Different dosage use of a single FSH application for superstimulation prior to ovum pick up in Holstein heifers

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ABSTRACT

The aim of this study was to determine the effect of different dosages of single FSH administration on superstimulation response, oocyte yield and *in vitro* embryo production prior to oocyte retrieval. The animal material for the study consisted of 10 healthy Holstein heifers. In group S75, 10 µg of GnRH was administered intramuscularly on a random day of the estrous cycle. At the same time, an intravaginal progesterone device was inserted into the vagina. On day 4, 25 mg of PGF2α was administered intramuscularly. On the 5th day, the intravaginal progesterone device was removed and at the same time, 75 µg Stimufol (1/8 of traditional superovulation dosage) was administered intramuscularly as a single dose. Oocytes were retrieved using transvaginal ultrasound, 2 days after FSH injection. The same protocol was applied in Groups S150 and S300, except for the FSH dosage. In the S150 group, 150 µg Stimufol (1/4 of traditional superovulation dosage), and in the S300 group, 300 µg Stimufol (1/2 of traditional superovulation dosage) were administered intramuscularly as a single dose. In the control group, oocytes were collected on a random day of the estrous cycle without hormone stimulation. Before oocyte retrieval, the number of medium-sized follicles (3-8 mm) in the ovary was found to be higher in the superstimulation groups (groups S75, S150 and S300) than in the control group ($P<0.05$). The study determined that the S300 group had a higher total number of oocytes, viable oocytes, and recovery rate compared to the control group ($P<0.05$). The number of blastocysts per OPU was higher in group S300 than in all the other groups ($P<0.05$). Furthermore, the number of blastocysts per OPU in Groups S75 and S150 was higher than in the control group ($P<0.05$). As a result, it was observed that success can be achieved with the use of a low dosage of FSH, but blastocyst yield increases with a higher dosage of FSH. Superstimulation applications with a single FSH before OPU were thought to increase the success of *in vitro* embryo production.

Key words: FSH dosage; Holstein heifer; *in vitro* embryo production; ovum pick-up; superstimulation

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Introduction

The world's rapidly increasing population is driving up demand for animal-based foods. Studies are being performed to enhance animal productivity in various fields. *In vitro* embryo production (IVEP) is an assisted reproductive technology that allows the production of many offspring in a short time from animals with high genetic capacity. Oocytes for bovine *in vitro* embryo production are obtained from slaughterhouse ovaries or from living donors by ovum pick up (OPU). (PIETERSE et al., 1988; BOLS and STOUT, 2018; WRENZYCKI, 2018). *In vitro* embryo production has become a serious alternative to traditional multiple ovulation and embryo transfer (MOET) when combined with the transvaginal follicular aspiration technique (FERRÉ et al., 2020). *In vitro* embryo production has recently observed a significant increase due to the rapid increase in high-yielding the number of animals obtained through OPU/IVEP. Approximately 70% of embryos worldwide are produced *in vitro*. According to the latest data published by the International Society for Embryo Technologies (IETS), 1,595,204 transferable embryos were produced worldwide, and 239 in Turkey, with OPU/IVEP in 2022. In 40.7% of the OPU sessions in which these embryos were produced, superstimulation with FSH was performed prior to OPU. This rate is even higher in regions with significant embryo industries, such as Europe and North America (88% and 69.2% respectively) (VIANA, 2023).

