FACULTY OF VETERINARY MEDICINE
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THE EFFECT OF A NUTRITIONAL SUPPLEMENT ON THE EFFICIENCY OF THE OPU-ICSI PROCEDURE

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SAMENVATTING

Deze studie evalueert het effect van voedingssupplementatie op de efficiëntie van Ovum Pick-Up (OPU) en Intracytoplasmatische Sperma Injectie (ICSI) bij paarden. Achtentwintig merries werden verdeeld in een behandelgroep (Groep 1, n=19) en een controlegroep (Groep 2, n=9). Groep 1 kreeg 15 gram van het supplement (Fresh & Fertile®, Curafyt) dagelijks gedurende ten minste één maand voor de eerste OPU-ICSI sessie in het seizoen 2023-24 (Jaar 2). Groep 2 kreeg geen supplementen. Het supplement bevat Foliumzuur, Betaïne, Choline Chloride, L-Cysteïne, Broccoli, Lijnzaad, Havermout, Vitamine B12 en natuurlijke Vitamine E. Belangrijke beoordeelde parameters waren onder andere het aantal follikelaspiraties, verkregen onrijpe oöcyten, gerijpte oöcyten, gesplitste geïnjecteerde oöcyten en geproduceerde embryo's. Binnen Groep 1 werden de gegevens van onbehandelde merries in het seizoen 2022-23 (Jaar 1) vergeleken met behandelde merries in Jaar 2, waarbij elke merrie als haar eigen controle diende. In de behandelde groep binnen Groep 1 werden in Jaar 2 significant meer embryo's per punctie verkregen vergeleken met Jaar 1 ($P<0.01$), gemiddeld één extra embryo per OPU-ICSI sessie per gemiddelde merrie. Deze preliminaire studie suggereert dat het supplement een positief effect heeft op de OPU-ICSI resultaten. Dit onderzoek is cruciaal voor het verbeteren van de *in vitro* embryo productie bij paarden en het optimaliseren van voedingssupplementstrategieën om de kwaliteit van oöcyten en embryo's te verbeteren.

Sleutelwoorden: Equine reproductie - *In vitro* embryo productie - Voedingssupplement – Oöcytkwaliteit - OPU-ICSI

ABSTRACT

This study evaluates the effect of nutritional supplementation on the efficiency of Ovum Pick-Up (OPU) and Intracytoplasmic Sperm Injection (ICSI) in horses. Twenty-eight mares were divided into a treatment group (Group 1, n=19) and a control group (Group 2, n=9). Group 1 received 15 grams of the supplement (Fresh & Fertile®, Curafyt) daily for at least one month before the first OPU-ICSI session in the 2023-24 season (Year 2). Group 2 received no supplements. The supplement contains Folic Acid, Betaine, Choline Chloride, L-Cysteine, Broccoli, Flaxseed, Oatmeal, Vitamin B12, and natural Vitamin E. Key parameters assessed, included the number of follicular aspirations, retrieved immature oocytes, matured oocytes, cleaved injected oocytes, and produced embryos. Within Group 1, data from untreated mares in the 2022-23 season (Year 1) were compared with treated mares in Year 2, with each mare serving as its own control. In the treated group within Group 1, significantly more embryos per punction were obtained in Year 2 compared to Year 1 ($P<0.01$), averaging one additional embryo per OPU-ICSI session per average mare. This preliminary study suggests that the supplement positively impacts OPU-ICSI results. This research is crucial for improving *in vitro* embryo production in horses and optimizing nutritional supplementation strategies to enhance oocyte and embryo quality.

Keywords: Equine reproduction – *In vitro* embryo production - Nutritional supplementation - Oocyte quality - OPU-ICSI

INTRODUCTION

OPU followed by ICSI is a well-established technique for commercial *in vitro* embryo production (IVEP) in horses. OPU involves transvaginal ultrasound-guided follicular aspiration to collect oocytes from the standing mare's ovaries. After in vitro maturation (IVM), oocytes are fertilized by injecting a single sperm cell using micromanipulation techniques (ICSI). The embryos are cultured for 6-8 days before being either transplanted into a recipient mare's uterus or cryopreserved for future use[1]. This process yields an average of 2.12 embryos per OPU session, significantly higher than traditional embryo transfer (0.4 embryos per session). Moreover, the OPU-ICSI procedure can be performed year-round and at any estrus cycle stage. Cryopreserved IVEP embryos give excellent pregnancy results, the need to synchronize the estrus cycles of recipient and donor mares[2].

