The procedure of inducing superovulation (SOV) in cattle has not changed significantly for years. The basic one, developed several decades ago, consists of a series of intramuscular injections of the aqueous solution of FSH (5, 6, 21, 27, 28). Knowledge of the follicle wave dynamics (3, 4) and the introduction of pharmaceutical substances (2, 3, 19, 22) to control the appearance of the first wave have enabled inducing SOV independently of the stage of the estrus cycle and timed artificial insemination (TAI), in this manner eliminating the workload associated with heat detection. This does not change the fact that a statistical donor receives in a relatively short period of time several injections of various endocrine substances. This system is laborious and could cause unnecessary stress in animals and also carries the risk of unintentionally omitting one of the injections. There have increasingly arisen studies on simplifying superovulation protocols. Unconventional, alternative methods of administration of gonadotropic preparations have been described and the effectiveness of these activities is evaluated (5, 6, 27). The aim of the study was to provide the evolution of superovulation protocols taking into account some changes in the composition of preparations containing pituitary extracts, the routes of their administration, and possible consequences.

**Conventional methods of inducing superovulation in cattle**

The pursuit of developing a unified SOV program started several decades ago. Most commonly used in classical polyovulation protocols are pituitary extracts (FSH, LH) of various domestic animals, equine chorionic gonadotropin (eCG) and human menopausal gonadotropin (hMG) (28). A scheme of an exemplary superovulation protocol is presented in Figure 1. The high variability of superovulation remains an important risk factor (26). Effectiveness of SOV depends

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**Summary**

The aim of this article is to set out certain possibilities related to the simplification of the superovulation protocols (SOV) in cattle. The traditional protocols, which have been in use for decades, include as standard several injections of FSH preparations. Due to the widespread increase in the importance of the animals’ welfare, alternative options for its administration are now being sought. Appropriate selection of gonadotropin as well as the dose and ratio of hormones makes it possible to limit undesirable effects such as prolonged ovarian stimulation. Adjuvants such as polyvinylpyrrolidone, polyethylene glycol, hyaluronic acid or aluminium hydroxide enable the extension of the absorption time of FSH preparations, thus reducing the amount of FSH injections. A single deposition of hormones dissolved in aqueous solutions is possible when it is administered via alternative routes, such as subcutaneous or epidural. Reduction of the amount of FSH injections may directly translate into a decrease of the stress level in animals treated by the SOV protocols and also to the improvement of the efficiency of embryo production. A significant risk factor remains the high variability of superovulation. Its effectiveness depends on a number of generally well known individual and environmental factors that should also be considered when joining SOV.

**Keywords:** superovulation, slow-release preparation, single dose of FSH, cows
on a number of – generally well known – individual and environmental factors. The type of gonadotropin used, the dosing period, the total dose applied and the degree of its reduction for multiple dosing are also significant (5, 28).

The earliest reports of superovulation in cattle and sheep came from Wisconsin, USA (5). The standard at that time was the use of equine chorionic gonadotropin (eCG), according to the previous nomenclature called pregnant mare’s serum gonadotropin (PMSG) (5). It is a glycoprotein with the activity of both pituitary hormones (FSH, LH). Its half-life is relatively long (approx. 40 hours) and determinable circulatory concentrations are observed up to 10 days after administration (34). A single administration was sufficient to induce superovulation (13). However, the long half-life of eCG carried the risk of prolonged stimulation of the ovaries. It caused some disturbances while folliculogenesis, leading to the appearance of ovarian cysts and resulting in a significant decrease in the quality of embryos collected (8, 15). It was the main reason for the gradual abandonment of the widespread use of eCG. The intravenous administration of anti-eCG antibodies at the time of prostaglandin administration or 6, 36, 48, 60 hours after or at the first insemination became a certain solution (2, 13, 15). Anti-eCG antibodies had an inhibitory effect on equine chorionic gonadotropin, shortening its half-life and thus eliminating unwanted ovarian stimulation.

At the turn of the 1970s and 1980s it was reported that folliculogenesis in mammals required both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) action and thus a stronger reaction to ovarian stimulation occurred after the pituitary extract administration than with equine chorionic gonadotropin (14, 28). It was also noticed that the percentage of cows from which more than three transferable embryos were collected was higher after FSH than after eCG administration (32). Some authors do not share this view, pointing out that there are no significant differences between the number of embryos collected after the use of protocols based on equine chorionic gonadotropin or follicle-stimulating hormone (16).