The success of OPU/IVEP programs is directly affected by the number and quality of oocytes collected. The oocyte yield in OPU applications depends on the number of follicles suitable for puncture (RIZOS et al., 2005; GETZ et al., 2011). Pre-OPU hormonal treatments are designed to increase the number of follicles retrieved per aspiration. FSH treatment prior to OPU is generally expected to increase the number of follicles of ideal diameter and oocyte quality. When the follicles in the bovine ovary reach a diameter of 3 mm, the oocytes gain the ability to develop into a blastocyst after fertilization (BLONDIN and SIRARD, 1995; HAGEMANN el al., 1999; DE WIT and KRUIP 2001; HUANG et al., 2023). Accordingly, it is stated that antral follicles ranging from 3-8 mm in size are the primary source of oocytes used in IVEP in cattle (HENDRIKSEN et al., 2004). Superstimulation before OPU is a commonly used practice to increase oocyte yield (BÓ and MAPLETOFT, 2020). For superstimulation prior to OPU, applications similar to conventional superovulation protocols are performed. However, their purpose is to create more and appropriately sized follicles, rather than stimulating multiple ovulation. In addition, as the dosage of FSH used increases, the endocrine changes that occur in the ovary increase and the time it takes to return to normal physiology is prolonged. Since the OPU technique can be applied to the same animal at short intervals, lower amounts of hormones and shorter duration superstimulation protocols are required. Therefore, modifications are necessary in the dosage and timing of the treatments administered (AERTS and BOLS, 2010; BOLS and STOUT, 2018). For this purpose, many methods are being tried in pre-OPU superstimulation protocols, such as single dose use of FSH, different application routes, reducing the amount of FSH applied, and dissolving it in different polymers (BÓ et al., 1994; CHAUBAL et al., 2007; ONGARATTO et al., 2010; VIEIRA et al., 2016).

In the majority of OPU sessions performed worldwide, superstimulation with FSH is performed. In addition, most pre-OPU superstimulation studies use repeated doses of FSH (NAWAZ et al., 2022). This results in an increased financial and labor burden for embryo production with OPU/IVEP. Embryo costs can be reduced by avoiding overuse use of FSH by determining the optimal dosage for single FSH applications. In this study, the aim was to determine the effect of different dosages on superstimulation response, oocyte yield and *in vitro* embryo production in a single FSH application for superstimulation.

Materials and methods

All animal applications were approved by the Selcuk University Local Ethics Committee for Animal Experiments (Approval Number: 2024/01/23). All applied methods are in accordance with the ARRIVE guidelines.

Animals. The study's animal material consisted of 10 healthy Holstein heifers selected from a farm where genomic selection was applied. Ultrasonographic examination of the animals revealed no pathology in the ovaries or uterus, and estrus had been observed at least once. The animals were 14-16 months old and their body condition scores ranged from 2.25 to 3.75. To ensure that individual differences in oocyte yield did not affect the results, four different OPU sessions were applied, and all the animals received all the treatments. Between sessions, the animals rested for one estrous cycle (21 days). The study was conducted as a cross-over design. Animals were fed ad libitum throughout the study. The ration included hay, maize silage, lucerne silage, concentrate feed, lucerne, vitamin and mineral supplements.

Experimental applications. S75 (n=10): On a random day of the estrous cycle, 10 µg GnRH (Buserelin, Receptal®, Intervet, Unterschleissheim, Germany) was administered intramuscularly. At the same time an intravaginal progesterone device (ClDR 1380®, Zoetis, Auckland, New Zealand) containing 1.38 g of progesterone was inserted into the vagina. On day 4, 25 mg PGF2α (Dinoprost, Dinolytic®, Zoetis, New Jersey, NJ, USA) was administered intramuscularly. On the 5th day, the intravaginal progesterone device was removed. At the same time, 75 µg Stimufol (Stimufol®, Reprobiol, Ouffet, Belgium) (1/8 of traditional superovulation dosage) was administered intramuscularly as a single dose. Oocytes were retrieved using transvaginal ultrasound, 2 days after FSH injection.

S150 (n=10): On a random day of the estrous cycle, 10 µg GnRH was administered intramuscularly. At the same time an intravaginal progesterone device containing 1.38 g of progesterone was inserted into the vagina. On day 4, 25 mg PGF2α was administered intramuscularly. On the 5th day, the intravaginal progesterone device was removed. At the same time, 150 µg Stimufol (1/4 of traditional superovulation dosage) was administered intramuscularly as a single dose. Oocytes were retrieved using transvaginal ultrasound, 2 days after FSH injection.