Despite its progress, the OPU-ICSI technique is not flawless. Expertise in OPU and ICSI is crucial for consistent, high-quality blastocyst production[2]. Breed influences success rates, with Arabian and Quarter horses showing lower cleavage and blastocyst rates than warmbloods[5,6]. Optimal embryo yields are seen in mid-aged mares (5-15 years) [7], while very young and older mares may have a low number of recovered oocytes and subsequently reduced embryo production per ICSI session[9]. Season has less impact than age and breed [7]. Mares identity and oocyte recovery significantly affect success within a commercial OPU-ICSI program. Mares with good results in one OPU-ICSI season often repeat their success, while those with poor results tend to continue having poor outcomes[7,10]. The individual stallions moderately influence efficiency, with blastocyst rates varying less than with mares (range: 15.6-26.8%) [9]. Ensuring oocyte quality is a major challenge, as poor quality reduces the number of oocytes reaching the blastocyst stage [11]. OPU-ICSI introduces oxidative, thermal, and mechanical stressors affecting *in vitro* oocyte maturation and embryo development. In human medicine, studies have demonstrated that nutritional supplements enhance gamete quality and the efficiency of artificial reproductive techniques (ART) efficiency, with periconceptional nutirition significantly influencing fertility and offspring health[12–19]. Studies have examined the influence of antioxidants, methyldonors, vitamins, minerals, and other nutrients on ART efficiency[20–25]. In equines, nutrition impacts fertility, oocyte quality, and development. Reproductive support supplements containing flaxseed oil, vitamins, minerals, amino acids, n-3 PUFAs, probiotics, prebiotics, and antioxidants enhance metabolic activity and reduce lipid content. Adequate nutrition, especially in well-fed mares, promotes follicular growth, with insulin-like growth factor 1 levels influencing oocyte maturation[26,27].

Table 1: List of Ingredients included in the supplement used in the present study with their potential benefits in ART Programs based on the existing literature in other species:

Nutrient	**Active ingredient**	**Effect**	**References**
Folic acid (=vitamin B11)	*Folic acid*	• Methyl group donor • ↑ Oocyte maturation • ↑ Blastocyst rate	16
Betaine/ trimethyl glycine	*Betaine/ Trimethyl Glycine*	• Methyl group donor • Anti-inflammatory effect	32
Choline chloride	*Choline chloride*	• Methyl group donor	33
L-cysteine	*L-cysteine*	• Enhances glutathione production	34
Vitamin E	*Vitamin E*	• Antioxidant	35,36
Vitamin B12	*Vitamin B12*	• Critical for methionine synthase • Crucial for DNA synthesis • Neural tube formation	33,34,37
Broccoli	*Di-Indolyl-Methane (DIM)* *Vitamin A* *Vitamin C* *Folic acid*	• Improves hormonal balance • Boosts mitochondrial function in aged oocytes • ↓ Chromosomal abnormalities in aged oocytes • Antioxidant • ↑ Maturation rate. • ↑ Mitochondrial function. • Antioxidant • Enhances oocyte maturation • *Mentioned above*	38 16 35
Flaxseed	*Lignans* *Alfa-linolenic acid (omega-3-fatty acid)* *Vitamin B* *Vitamin A, E, C*	• Balances estrogen levels • ↑ Number and recovered oocytes • Enhances oocyte cleavage and blastocyst formation post-IVF • ↑ Embryo quantity • ↑ Embryo quality before implantation • ↑ Follicle development • ↑ Cleavage rate • ↑ Oocyte quality • ↑ Small follicles • ↑ Oocytes and embryo quality • Mentioned above	29 39 40,41 42 43,44 45
Oatmeal	*Zinc* *Selenium* *Iron* *Vitamin A, B, E*	• ↑ Oocyte maturation • ↑ Oocyte quality • Antioxidant activity • Essential for hormone balance and follicular development *Mentioned above*	44 46 31,47,48 45

Oocyte maturation is influenced by DNA methylation and oxidative stress. DNA methylation, an epigenetic mechanism, involves DNA and histone modifications that regulate gene expression[49–51]. Homocysteine, a byproduct of DNA methylation, must be converted to methionine to prevent methylation failure, crucial for pre-implantation embryos. Methyl donors like folic acid, choline chloride, and betaine are essential for this conversion[33,34,37]. Homocysteine is also a precursor for cysteine, vital for oocyte maturation and glutathione synthesis. Glutathione neutralizes reactive oxygen species (ROS), maintaining oocyte health[34]. Its depletion in follicular and cumulus cells impairs oocyte quality and early embryonic development [52]. Increased glutathione boosts blastocyst formation and cell count[34].

Vitamin B12, along with folic acid, facilitates the methylation of homocysteine to produce methionine[33,34,37].

Folic acid (FA, Vitamin B11) is essential for DNA methylation and must be obtained through diet. Maternal FA intake enhances oocyte maturation, embryo development, and blastocyst formation while reducing ROS levels, potentially preventing epigenetic abnormalities in offspring[53,54]. FA supplementation is dose-dependent: moderate levels improve embryo production, while high doses reduce it. Different FA concentrations affect bovine embryo production, gene expression, and oocyte quality[53,55].