Currently, the most popular method of inducing superovulation in cattle is the administration of pituitary extracts of porcine FSH (5, 6). However, the primary, uncleared preparations contained a large amount of LH that could hinder the response to stimulation. This was demonstrated by an experiment (32) in which three groups of dairy cows with the same dose of pure FSH (450 µg) to all cows tested, and different doses of LH in each group (4500 µg LH in group A, 900 µg LH in group B and 50 µg LH in group C). The average number of recovered ova/embryos or the number of transferable embryos increased with the dose of LH decreasing. Perhaps the level of endogenous LH is sufficient for the growth of pre-ovulatory follicles (25). Studies on the use of pure bovine FSH have also shown that a slight presence of LH is not necessary to induce a strong superovulatory response. However, this is not confirmed by the results of field studies comparing commercially used gonadotropic preparations with different LH content. There were no statistically different results the efficiency of superovulation between the groups receiving various doses of LH (31). Conversely, the dominant ovarian follicles have receptors of LH hormone, which is responsible for the course of the ovulation process (30).

As a result, the hypothesis was made that in order to improve the results of multiple ovulation it ought to support the administration of FSH by injections of eCG (with LH properties). Some studies also indicate that the application of eCG causes increased angiogenesis, which may have a beneficial effect on the blood supply to the luteal tissue (33). However, that effect has not yet been confirmed (36).

Equally important as the proportion of FSH to LH in the gonadotropic preparation is the dose size of FSH. It was found that increasing doses of FSH did not cause a proportional increase in the number of corpora lutea and embryos collected (21). Due to the short half-life of FSH in cattle (approx. 5 hours) it is necessary to administer it several times (32). Using an infusion pump for repeated administration of FSH may be a solution (20). Experimental studies have shown that four applications of 30 units of pFSH per day administered with an infusion pump had a similar superovulation efficiency to the conventional protocol of two manual injections per day, applied for four days. However, it seems that for economic reasons, the introduction of this type of equipment on a wider scale may be associated with some difficulties.

Other studies compared the efficacy of porcine FSH administration to a single-dose of recombinant bovine FSH (9). The donors received accordingly: 300 mg of pFSH in eight decreasing, intramuscular doses over 3.5 days (group A), a single injection of 50 µg A-rbFSH (group B), a single injection of 100 µg A-rbFSH (group C), or a single injection of 50 µg B-rbFSH (group D). A single injection of rbFSH long-acting preparation provided results similar to an application of pFSH administered according to the conventional protocol. However, it was emphasized that further studies were necessary due to ambiguous results.
There are reports of attempts to use human menopausal gonadotropin (hMG) to induce polyovulation (29, 40). Despite satisfactory results comparable to those achieved after the use of FSH, hMG is not widely used in veterinary practice due to its high costs (5).

The knowledge of the follicular wave dynamics led to the introduction of dominant follicle growth control methods into SOV protocols to facilitate the synchronization of the procedure in many animals (3, 4, 7, 17, 22, 35, 44). Initially, the possibility of mechanical removal of large follicles was used (44). The proposal to use ultrasound ablation of the dominant follicle requiring the use of specialized equipment and trained personnel was not accepted in field practice (35). Exogenous GnRH or LH was used to synchronize the appearance of the follicular wave and induce the ovulation with different results (3, 4). Research on the use of antagonadoliberin preparations allowing for inhibiting the wave growth of follicles, thus enabling the synchronization of donors is also underway (7, 17, 22).

**Attempts to reduce the number of FSH injections, slowing down absorption, different routes of administration**

The use of the conventional method of inducing superovulation by repeated administration of FSH is laborious and stressful for cattle. Research on alternative methods has been carried out for a long time due to a growing awareness of the above and thus a general obligation to ensure the welfare of farm animals (5, 6, 28). In recent years, attention has been focused on the possibility of inducing SOV by a single injection of FSH or different routes of the follicle-stimulating hormone administration. In order to reduce the number of injections needed to achieve the desired effect, studies have been conducted to extend the absorption time of FSH after a single injection. The first studies focused on the evaluation of the effect of a mixture of hMG or porcine FSH and polyvinylpyrrolidone (PVP) (40, 41, 43). Superoovulation efficiency was measured after single intramuscular (40, 43) and subcutaneous (41) administration. In the study of Sugano et al. (40) 48 cows divided into 3 study groups were examined and applied intramuscularly respectively: 450 IU hMG, 600 IU hMG or 30 mg FSH dissolved in 30% PVP solution. In the control group the total dose of 600 IU hMG was administered intramuscularly in six decreasing doses for 3 days. Yamamoto et al. (43), on 77 HF cows, compared the effect of intramuscular injection of 30 mg FSH dissolved in 30% PVP with both single administration of 30 mg FSH in saline solution and a traditional model (28 mg of FSH in decreasing doses for 4 days). Takedomi et al. (41) studied 19 Holstein-Friesian cows, evaluating the effect of PVP concentration on superovulation efficiency after single subcutaneous injections. Animals from the study groups were given 30 mg of FSH in PVP solution at 25% or 50% concentration. The control group received a total dose of 30 mg FSH in saline solution intramuscularly in decreasing doses for 3 days. Despite satisfying results (a large number of corpora lutea and transferable embryos collected), PVP seemed to be an adjuvant with some defects. The FSH suspension in PVP characterized with high viscosity, which created some difficulties to achieve a homogeneous mixture and limited its practical application (6, 23). However, as no other undesirable effects have been demonstrated, PVP is still used in some countries as an adjuvant in commercial FSH preparations (10).