S300 (n=10): On a random day of the estrous cycle, 10 µg GnRH was administered intramuscularly. At the same time an intravaginal progesterone device containing 1.38 g of progesterone was inserted into the vagina. On day 4, 25 mg PGF2α was administered intramuscularly. On the 5th day, the intravaginal progesterone device was removed. At the same time, 300 µg Stimufol (1/2 of traditional superovulation dosage) was administered intramuscularly as a single dose. Oocytes were retrieved using transvaginal ultrasound, 2 days after FSH injection.

Control (n:10): Superstimulation was not applied to the animals. Oocytes were collected on a random day of the estrous cycle.

A schematic representation of the OPU/IVEP experimental groups and superstimulation protocols is shown in Fig. 1.

Oocyte retrieval and classification of the cumulus oocyte complex. During oocyte collection, a catheter-aspiration device (aspiration pump for bovine OPU, 230 V, Minitube, Germany) combination, 4.0-9.0 MHz microconvex probe (SC3123 VET, Esaote, Italy) and real-time ultrasonography device (Esaote MyLab TwiceVet, 5001, Italy) were used. Aspiration vacuum pressure was 80-90 mm/Hg during oocyte retrieval. The follicles in the ovary were classified on the basis of their diameter as small (0-3 mm), medium (3-8 mm), and large (>8 mm). All the follicles that could be aspirated from the ovary were aspirated using a catheter fitted with a 20-gauge needle, accompanied by a special microconvex vaginal probe.

The collected oocytes were examined under a stereomicroscope (S ApoE, Leica, Germany). The morphological characteristics were used to classify the cumulus oocyte complexes (COCs). The oocytes were evaluated for morphological features, including cytoplasm homogeneity, cumulus cell compactness, and cumulus cell layer. The oocytes were classified on the basis of their quality, which was graded as Grade A (very good), Grade B (good), Grade C (fair), or Grade D (poor) (PETYIM et al., 2003; GORDON, 2003). Grade A, B and C quality oocytes were evaluated as viable for *in vitro* embryo production.

In vitro embryo production. Mediums prepared by a commercial company (IVF Bioscience, Unit-