Betaine, a stable and nontoxic methyl donor found in animals, plants, and microorganisms, is also synthesized from choline metabolism. Oral betaine intake can potentially lower plasma homocysteine levels. As a crucial methyl donor, it restores cellular methionine balance and significantly enhances the antioxidant defense system, playing a key anti-inflammatory role by neutralizing ROS generated during biological energy reactions[32,33].[32]

Choline chloride is essential for cell function and overall health. While the liver synthesizes choline endogenously through phosphatidylcholine de novo synthesis, dietary choline is still important. Choline is a key methyl donor necessary for DNA methylation in all cells, including oocytes. Betaine, derived from choline via choline dehydrogenase, also acts as a methyl donor[33].

L-cysteine enhances glutathione production, improving embryonic development by increasing the blastocyst formation rate and cell count per blastocyst[34].

Vitamin A a fat-soluble micronutrient, includes retinol, retinal, and retinoic acid (RA). It regulates immune function, follicular growth, oocyte maturation, and embryo development[44,45]. In ovarian follicles, it is metabolized and transported to oocytes and cumulus granulosa cells, with RA acting as a gene expression regulator[56]. In ARTs, RA promotes bovine oocyte maturation by modulating gonadotropin receptors, cyclooxygenase 2, and nitric oxide synthase expression in cumulus cells. Beta-carotene may reduce oxidative stress-related damage to oocyte maturation and development[57]. Oxidative stress, due to excess ROS, hinders oocyte development in IVEP[35,36].

Vitamin C (L-ascorbic acid), is a vital low molecular weight antioxidant essential for many enzymes. It protects cells from ROS, toxins, and pollutants. As a potent antioxidant and enzymatic cofactor, vitamin C influences gene expression and reduces cell damage. Maintaining optimal concentrations is crucial to avoid adverse effects[35].

Vitamin E (alpha-tocopherol) and its derivatives are lipid-soluble, non-enzymatic antioxidants. They protect against lipoperoxidation by neutralizing peroxyl and alkyl radicals, forming a less reactive tocopheryl radical[35,36].

Diindolylmethane (DIM) improves mitochondrial function in aged oocytes, enhancing quality and reducing chromosomal abnormalities by decreasing DNA damage. DIM supplementation may delay reproductive aging by maintaining germ cell apoptosis levels, which are linked to improved oocyte quality[38]. DIM also rebalances hormones by altering the ratio of harmful to beneficial estrogens and inhibits aromatase activity, preventing testosterone conversion to estrogen[59] .

To the best of our knowledge, the effect of nutritional supplementation in mares undergoing OPU-ICSI remains unexplored. In this study, oocyte quality in nutritional supplemented donor mares is assessed based on the percentage of oocytes successfully matured *in vitro*, the ratio of matured oocytes that undergo cleavage following in vitro fertilization, and embryos reaching the blastocyst stage. By investigating the potential benefits of nutritional supplementation in equine OPU-ICSI, this study endeavors to enhance our understanding and optimize the outcomes of this ART.

MATERIALS AND METHODS

Animals And Experimental Design

Twenty-eight mares aged between 4 and 24 years, were included in the study. The mares were randomly assigned to two groups: a treatment group (Group 1, n=19; 15 Warmblood horses and 4 Arabian horses), and a control group (Group 2, n=9; 8 Warmblood horses and 1 Arabian horse). The only selection criteria was that the mare produced one or more embryos by OPU-ICSI in Year 1 of the study (August 2022 to April 2023). As this is a field study, stallion selection is at the owners' discretion; however, the same stallions were often used.

Mares in the treatment group (Group 1) received a daily dosage of 15 grams of a nutritional supplement (Fresh & Fertile® Curafyt, Belgium) starting at least one month before the first OPU-ICSI session in Year 2 (August 2023 to April 2024). Supplement ingredients are listed in Table 1.

To assess the supplement's effect on OPU-ICSI efficiency, various criteria were compared between Group 1 and Group 2. These criteria included the number of follicles aspirated, the number of immature oocytes collected, number of matured/injected oocytes, number of cleaved injected oocytes, and number of produced embryos. In Group 1, data from untreated mares in Year 1 were compared with treated mares in Year 2, with each mare functioning as its own control. To account for seasonal variation and technical changes, data of nine non-supplemented mares (Group 2) were compared between Year 1 and Year 2. An overview of the study design is shown in Figure 1a,b.