Some hopes were connected with FSH preparations containing polyethylene glycol (PEG) (11, 12). Choi et al. carried out the first research with its use in 2002 (12). Cows from 3 groups received a mixture of different variants of the FSH concentration dissolved in 30% solution of PEG (first group – single injection of 400 mg FSH; second group – single injection of 200 mg FSH; third group – two injections of 200 mg FSH). Cows from the control group were injected intramuscularly with 50 mg of FSH twice daily for 4 consecutive days. The average number of corpora lutea were 19.6, 11.1, 13.4 and 7.6, respectively. Interestingly, there were no differences between the studied groups in terms of the average number of total embryos and the number of transferable embryos collected. Subsequent studies proved that FSH with the addition of polyethylene glycol can be an effective alternative to conventional SOV protocols (11).

Another carrier slowing down the release of FSH is hyaluronian. It is a widely distributed glycoaminoglycan which is one of the components of extracellular matrix (ECM) of many tissues and organs playing an important role in the regulation of their metabolism. Satisfactory results of SOV were ensured by a single intramuscular administration of the preparation containing 400 mg FSH mixed with 2% hyaluronate (6, 42); however, similarly to PVP, it showed significant viscosity and consequently difficulty in obtaining a homogeneous form. The high density of the mixture generated problems with the injection (the necessity of using thick needles). Although lower concentrations of hyaluronate facilitated the achievement of a homogeneous mixture, two doses of FSH applied at an interval of 48 hours were necessary to induce superovulation (42). Currently, a commercial preparation of pFSH with 0.5% hyaluronate solution (Folltropin V, Vetoquinol) is used to obtain polyovulation after a single injection (1).

A macromolecular adsorbent that slows down the absorption of FSH is a recently described aluminum hydroxide gel (AH-gel – aluminum hydroxide gel), widely used as an adjuvant in various vaccines. The characteristics of the aluminum hydroxide gel structure, such as a fibrous structure and low viscosity, make it an excellent carrier, significantly extending the absorption time of FSH. *In vivo* studies compared
the efficacy of SOV following a single intramuscular administration of 30 mg pFSH in a mixture with AH-gel to the conventional protocol of supply of decreasing doses (6-2 mg pFSH dissolved in natrium chloride solution) administered at 12-hour intervals for 4 consecutive days (24). It was shown that for cattle receiving single intramuscular doses of 30 mg of pFSH with 5 ml of aluminum hydroxide gel, the number of corpora lutea, the total number of ova and the number of transferable embryos collected did not differ significantly from the conventional pFSH treatment. However, the disadvantage of AH-gel applied intramuscularly were pathological changes in the site of administration in the form of granulomas and swelling accompanied by a significant accumulation of macrophages, monocytes and giant cells (23). These pathological changes at the place of injection in meat cattle caused significant economic losses and growing consumer dissatisfaction. For this reason, other routes of administration of FSH were searched for. It was hypothesized that subcutaneous administration would provide the possibility to decrease the amount of Aluminum contained in AH-gel and consequently reduce adverse reactions (23).

An important factor determining the rate of the absorption process is the body condition score of the animal (BCS), thus the amount of fat tissue at the place of deposition of the drug (6, 18, 43). It was shown that the reaction to a single dose of the preparation in the neck area was weaker than after its administration in the shoulder region in cows in insufficient condition. However, while the animal was in good condition or overweight, the response was satisfactory regardless of the place of administration. Since subcutaneous injection behind the shoulder generated a comparable response regardless of the BCS value, a hypothesis was made that the amount of fat tissue and the place of deposition could be critical points in the process of slowing down the absorption of pFSH (18, 43). For this reason, deposition of the drug in animals with lower BCS values or genetically predisposed to lower fat cover is suggested in the shoulder region.