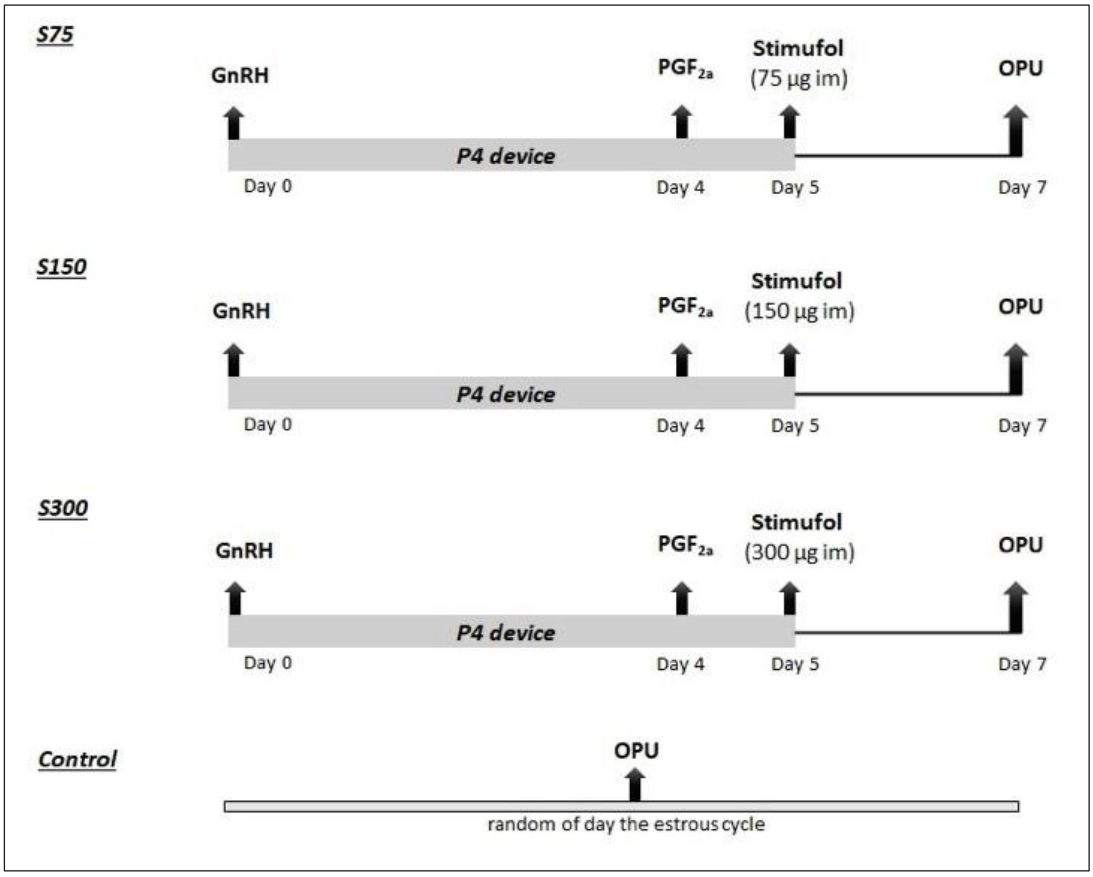

Fig. 1. Superstimulation protocols and experimental groups applied prior to OPU
(P4: progesterone device; PGF2α: dinoprost; GnRH: buserelin acetate; Stimufol: 500 µg Porcine FSH and 100 µg Porcine LH; OPU: Ovum - Pick Up)

ed Kingdom) were used during the *in vitro* embryo production stages. The quality of the COCs was evaluated after aspiration, and they were washed three times in oocyte washing medium (BO-Wash). Afterwards, COCs were placed in an *in vitro* maturation environment (BO-IVM) and incubated at 38.5°C and 5.5% CO2 for 20-22 hr. After *in vitro* maturation, the oocytes were transferred to the medium for *in vitro* fertilization. Semen of the same sire was employed for IVF throughout the study. In semen preparation, the sperm washing proce- dure was performed using the semen preparation medium (BO-SemenPrep). After completing the process, the number of spermatozoa was calculated to be 1 million per mL. These were then added to the *in vitro* fertilization media containing cumulus-oocyte complexes (Incubated for 20 hr at 38.5°C, 5% CO2). After fertilization, the oocytes were vortexed to remove the cumulus cells. The zygotes were transferred to an *in vitro* culture medium (BO-IVC) coated with mineral oil (BO-Oil). Zygotes were cultured *in vitro* in BO-IVC medium at 38.5°C, 6% CO2, and 6% O2 for 7 days. The embryos' quality and developmental stages were evaluated according to the International Embryo Transfer Society (IETS) (BÓ and MAPLETOFT, 2013; ALKAN et al., 2023).

Statistical analysis. The statistical evaluation of the data was performed using the SPSS 25.0 program (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). The homogeneity of variances and normality prerequisites of the variables were checked using the Kolmogorov-Smirnov test. The mean ± standard deviation (SD) of normally distributed variables was found. The variables were evaluated using One-way ANOVA (one-way analysis of variance) and the post-hoc Tukey HSD test.

Proportions were analysed by the Chi-square test. The differences were considered statistically significant at the 95% confidence level (P<0.05).

Results

The number of follicles of various diameters, as determined by ultrasound prior to aspiration, is presented in Table 1. There was no statistically significant difference in the mean number of antral follicles during OPU between the groups. The control group had a higher number of small follicles (0-3mm) compared to all the other groups (P<0.05). The study found that the superstimulation groups (S75, S150, S300) had a higher number of medium diameter follicles (3-8mm) compared to the control group (P<0.05). The number of medium-sized follicles was similar in all the superstimulation groups (S75, S150, S300). No statistically significant difference was detected between the groups in terms of the number of large diameter follicles (>8 mm).