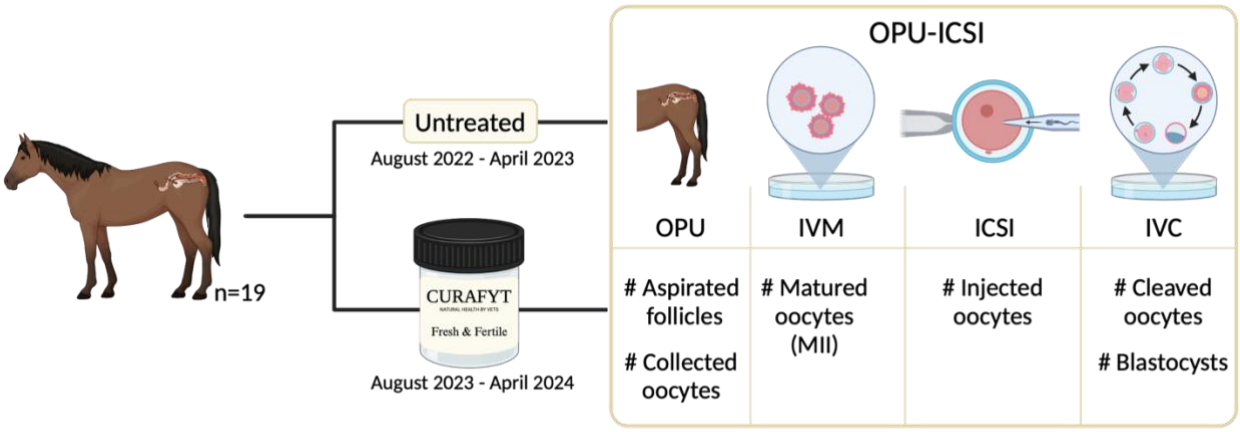

Figure 1a: GROUP 1 - Study Design Overview (Margo Verstraete, 2024) (IVC= in vitro culture; # = number)

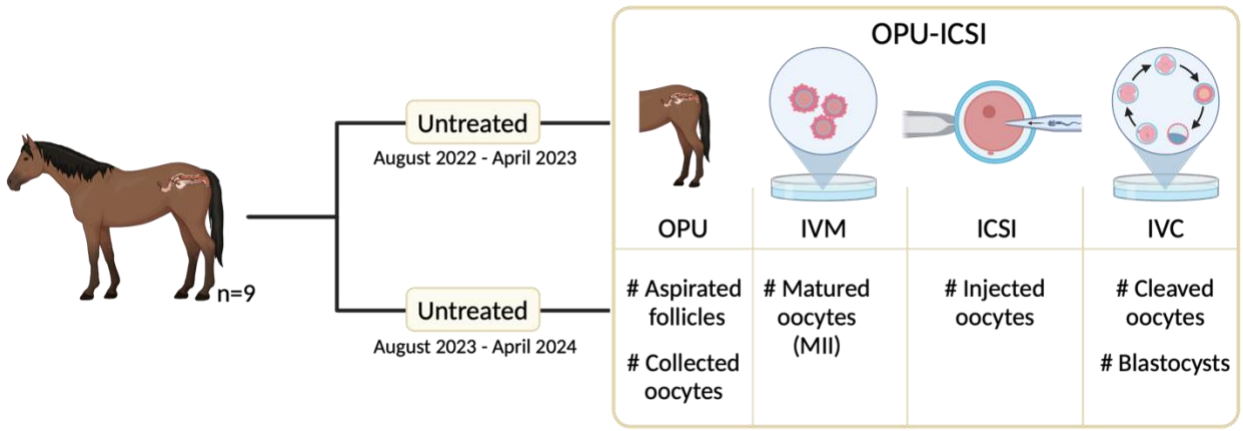

Figure 1b: GROUP 2 – Study Design Overview (Margo Verstraete, 2024) (IVC= in vitro culture; # = number)

OPU-ICSI Procedure

All OPU procedures were performed by the same team at the same facilities using standard procedures. Immature oocytes were collected, processed, and shipped to the same ICSI laboratory. The following parameters were recorded for each OPU-ICSI session: ID of the donor mare, number of follicles punctured, oocytes retrieved, matured oocytes, injected oocytes, blastocysts, and cryopreserved embryos as well as the stallion used per oocyte.

Statistical Analyses

First, a descriptive analysis was performed, calculating the mean value per mare and OPU season. Results are presented in boxplots and descriptive statistics (mean, median, standard deviation, minimum and maximum). Next, differences between OPU-seasons were analyzed using linear mixed regression models, with mare added as random effect to correct for clustering within mares. Separate models were fitted Group 1 and Group 2. Residual plots checked model fit, and differences with a P-value below 0.05 were considered as statistically significant.

RESULTS

The study assessed the effect of the Fresh&Fertile® supplement on fertility in horses across four groups of mares. Table 2 provides details on the number of mares, their age, breed, and the number of OPU-ICSI sessions. Table 3 shows the number of OPU-ICSI sessions, aspirated oocytes, and the ratio of aspirated oocytes per session for each group.

