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Controlled delivery of follicle-stimulating hormone in cattle

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Abstract

Embryo transfer in cattle is a key issue for *in vivo* production of several mature follicles as opposed to the normal production of only one. *In vivo* produced embryos can then be transferred to recipient cows for gestation to occur. To obtain a large number of transferable embryos, the superovulation step is crucial. To allow the growth of ovarian follicles, the most commonly used protocol consists of 2 intramuscular injections per day over 4 days of a saline solution of the follicle-stimulating hormone. To reduce workload, technical errors in the injected dose and animal stress, different strategies have been investigated to sustain the release of this hormone over 4 days in 1 or 2 injections. This review introduces the physicochemical properties of the follicle-stimulating hormone and discusses the limitations of marketed products and all the research that has been conducted to overcome these limitations. In particular, the field of subcutaneous administrations, the development of new formulations such as viscous solutions, implants and microspheres and the modification of the structure of the follicle-stimulating hormone are overviewed and discussed.

Keywords

Follicle-stimulating hormone (FSH); Drug-delivery; Formulation; Protein; Superovulation (SOV); Sustained-release, veterinary field.

Abbreviations

Al-gel, Aluminum oxyhydroxide/hydroxide gel; API, Active pharmaceutical ingredient; BCS, Body condition score; bFSH, Bovine follicle-stimulating hormone; CMC, Carboxymethylcellulose; CTP, Carboxy-terminal peptide: glycosylation sites; FDA, Food and drug administration; FSH, Follicle-stimulating hormone; HA, Hyaluronic acid; hFSH, Human follicle-stimulating hormone; IM, Intramuscular; IVP, *In vitro* embryo production; LH, Luteinizing hormone; MOET, Multiple ovulation and embryo transfer; MM, Molar mass; ND, Not determined; PEG, Polyethylene glycol; pFSH, Porcine follicle-stimulating hormone; pFSH $\alpha\beta$, Porcine follicle-stimulating hormone alpha and beta heterodimer; pFSH α , Porcine follicle-stimulating hormone alpha subunit; pFSH β , Porcine follicle-stimulating hormone beta subunit; pLH, Porcine luteinizing hormone; PLA, poly(lactic acid); PLGA, Poly(lactic-co-glycolic acid); PMSG, Pregnant mare serum gonadotropin; PVP, Polyvinylpyrrolidone; rec-bFSH, Recombinant bovine follicle-stimulating hormone; rec-FSH, Recombinant follicle-stimulating hormone; SOV, Superovulation; SC, Subcutaneous; TSH, Thyroid-stimulating hormone

1. Introduction

If no fertilization occurs, the reproductive cycle in cows lasts 21 days. Two components are involved during this phase: the anatomical structures such as the hypothalamus, pituitary gland and ovaries, and the hormones that allow interaction between these different anatomical structures. Briefly, the hypothalamus produces the gonadotropin-releasing hormone which interacts directly with the pituitary gland. The pituitary gland produces follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that migrate *via* the bloodstream and act on the ovaries. In response, the maturation of several follicles (immature follicles) within the ovaries is observed and followed by the production of estradiol which displays a positive feedback on the pituitary gland. At this point, a surge of LH is produced and causes the maturation of a single follicle (dominant follicle). After 21 days, ovulation of the dominant follicle is observed and, if fertilization occurs, the cycle continues with gestation, which lasts approximately 280 days (Ball *et al.*, 2004).

To be economically viable, a one-year calving interval needs to be maintained. The pregnancy needs, therefore, to be ensured and consequently optimized. The first optimization method, called *in vitro* embryo production (IVP), is based on the collection of immature follicles from a donor cow (Boni, 2012) followed by the maturation and *in vitro* fertilization in a laboratory. The embryos produced *in vitro* are then implanted into recipient cows to allow gestation (Bousquet *et al.*, 1999). The second method to optimize the reproductive cycle called multiple ovulation and embryo transfer (MOET), is based on superovulation (SOV) (Bó & Mapletoft, 2014) followed by artificial or natural insemination. SOV can be summed up as the *in vivo* production of several mature follicles as opposed to the normal production of only one. These *in vivo* produced embryos can then be transferred to recipient cows as described in the first method (Hasler, 2014).

Both techniques provide the transfer of a large number of embryos and rapidly improve the genetic potential in one or more herds. According to a report from the International Embryo Technology Society, embryos from cattle represent more than 95% of the world production. The total production in cattle is increasing from year to year and around 1.5 million embryos have been transferred in 2018 (Viana, 2018). IVP is mainly practiced by the North American industry and more used than MOET since 2016. However, MOET is cheaper because it does not require specific infrastructure (*e.g.* laboratory) and is still preferred in Europe and Africa (Viana, 2018).