The results of OPU/IVEP among the experimental groups are presented in Table 2. The recovery rate and total oocyte number obtained was found to be higher in the S300 group than in the control group (P<0.05). Additionally, the total number of oocytes was similar between superstimulation groups (S75, S150, S300). The number of viable oocytes was found to be higher in the S300 group than in the control group (P<0.05). There was no statistically difference in the num- ber of viable oocytes among the superstimulation

Table 1. Number of follicles according to their diameter in the ovary during oocyte retrieval between groups (mean ± standard deviation)

	S75	S150	S300	Control
0 - 3 mm / %	7.40 ± 3.53^{a} / 27.62	7.50 ± 3.50^{a} / 29.41	7.70 ± 2.36^{a} / 26.46	10.90 ± 3.81^{b} / 49.54
3 - 8 mm / %	18.80 ± 12.82^{a} / 70.15	16.90 ± 8.61^{a} / 66.27	19.80 ± 16.43^{a} / 68.05	9.90 ± 2.96^{b} / 45.01
> 8 mm / %	0.69 ± 0.60 / 2.23	1.10 ± 0.73 / 4.32	1.60 ± 1.57 / 5.49	1.2 ± 0.78 / 5.45
Total	26.80 ± 16.29	25.50 ± 10.86	29.10 ± 16.72	22.00 ± 5.14

^{a,b} The statistical difference within rows (P<0.05)

Table 2. Results of OPU/IVEP between groups after applications (mean ± standard deviation)

	S75	S150	S300	Control
Recovery Rate (%)	55.22^{ab}	48.23^{ab}	67.69^{b}	35.90^{a}
Total COCs / OPU	14.80 ± 8.31^{ab}	12.30 ± 7.08^{ab}	19.70 ± 16.11^{a}	7.90 ± 4.43^{b}
Viable COCs / OPU	8.70 ± 4.81^{ab}	9.00 ± 6.89^{ab}	12.40 ± 9.95^{a}	5.20 ± 2.74^{b}
Cleaved oocytes / OPU	6.30 ± 3.86^{a}	7.10 ± 6.52^{a}	9.40 ± 8.31^{a}	3.70 ± 2.98^{b}
Cleavage rate (%)	72.41	78.88	75.80	71.15
Blastocysts/OPU	2.70 ± 0.82^{b}	2.90 ± 0.87^{b}	4.80 ± 1.31^{a}	1.50 ± 0.52^{c}
Blastocyst rate (%)	31.03^{a}	32.22^{a}	38.70^{b}	28.84^{a}

$^{a-c}$ The statistical difference within rows (P<0.05)

groups (S75, S150, S300). The recovery rate, total and viable oocyte numbers in the S75 and S150 groups were similar to the S300 and control groups (P>0.05).The number of cleaved oocytes was lower in the control group than in all other groups (P<0.05).

The number of cleaved oocytes was lower in the control group than in the other groups (P<0.05). There was no statistical difference between the superstimulation groups (S75, S 150, S 300) in the number of cleaved oocytes. Additionally, no statistical difference was detected between the groups in terms of cleavage rate. The number of blastocysts per OPU was higher in the S300 group compared to the other groups (P<0.05). In addition, the number of blastocysts per OPU was higher in the S75 and S150 groups than in the control group. The blastocyst rate was found to be statistically higher in the S300 group than in all the other groups.

Discussion

Although many studies have shown that the use of FSH prior to oocyte retrieval increases the success of *in vitro* embryo production, there is still debate about the appropriate dosage of FSH to use in superstimulation (NAWAZ et al., 2022). In this study, the effect of single FSH application dosage in pre-OPU superstimulation protocols on superstimulation response, oocyte yield, recovery rate and IVEP was evaluated.

