta-analysis by Hamdine et al. (2014) they state that the clear impact of elevated progesterone levels before the initiation of ovarian stimulation with GnRH antagonist co-treatment on clinical outcome remains controversial. Further they stated that although a number of treatment options have been proposed, there is still a lack of high-quality evidence in regards to management of women with elevated baseline progesterone levels to optimize or normalize clinical outcomes (Hamdine et al. 2014).

However, there are also reports that showed that increased plasma progesterone level during the early follicular phase may adversely affect follicular development, oocyte quality and success rate of the cycle (Mahapatro and Radhakrishan 2017). A study from Brussels, Belgium, done by Kolibianakis et al. (2004) defined a high progesterone value as >1.6 ng/mL and reported 20 patients with high progesterone in their study out of 410 patients. Due to those findings, initiation of stimulation was postponed for 1-2 days in the elevated progesterone group and started if the progesterone was normal. Regardless of the delay in protocol initiation, their study found that a significantly lower chance of achieving an ongoing pregnancy is present in patients with high progesterone level on day 2 of a GnRH antagonist cycle. They suggested that in cases of high progesterone,

the cycle should be cancelled, stating that it might represent a coincidental event that should not reoccur in the next cycle (Kolibianakis et al. 2004). Although this current study had fewer participants overall, more cases had a high progesterone on day 2 than Kolibianakis et al. (2004), 55 versus 20. As well in this study a lower cut-off for high progesterone was used than in Kolibianakis et al. (2004). These differences in in the study criteria may have lead to the different conclusions that were reached.

Panaino et al. (2017) looked at progesterone levels at the beginning and end of the follicular phase. Unlike the results of our study, their study found that there is an impact of early progesterone levels in antagonist cycles on pregnancies, mainly when the higher progesterone level (>1.5 ng/mL) is also present at the end of the stimulation period. They also found that there were significantly better live birth rates in women with normal progesterone levels than those with elevated levels, either in the beginning or at the end of the COS (Panaino et al. 2017).

Tang et al. (2007) also examined the effects of elevated progesterone levels early in the follicular phase, however they tested the progesterone on day 4 of the cycle. Their results showed that in patients undergoing GnRH agonist down-regulation short protocols for IVF, if the progesterone level is significantly elevated (>3 ng/mL) on day 4 of stimulation, then the chances of pregnancy are decreased (Tang et al. 2007). Likewise, the study done by Bushaqer et al. (2017) looked at how different IVF stimulations were affected by an increased progesterone level and they found that in the short and long GnRH agonist protocols, high progesterone level (>1.5 ng/mL) did not affect clinical pregnancy while in a fixed GnRH antagonist protocol, high progesterone level (>2 ng/mL) affected the clinical pregnancy adversely.

There are a few limitations to the present study. One limitation includes the small sample size, which could lead to imprecise effect size estimates. Another limitation may be that we did not limit our patients to only one IVF/ICSI case in the study, meaning that some of the cases belong to the same patient. Some other studies only entered their patients into the study once, so when comparing their studies to ours it may affect the end result of each study, making the conclusions different. Another limitation in comparing our study with others is the fact that the definition for a high progesterone level is not consistent. In order to be able to

compare our study to other studies, we chose a high progesterone value of >1.5 ng/mL as the threshold. However, for instance if our threshold for a high progesterone level was changed to >1.6 ng/mL, then maybe we would have gotten different results in our study. Finally, a limitation of this study may be that different ART protocols were used on the patients and as a result there may be some protocols that have a better success rate than others when the progesterone level is increased. However, the data in this study was not analyzed to that degree.

7. Conclusion

During the past few years, discussions have been centered in both tailoring and individualizing protocols and costs, as well as patient convenience during ART cycles (Panaino et al. 2017). In conclusion, this study found that an elevated day-2 serum progesterone level does not have a statistically significant difference on the clinical outcome of IVF/ICSI.

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10. Biography

Ana Mičić was born on November 1, 1992 in Supetar, Croatia. Due to the homeland war at the time, at the age of two, she and her family moved to Canada. After completing secondary school, she attended the University of Western Ontario in Canada where she earned a Bachelors of Science, Honors Specialization in Biology, in 2014.

In the fall of 2014, she enrolled into the University of Zagreb School of Medicine, Medical Studies in English. During her studies in Zagreb, Ana strived to be an active part of the student body and she was elected as class representative from 2nd year to the final year of her studies. As a class representative, Ana was an active member of the student council for the English program (eMed), helping with various projects to improve the program. During her final year in medical school, while acting as a student representative, she was also elected to be the secretary of eMed.

Ana's interest in gynecology and obstetrics started in secondary school when she did a co-op course and spent time in a birth unit. Since then her fascination with the specialization and IVF has grown, leading to the interest in doing this research. As well, during the summer between 5th and 6th year, Ana had the opportunity to do a clinical rotation in neonatology at the Health Sciences Centre in Winnipeg. That was a very rewarding experience for her, which also verified her interest in pediatrics. In the future, Ana hopes to practice in gynecology or pediatrics.