To obtain a large number of embryos *in vivo*, the SOV step is crucial. For this purpose, FSH is the hormone responsible for the growth of ovarian follicles. The exogenous administration

of FSH (natural or recombinant protein) in cows or mature heifers for reproduction during the follicular wave, stimulates the growth of a large number of follicles, which would normally be lost by atresia during the estrus cycle. The development of multiple follicles requires stimulation with FSH until maturation has progressed enough to respond to LH for the final phase of ovulation. This takes about 4 days. SOV occurs when the number of transferable embryos obtained after fertilization is greater than 6 (Bó & Mapletoft, 2014).

SOV can also be induced by equine chorionic gonadotropin also known as pregnant mare serum gonadotropin (PMSG) (Monniaux *et al.*, 1983). However, FSH is more commonly used due to a higher ovarian response despite a shorter half-life (FSH, 5h versus PMSG, 120h) (Laster, 1972; Monniaux *et al.*, 1983). Injection after 96h (4 days) of an antibody against PMSG was used to rapidly decrease the plasmatic concentration of PMSG and restore a biological efficacy similar to FSH injections (Dieleman, *et al.*, 1993). The marketing of this antibody was stopped in the 1990s and favored the FSH treatment (Bó & Mapletoft, 2020).

SOV is part of an overall process of optimizing cow reproduction (Bó & Mapletoft, 2014; Hasler, 2014). To reach this goal, a sufficient plasmatic concentration of FSH over 4 days is necessary. Driven by the need for a high fertilization rate and considering the fast elimination of FSH, different strategies have been investigated to obtain easy-to-use products. This review presents FSH, the hormone used to induce SOV in cattle and focuses on the marketed products containing FSH, their limitations, and the research that has been done to overcome these limits. In particular, studies about the route of administration, the development of new FSH formulations and the modification of FSH structure are overviewed and discussed in this review.

2. Porcine FSH

As previously mentioned, porcine FSH (pFSH) is synthesized and secreted by the anterior part of the pituitary gland and is directly involved in the growth of immature follicles. This hormone is a heterodimer (pFSH $\alpha\beta$) belonging to the family of glycoprotein hormones. The two subunits, pFSH α , and pFSH β are structurally different and are linked by interchain hydrogen bonds (Closset & Hennen, 1978; Whitley *et al.*, 1978; 1981). FSH α subunit is common to different hormones such as LH and thyroid-stimulating hormone (TSH) while pFSH β subunit is specific to pFSH and confers its biological activity (Pierce & Parsons, 1981; Combarrous, 1992). After heterodimer assembly, glycosylation reactions occur to give the final native and functional form of pFSH $\alpha\beta$. Within the same species, glycosylation steps may

be slightly different (*e.g.* addition of a different number of uncharged or charged glycans). Glycosylation is therefore responsible for mass and charge heterogeneity and all these glycoprotein hormones are called pFSH isoforms (Carvalho *et al.*, 2009). Each isoform exhibits biological differences in terms of association with its receptor, activity, or even clearance rate (Ulloa-Aguirre *et al.*, 1999; Bousfied *et al.*, 2018).

pFSH has a mean isoelectric point of about 5 and thus a negative overall charge at a pH above 5. Indeed, the different isoforms of the pFSH have slightly different isoelectric points ranging from 4.8 to 5.2 (Saxena & Rathnam, 1978). The molar mass of pFSH is about 34,000Da. The molar mass of the pFSH α subunit is 15,500Da whereas that of pFSH β is 18,500Da (Closset & Hennen, 1978). pFSH molar mass appears to be more or less identical to that of human FSH (hFSH) (Ryan *et al.*, 1971). A strong genetic similarity exists between FSH of different species. However, differences in the amino acid sequence are still observable. The three-dimensional structure of pFSH has not been studied by X-ray diffraction but the ribbon diagram of hFSH is shown Figure 1.