Table 2: Demographic characteristics of the groups of mares

Group	Number mares	Mean age	Breeds	Mean No. of OPU-ICSI sessions/ mare
Group 1 - untreated	19	12	15 Warmblood horses 4 Arabian horses	3
Group 1 - treated	19	13	15 Warmblood horses 4 Arabian horses	2.7
Group 2 - untreated	9	14	8 Warmblood horses 1 Arabian horse	3.3
Group 2 - untreated	9	15	8 Warmblood horses 1 Arabian horse	2.7

Table 3: Groups of mares and their procedural outcomes

Group	OPU-ICSI season	No. OPU-ICSI sessions	No. of oocytes	No. oocytes/OPU-ICSI sessions
Group 1 – treated	Year 1	53	1546	29.2
Group 1 – untreated	Year 2	51	1528	29.9
Group 2 – untreated	Year 1	30	684	22.8
Group 2 – untreated	Year 2	24	872	36.6

Table 4: Average OPU-ICSI parameter outcomes per group and per season in Group 1 and Group 2

GROUP		Number of aspirated follikels		Number of retrieved oocytes		Number of matured oocytes	
		mean	SE	mean	SE	mean	SE
Group 1 n = 19	Year 1	27.1	17	15.0	12	7.7	6
	Year 2	30.3	21	17.5	14.0	10.0	8
Group 2 n = 9	Year 1	34.2	11.5	19.2	8.1	9.6	3.7
	Year 2	35.9	10.0	21.7	6.8	11.4	3.7

GROUP		Number of cleaved fertilized oocyted		Number of embryos	
		mean	SE	mean	SE
Group 1 n = 19	Year 1	15.9	5.0	2.0	3.0
	Year 2	8.0	7.0	2.9	4.0
Group 2 n = 9	Year 1	8.0	3.2	2.7	1.3
	Year 2	8.2	2.7	2.0	1.7

The descriptive analysis of OPU-ICSI parameters (Figure 2) demonstrated several trends: There is an increase in the following criteria: number of aspirated follicles, number of retrieved oocytes, number of matured oocytes, and number of cleaved fertilized oocytes in Year 2 compared to Year 1 in both Group 1 and Group 2. However, the increase in these parameters in Year 2 appears descriptively higher in Group 1 compared to Group 2. The average number of embryos in Year 2 shows a descriptive increase in Group 1, whereas it exhibits a substantial decrease in Group 2.

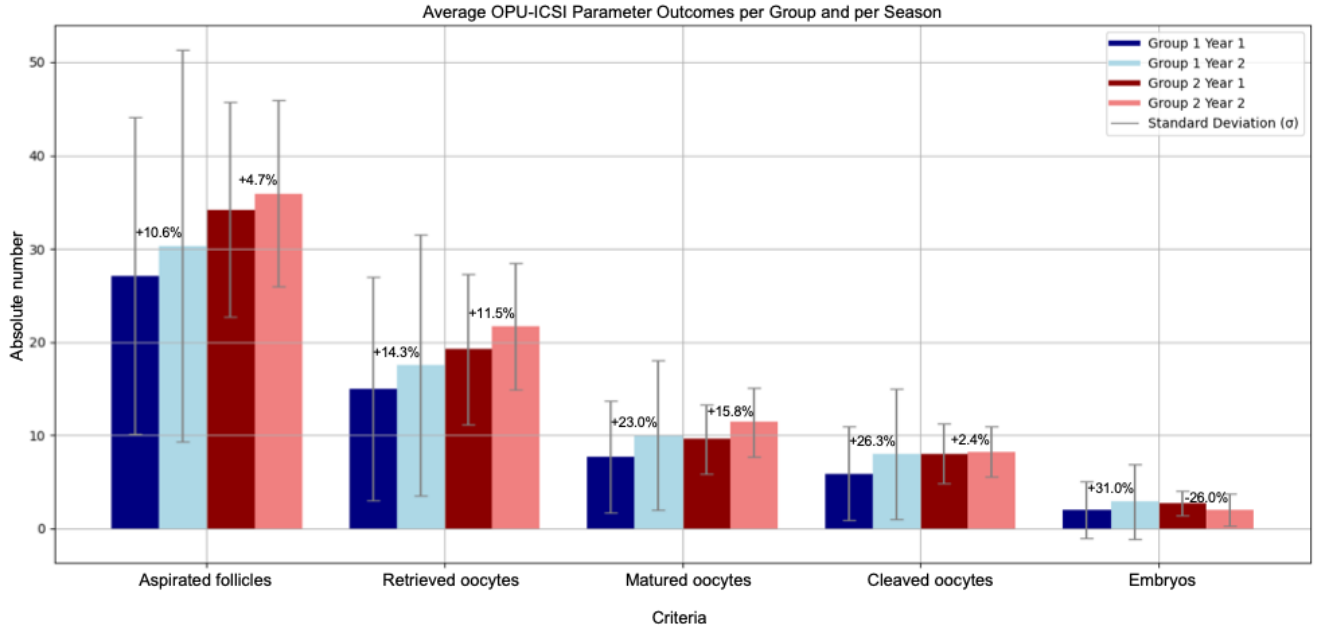

Figure 2: A graphical representation, namely a grouped bar chart, of the descriptive analysis of the following criteria presented in absolute numbers: number of aspirated follicles, number of retrieved oocytes, number of matured oocytes, number of cleaved fertilized oocytes, and number of embryos. The error bars represent the standard deviations for each group and parameter. The percentages indicated in the chart represent the increase or decrease, comparing Year 1 and Year 2 for Group 1 and Group 2, respectively.