The antral follicle count (AFC) during OPU applications provides information about the ovarian reserve and primordial follicle number of donor animals. Donors with high AFC exhibit higher superstimulation response and oocyte yield (IRELAND et al., 2007; IRELAND et al., 2008). However, hormonal treatments administered before OPU have been reported to affect the diameter of follicles, but not the number of antral follicles. The number of antral follicles is generally influenced by fac- tors such as nutrition, breed, and temperature (DE ROOVER et al., 2005; WALSH et al., 2014). In the present study, no statistical difference was detected between the groups in terms of the total number of antral follicles during oocyte retrieval. The reason for this may be that the animals were kept in a facility where genomic selection is carried out and under similar environmental conditions.

The size of the follicles in the ovary is an important factor that affects the efficiency of IVEP during OPU procedures (VASSENA et al., 2003). It is stated that COCs obtained from medium-sized follicles (3-10 mm) have higher developmental competence and the ability to reach the blastocyst. Therefore, superstimulation applications prior to OPU are expected to increase the number of medium-sized follicles (DIELEMAN et al., 2002; DE ROOVER et al., 2005; SIRARD, 2012). VIEIRA et al. (2016) stated that superstimulation application before OPU increases the number of medium-sized follicles (6-10 mm). The same study found no correlation between FSH dosage and the number of medium-sized follicles. In another study conducted on Holstein cattle, it was found that superstimulation increased the number of medium-sized follicles (6-10 mm). They also found that the superstimulation response increased in parallel with the FSH dosage (HAYDEN et al., 2023). This study found that pre-OPU superstimulation increased the number of medium diameter follicles (3-8 mm). However, the number of medium-sized follicles was not statistically different between the superstimulation groups using different dosages of FSH.

In OPU/IVEP programs, superstimulation applications are used to increase oocyte yield and the number of follicles of appropriate diameter. Increasing oocyte yield allows obtaining more blastocysts. Many studies have shown positive effects of FSH superstimulation on the number of cleaved oocytes and blastocyst yield (GOODHAND et al., 1999; VIEIRA et al., 2014; 2016; EGASHIRA et al., 2019). Repeated doses of FSH applications are generally used for ovarian superstimulation. However, as OPU can be applied at short intervals, shorter superstimulation protocols are provided with a single FSH application (SILVA et al., 2019). ONGARATTO et al. (2020) state in their study that a single application of FSH is sufficient for superstimulation. In addition, the same study reported that superstimulation with a single FSH application also increased the total number of oocytes, viable oocytes and blastocysts. A different study stated that superstimulation with a single FSH injection

prior to oocyte retrieval increased oocyte and blastocyst yields (SANTOS et al., 2021). In a study conducted to determine the optimal FSH dosage for superstimulation applications, it was reported that as the amount of FSH increased, the superstimulation response and the number of blastocysts also increased (BÓ et al., 1994). In another study investigating the effect of FSH dosage in superstimulation before OPU, it was found that increasing the FSH dosage had no effect on the recovery rate and the number of viable oocytes. However, it stated that the number of dividing oocytes and blastocysts increased in parallel with increasing FSH dosage (HAYDEN et al., 2023). LOLLATO et al. (2022) reported in their study that increasing the FSH dosage used in superovulation increased the embryo yield. In a study investigating the effect of FSH dosage in superovulation, it was found that more embryos were obtained in the group using high-dose FSH (DELL'EVA et al., 2019). This study found that total and viable oocyte counts were higher in the S300 group than in the control group. However, similar results were obtained between the superstimulation groups (S75, S150, S300) in terms of total and viable oocyte numbers. The number of cleaved oocytes in the superstimulation groups was found to be higher than in the control group. The number of cleaved oocytes was not statistically different between superstimulation groups according to FSH dosage. The number of blastocysts per OPU was higher in the S300 group than in all other groups. It was determined that the average number of blastocysts in the S75 and S150 groups was higher than in the control group.