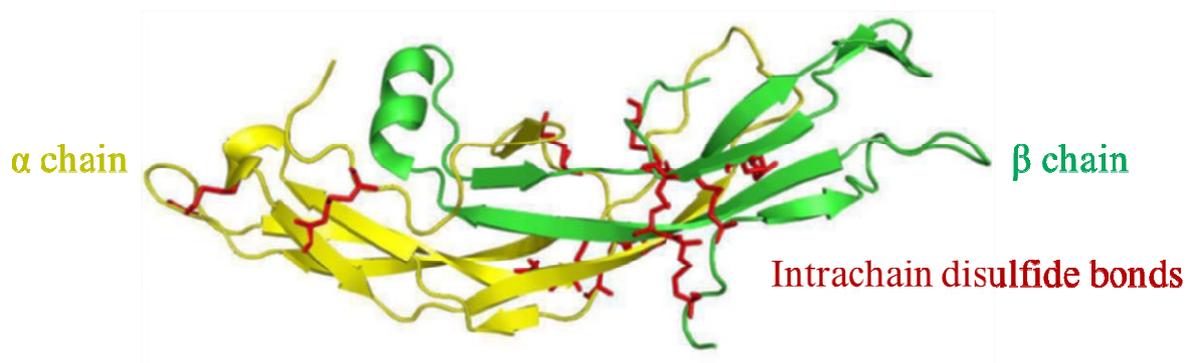


Figure 1 – Three-dimensional structure of hFSH obtained by X-ray diffraction. Protein data bank (PDB 1fl7; Fox *et al.*, 2001) and PyMOL software (ribbon diagram).

Some antibodies specific to the hFSH α subunit recognize also the ovine and bovine FSH α while they do not recognize the equine and porcine ones. This implies a difference in the three-dimensional structure (Haj Hassan *et al.*, 2015).

Based on a purification process described by Closset and Hennen in 1978, pFSH is obtained from the pig pituitary extract through several steps including protein precipitation, chromatographic separation (ion-exchange, adsorption, exclusion) and filtration. The final powder obtained by this method has shown a high biological potency but the purity of pFSH compared to other unknown proteins has not been determined (Closset & Hennen, 1978).

3. Commercial formulations

Currently, several products containing FSH are available on the veterinary market (United States, Europe, and Japan) to induce SOV in cattle. Their composition and the standard protocol used are summarized in Table 1.

Table 1 – Marketed products containing FSH available in the United States, Europe, France, and Japan to induce SOV in cattle. (API: active pharmaceutical ingredient, ND: not determined)

Product	API	Origin	Excipients and solvent	Standard protocol
Super-OV [®] (United States)	FSH (powder)	Porcine	NaCl 0.9%, preservative (ND), water for injection (10mL)	1 injection (1.6mL) / 12h 3 days (6 injections) Intramuscular
Folltropin-V [®] (Europe and United States)	FSH (powder)	Porcine	NaCl 0.9%, benzyl alcohol, NaOH, HCl, water for injection (20mL)	1 injection (2.5mL) / 12h 4 days (8 injections) Intramuscular
Pluset [®] (Europe)	FSH (powder)	Porcine	NaCl 0.9%, chlorocresol, water for injection (20mL)	1 injection (1-3mL) / 12h 4 days (8 injections) Intramuscular
Stimufol [®] (France)	FSH (powder)	Porcine	NaCl 0.9%, methyl parahydroxybenzoate and propyl parahydroxybenzoate, water for injection (10mL)	1 injection (1.25mL) / 12h 4 days (8 injections) Intramuscular
Antrin-R10 [®] (Japan)	FSH (powder)	Porcine	NaCl 0.9%, preservative (ND), water for injection (25mL)	1 injection (1-3mL) / 12h 4 days (8 injections) Intramuscular
Antrin-R10 Al [®] (Japan)	FSH (powder)	Porcine	Solvent: NaCl 0.9%, water for injection (2.5mL) + Sustained-release solution: aluminium oxyhydroxide 0.05%w, water for injection (0.5mL)	1 injection (3 mL) Subcutaneous

As shown in Table 1, the pFSH is used as an API for all the veterinary products. The amount of pFSH depends on the standard used for the measurement. The dose is mainly given in NIH-FSH-P1 equivalent, armour units or international units. Then, the true amount of pFSH is difficult to assess and compare. Indeed, the therapeutic window of pFSH required to induce a SOV in cows is unclear but a deleterious effect on the hormonal response was shown with too high or too low FSH peaks in plasma (Bó & Mapletoft, 2020). Nevertheless, each product contains a standardized dose of pFSH inducing SOV in cattle with a defined protocol.

Purification methods for commercial products are confidential but are probably based on the optimization of the method mentioned above (Closset & Hennen, 1978). In the case of veterinary products containing pFSH, each company balances biological efficacy with the cost of purification. The products are therefore not pure and generally contain other proteins such as TSH and LH. Among the residual impurities, LH-content is important to control because a negative impact of LH was demonstrated (Murphy *et al.*, 1984; Chupin *et al.*, 1984; Donaldson & Ward, 1986; Herrler *et al.*, 1991). The FSH/LH ratio must be high to induce a strong and reproducible SOV, but a small quantity of LH improves ovarian response in cows (Herrler *et al.*, 1991). As seen before with pFSH, pLH-content is difficult to assess and compare because the amount depends on the standard used for the measurement. According to the good efficacy and the cost balance, pFSH powder of marketed products is not pure but is relatively high-purified against pLH. Depending on the purification method used, some small differences could also be visible on the pFSH (isoforms).