The descriptive analysis (Figure 3) revealed several trends: In Year 2, the number of matured oocytes increased in Group 1 compared to Year 1, but remained constant in Group 2. Cleavage rates out of matured oocytes declined in Group 2 but remained constant in Group 1. Both groups showed a decrease in embryos formed from well-cleaved fertilized oocytes. Ultimately, Group 1 had more embryos in Year 2, while Group 2 showed a slight decrease. Linear regression will test if the nutrient blend supports oocyte quality, maturation, and early embryonic development.

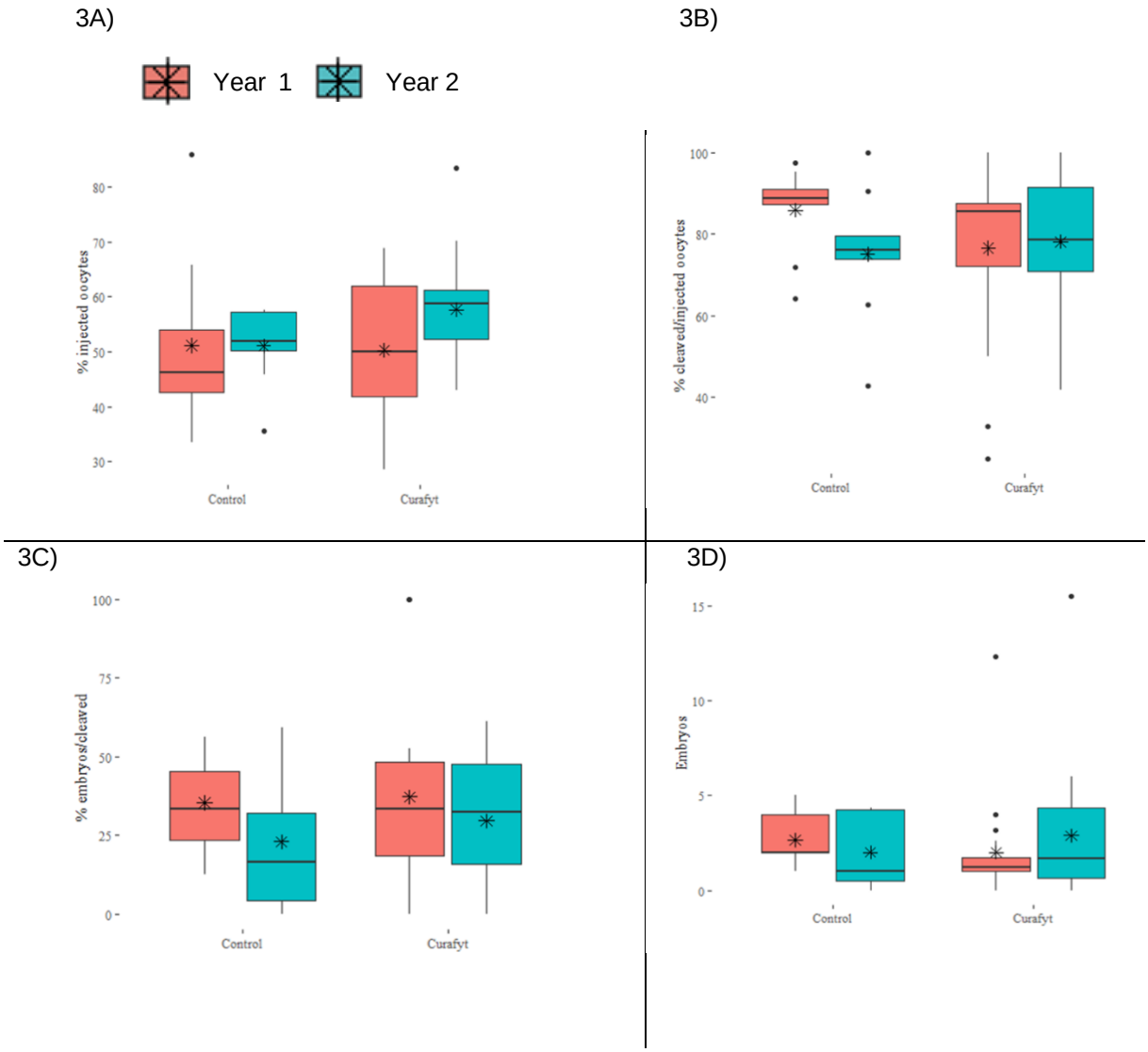

Figure 3A,B,C,D: Graphical representations, namely boxplots, of the descriptive analyses of following criteria: 2A) %injected oocytes (=matured oocytes), 2B) %cleaved/injected oocytes, 2C) %embryos/cleaved and 2D) No. embryos.

Oocyte Maturation: No significant differences were observed between Year 1 and Year 2 in both groups. However, Group 1 showed a numerical increase in the treated group compared to the untreated group, while Group 2 remained constant. Statistical analysis results are summarized in Table 5.