Interesting research results have recently been presented by Hiraizumi et al. (18). They compared the efficacy of a single subcutaneous administration of pFSH suspended in aqueous solution of 0.9% NaCl in Black Japanese cows to the classic SOV protocol. The cows were divided into four study groups and two control groups. Animals from group A (n = 14) received 20 pFSH units dissolved in 10 ml NaCl sol., from group B (n = 14) 20 pFSH units dissolved in 50 ml NaCl sol., from group C (n = 15) 30 pFSH units dissolved in 10 ml NaCl sol. and from group D (n = 15) 30 pFSH units dissolved in 50 ml NaCl sol. Animals from both control groups (n1 = 14, n2 = 15) received 20 units of pFSH twice daily in decreasing doses intramuscularly for 4 days. In experimental groups there were no significant differences in the number of corpora lutea, ova and embryos collected. There were also no differences in serum pFSH concentrations in the tested animals qualified to experimental groups. It was concluded that a single administration of pFSH dissolved in aqueous saline solution makes it possible to achieve a comparable reaction to that recorded after follicle-stimulating hormone administration twice a day for several days by the intramuscular route (18).

Recently, promising studies on the possibility of the administration of aqueous FSH solutions to the epidural space have arisen. Taking into account the results, these studies seem to be particularly interesting. The first report on gonadotropin application in this way appeared a few years ago and concerned the treatment of ovarian cysts using GnRH (37, 38). The results of recent studies by Sakaguchi et al. (39) indicate that a single application of 20 AU of FSH in aqueous solution into the epidural space between the caudal vertebrae made it possible to reduce the number of injections performed and, moreover, to significantly reduce the amount of the drug administered. The epidural administration ensured the efficiency of superovulation comparable to intramuscular application of a series of FSH injections (a total dose of 20 AU in 6 decreasing doses for 3 days). It was also found in subsequent studies that the percentage of in vitro cultured blastocysts from oocytes obtained by OPU from donors stimulated by single injections of FSH in the epidural space was higher than after several injections (39). On the one hand, the above studies indicate the possibility of replacing the standard program of inducing superovulation and, on the other hand, the possibility of increasing the effectiveness of OPU procedures and in vitro embryo production (IVP). A comparative analysis of the results

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**Tab. 1. Comparison of the effectiveness of individual FSH carriers (own work for Yamamoto et al. (43), Takedomi et al. (41),**

| Route of administration | Carrier of FSH | PVP sol. 30% | PVP sol. 25%/50% | PEG sol. 30% |
|-------------------------|----------------|-------------|-----------------|-------------|
| 1 x im                  | 1 x sc         | 1 x sc/2 x sc | 8 x im         |
| 28 mg²                  | 6 x im         | 10.5        | 8 x im         |
| Group CL                | 1.73           | 12.3        | 8.15           |
| Ova/embryos collected   | 8.3            | 10.1        | 7.55           |
| Transferable embryos    | 6.2            | 5.8         | 4.65           |

Explanations: * averaged research results on several groups)

The cows were divided into four study groups and two control groups. Animals from group A (n = 14) received 20 pFSH units dissolved in 10 ml NaCl sol., from group B (n = 14) 20 pFSH units dissolved in 50 ml NaCl sol., from group C (n = 15) 30 pFSH units dissolved in 10 ml NaCl sol. and from group D (n = 15) 30 pFSH units dissolved in 50 ml NaCl sol. Animals from both control groups (n1 = 14, n2 = 15) received 20 units of pFSH twice daily in decreasing doses intramuscularly for 4 days. In experimental groups there were no significant differences in the number of corpora lutea, ova and embryos collected. There were also no differences in serum pFSH concentrations in the tested animals qualified to experimental groups. It was concluded that a single administration of pFSH dissolved in aqueous saline solution makes it possible to achieve a comparable reaction to that recorded after follicle-stimulating hormone administration twice a day for several days by the intramuscular route (18).
of the effectiveness of FSH preparations dissolved in particular types of carriers and aqueous solution have been presented in Table 1. It shows that the use of the latter brings equally satisfactory results. However, it is necessary to confirm the results on a more numerous group of animals.

In recent years, a great deal of effort has been put into improving the standard SOV protocols. These include a number of additional hormonal interventions to control the development of follicles and timed artificial insemination. Their aim was to increase the efficiency of superovulation. Considering the welfare of animals, this fact is significant. There is a need to minimize the stress associated with the preparation of donor females. Satisfactory results of superovulation are now possible thanks to the prolonged time of hormone release through the use of its original carriers and the use of alternative routes of administration. The most recent reports indicate the effectiveness of single injections of aqueous FSH solutions is comparable to preparations containing various adjuvants slowing hormone release through the use of its original carriers and the use of alternative routes of administration. The form of FSH application seems to be preferred for both its lesser labor intensity and for economic reasons.

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