Conclusions

Superstimulation prior to oocyte retrieval had no effect on AFC. Superstimulation application increased the number of medium-sized follicles (3-8 mm) at all FSH dosages. In the pre-OPU superstimulation groups, the number of total, viable and cleaved oocytes did not differ depending on the FSH dosage. The number of blastocysts per OPU was highest in the group receiving high dosage FSH. It was observed that success can be achieved with the use of low dosage of FSH, but blasto- cyst yield increases with high dosage of FSH. It is thought that superstimulation applications before OPU will have a positive effect on IVEP success.

Ethics approval

This study was approved by the Local Ethics Committee (Selcuk University Local Ethics Committee for Animal Experiments, Approval Number: 2024/01/23). All methods applied are in accordance with the ARRIVE guidelines.

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Authorship contribution statement

MFC: conceptualization, methodology, formal analysis, investigation, writing - original draft, supervision; **OFY:** methodology, investigation; **MG:** methodology; **AS:** methodology; **SUC:** methodology; writing - review & editing; **DAD:** conceptualization, methodology, supervision.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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SAŽETAK

Cilj je istraživanja bio ustanoviti učinak različitih doza jednokratne primjene FSH-a prije aspiracije jajnih stanica. Kod 10 zdravih junica holštajnske pasmine navedeni učinak analiziran je na temelju superstimulacijskog odgovora, broja oocita i proizvodnje embrija *in vitro*. U skupini S75, na nasumično odabrani dan estrusnog ciklusa, primjenjeno je 10 µg GnRH intramuskularno. Junicama je istodobno u vaginu stavljen umetak s progesteronom. Četvrti je dan intramuskularno primijenjna doza od 25 mg PGF2α. Peti je dan intravaginalni umetak s progesteronom uklonjen te je istodobno primijenjeno 75 µg Stimufola (1/8 uobičajene doze za superovulaciju), intramuskularno u pojedinačnoj dozi. Uz pomoć transvaginalnog ultrazvuka, 2 dana poslije injekcije FSH-a, provedena je aspiracija oocita. Isti je protokol primijenjen u skupinama S150 i S300, osim doziranja FSH-a. U skupini S150 primijenjeno je 150 µg Stimufola (1/4 uobičajene doze za superovulaciju) u pojedinačnoj dozi intramuskularno, a u skupini S300 intramuskularno u jednokratnoj dozi primijenjeno je 300 µg Stimufola (1/2 uobičajene doze za superovulaciju). U kontrolnoj su skupini jajne stanice aspirirane na nasumično odabrani dan estrusnog ciklusa, bez stimulacije hormonima. Prije aspiracije jajnih stanica, broj folikula srednje veličine (3 – 8 mm) u jajniku bio je veći u skupini junica koje su tretirane s ciljem superstimulacije (skupine S75, S150 i S300) nego u kontrolnoj skupini ($P<0,05$). Istraživanjem je ustanovljeno da skupina S300 ima veći ukupan broj jajnih stanica, vijabilnih jajnih stanica i veću stopu oporavka u usporedbi s kontrolnom skupinom ($P<0,05$). Broj blastocista pri aspiraciji jajnih stanica bio je veći u skupini S300 nego u drugim skupinama ($P<0,05$). Osim toga, broj blastocista za aspiraciju jajnih stanica u skupinama S75 i S150 bio je veći nego u kontrolnoj skupini ($P<0,05$). Zapaženo je da se manjom dozom FSH-a može postići superstimulacija, međutim broj blastocista raste s većom dozom FSH-a. Smatra se da superstimulacija provedena jednokratnom primjenom FSH-a prije aspiracije jajnih stanica povećava uspjeh proizvodnje embrija *in vitro*.

Ključne riječi: doziranje FSH; junice holštajnske pasmine; proizvodnja embrija *in vitro*; aspiracija jajnih stanica; superstimulacija