Oocyte Cleavage after ICSI: No significant differences were observed between Year 1 and Year 2 in Group 1 (% cleaved/ total oocytes; Table 5). Group 1 results remained consistent, while Group 2 showed a numerical decline in Year 2 compared to Year 1.

Table 5: Statistical analysis results for various OPU-ICSI parameters in Group 1 and Group 2 across different seasons (LSM = least square means, SE = standard error).

GROUP		% matured oocytes			% cleaved /total oocytes			% cleaved/matured oocytes			% embryos/total oocytes		
		LSM	SE	P-value	LSM	SE	P-value	LSM	SE	P-value	LSM	SE	P-value
Group 1 n = 19	Untreated (August 2022 – April 2023)	51.3	2.6	Ref.	39.1	2.8	Ref.	77.6	4.1	Ref.	12.8	2.1	Ref.
	Treated (August 2023 – April 2024)	56.3	2,6	0,102	43.4	2.8	0.143	77.4	4.1	0.962	14.0	2.2	0.531
Group 2 n = 9	Untreated (August 2022 – April 2023)	50.1	3.7	Ref.	42.6	3.8	Ref.	85.5	3.8	Ref.	14.1	2.5	Ref.
	Untreated (August 2023 – April 2024)	50.9	4.0	0.868	40.5	4.0	0.620	77.4	4.0	0.076	10.0	2.6	0.078

Embryo Development: No significant increase in embryo development percentages was observed in Group 1 between Year 1 and Year 2 across three columns. Group 2 showed a tendency for lower results in Year 2 compared to Year 1 (Table 6).

Overall Embryo Production per OPU Session: Group 2 showed no significant difference between Year 1 and Year 2. In Group 1, significantly more embryos per session were obtained in Year 2 ($P < 0.01$) (Table 6), averaging one additional embryo per OPU-ICSI session per mare (Figure 4).

Table 6: Statistical analysis results for embryo production in Group 1 and Group 2 across different seasons (LSM = least saure mens, SE = standard error).

GROUP		% embryos/ matured oocytes			% embryos/cleaved			Average #embryos per OPU-ICSI session per average mare		
		LSM	SE	P-value	LSM	SE	P-value	LSM	SE	P-value
Group 1 n = 19	Untreated (August 2022 – April 2023)	25.7	4.3	Ref.	33.53	4.72	Ref.	1.8	0.7	Ref.
	Treated (August 2023 – April 2024)	25.1	4.3	0.874	29.49	4.79	0.443	2.8	0.7	0.006
Group 2 n = 9	Untreated (August 2022 – April 2023)	31.4	5.6	Ref.	35.34	6.28	Ref.	2.5	0.5	Ref.
	Untreated (August 2023 – April 2024)	19.1	5.8	0.026	24.75	6.49	0.069	2.2	0.5	0.616

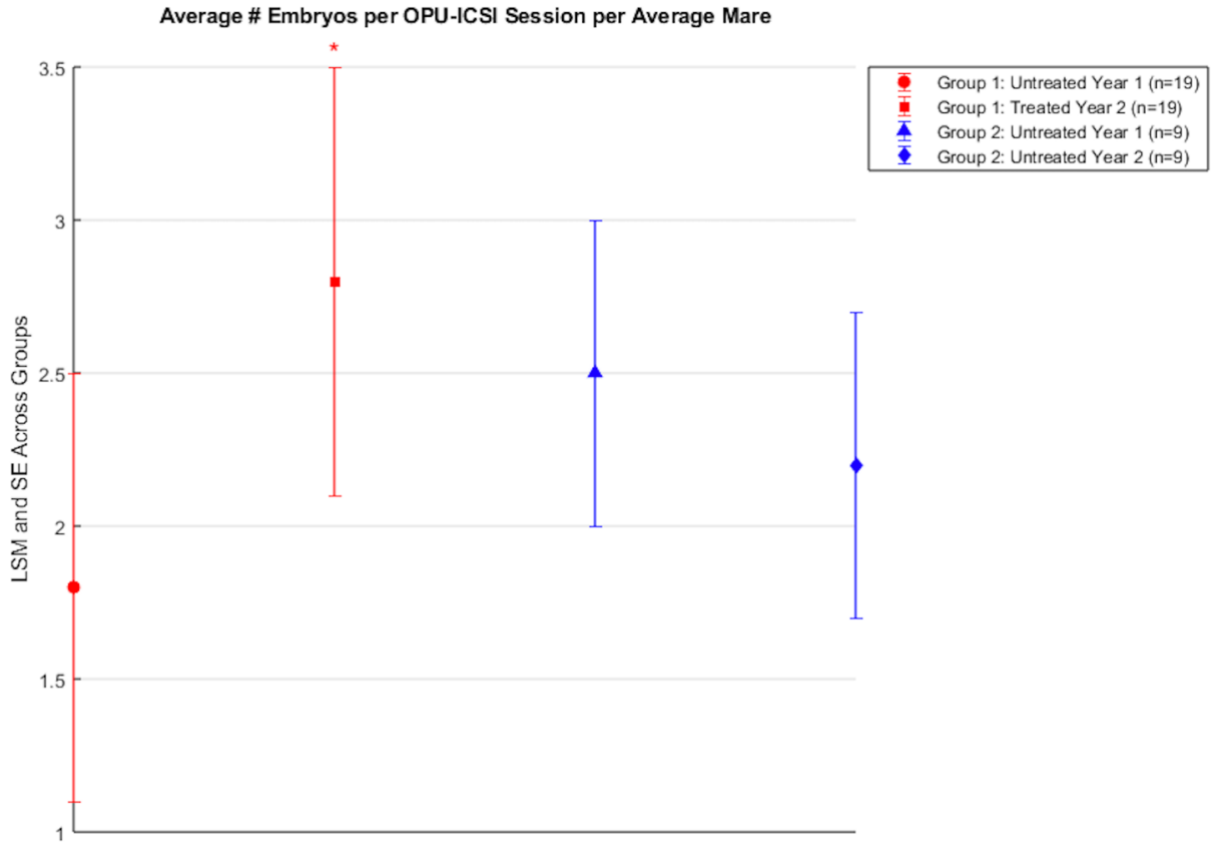

Figure 4: Graphical representation of the statistical analysis for the average number of embryos per OPU-ICSI session per mare, including LSM (Least Squares Mean) and SE (Standard Error) across four groups. Red asterisk (*) indicates statistical significance (P < 0.01).

DISCUSSION AND CONCLUSION

The descriptive analysis of this study shows that in Year 2 there is an increase in both Group 1 and Group 2 in the categories of: average number of follicles aspirated, average number of retrieved oocytes, average number of matured oocytes, and average number of cleaved fertilized oocytes. This may indicate that seasonal influences and/or improvements in techniques had a positive effect in Year 2. Since the increase appears to be greater in Group 1, this could suggest a positive effect of the supplement on OPU-ICSI outcomes. Additionally, the data reveals that there seems to be an increase in the number of embryos in Group 1 in Year 2, while there appears to be a decrease in Group 2. A possible explanation for this could be that the supplement supports the development of the embryo to the blastocyst stage.

Our study shows that the nutrient blend Fresh&Fertile® from Curafyt significantly increases the number of embryos (LSM = +1) in mares undergoing OPU-ICSI. This suggests that specific nutrients in the blend improve oocyte quality and support early embryonic development. Consistent with previous research, nutritional supplementation can enhance ART outcomes. The rate of embryo division relative to matured oocytes declined in Group 2 but remained stable in Group 1, potentially indicating a protective effect of the supplement against seasonal influences or technical changes. The percentage of matured oocytes increased in treated mares (Year 2) compared to non-treated mares (Year 1) within Group 1, suggesting a positive influence on oocyte quality. Further research with larger groups is needed to confirm these findings and determine the supplement's most impactful stage in the OPU-ICSI process.

This study has strengths and limitations. We used a moderately heterogeneous group of mares in terms of age and breed, which may act as confounding factors. The small sample size limits our ability to determine the supplement's effect on subpopulations with low (1), average (2), and high (3) success in OPU-ICSI. To mitigate seasonal and technical variations, we included a control group (Group 2), but this may still introduce variability. Fonte et al. (2024) found no significant seasonal impact on OPU-ICSI results, but our study only compared two seasons, resulting in a large interval. Including more seasons in future studies would reduce this interval and

variability, enhancing the reliability of conclusions. While we standardized puncture clustering, an optimal design would puncture all mares at synchronized time points. We also assessed changes in the number of follicles at the start of each OPU-ICSI session compared to the previous season, adjusting the difference between Year 1 and Year 2 using ratios instead of absolute values.

On the other hand, the present study has also several strengths. Consistent puncture procedures were conducted by the same individual, and oocytes were fertilized by the same laboratory. Despite a wide age range, the average age across the four groups was between 12 and 15 years. Mares in the supplemented group served as their own controls, with stable management and feeding practices maintained by the same owner as the previous year. A control group was included to account for seasonal and technical variations, partially mitigating these effects.

Identifying the specific components of the supplement that contribute to reproductive outcomes is crucial. Detailed analysis of the active ingredients and their mechanisms will help refine the supplement blend to maximize its efficacy.

In conclusion, this study demonstrates that the Fresh&Fertile® nutrient blend from Curafyt significantly increases the number of embryos in mares undergoing OPU-ICSI. The positive effect on embryo yield suggests that specific nutrients in the blend may improve oocyte quality and early embryonic development. Despite the small sample size and heterogeneity of the mares, the findings align with existing evidence on the beneficial impact of nutritional supplementation on ART outcomes. Further research with larger sample sizes and more seasons is necessary to confirm these preliminary findings and to identify the specific components of the supplement responsible for the observed effects.

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