The 1st generation of veterinary products based on pig pituitary extract were marketed in the 1970s (*e.g.* FSH-P[®]) and the 2nd generation (current pFSH powder vial) with a controlled pLH-content appeared in the 1990s (Bó & Mapletoft, 2014).

Except for Antrin-R10 AI[®], these products are distributed in 2 parts: one containing pFSH in powder form and another for the solvent used to dissolve the pFSH at the time of the first injection (Table 1). The volume after reconstitution of the total dose of pFSH is 10mL (Super-OV[®], Stimufol[®]), 20mL (Folltropin-V[®], Pluset[®]) or 25mL (Antrin-R10[®]) and, in the standard protocol, each injection contains 1 to 3mL. Usually, the volume injected is used to adjust the dose of pFSH administered to each cow if corrections from the standard protocol are necessary. The solvent is composed of a saline solution containing a preservative to prevent, after reconstitution, microbial contamination during the time of treatment (3-4 days) (Table 1). To obtain SOV, the current protocol consists, after reconstitution of the lyophilized powder using a saline solution, of intramuscular (IM) administration twice daily for 3-4 days (Table 1). As seen before, the half-life of pFSH is about 5 hours (Laster, 1972; Monniaux *et al.*, 1983) which justifies treatment with several injections.

Antrin-R10 AI[®] is the latest product commercialized (3rd generation) on the veterinary market and is different from other products such as Antrin-R10[®] (Table 1). Indeed, the induction of SOV is reached after only one subcutaneous (SC) injection versus 6 to 8 IM ones. pFSH powder is first dissolved in a saline solution and then diluted with the sustained-release solution containing an aluminum oxyhydroxide gel, also known by misnomer as aluminum

hydroxide gel (Al-gel) (Kimura, 2016). Electrostatic interaction between positive Al-gel and negatively charged pFSH at physiological pH has been demonstrated (Chang *et al.*, 1997) and leads, in this case, to the adsorption of 99.9% of the pFSH (Aoki *et al.*, 2004; Kimura *et al.*, 2007). The release is, therefore, prolonged over 4 days due to electrostatic interactions rather than to the viscosity of the solution. Aluminum salts are usually used as adjuvants in vaccines (Baylor *et al.*, 2002) and can promote the appearance of granuloma (Kimura, 2016). At the low concentration used in Antrin-R10 Al[®] and using SC injection, the inflammatory response is limited (Kimura, 2016). Marketed only in Japan, a worldwide patent application (WO2015011977 A1) was filed in 2015 for commercial use in Europe and America (Kimura *et al.*, 2015).

For several years now, researchers have been interested in reducing injections while keeping the same efficacy. Thus, workload, stress in the animal (Biancucci *et al.*, 2016) and technical errors in the injected dose would be reduced. Three approaches were investigated: i) the modification of the multi-injection protocol from marketed products by changing the route of administration (Table 2); ii) the modification of pFSH formulation to induce SOV in cattle in 1 or 2 injections (Tables 3 and 4) and iii) the structural modification of the hormone.

4. Modification of the current multi-injection protocol from marketed products by changing the route of administration

Some scientists have tested the induction of SOV by Folltropin-V[®] or Antrin-R10[®] reconstituted in a saline solution in a single SC injection instead of several IM injections (Table 2). Indeed, SC tissue is well-known to prolong the release by slowing the rate of diffusion of molecules (Ashai, *et al.*, 1993; Kim *et al.*, 2017). The single SC injection behind the shoulder of pFSH in Rubia Gallega (Fernández *et al.*, 1993), Fleckvieh (Schallenberger, *et al.*, 1994) or cross-breed beef cows (Bo *et al.*, 1994) showed that the SOV was not significantly different from those using the protocol with 8 IM injections for 4 days. However, the same SC injection in Holstein cows produced a lower SOV efficacy than the multi-injection protocol (Lovie, *et al.*, 1994; Takedomi, *et al.*, 1995). In Holstein cows, giving the SC injection in two steps (75% of the total dose at day 0 and 25% at day 2) improved the hormonal stimulation but SOV was still slightly lower compared to the treatment with 8 IM injections (Lovie, *et al.*, 1994). These results show that there is significant variability in pFSH response between different species of cows with a single SC injection.

Besides, variability in the SOV response has also been demonstrated within breeds depending on the site of injection. Indeed, SC injection in the neck (low-fat content) of cross-breed cows does not induce SOV consistently whereas injection behind the shoulder (high-fat content) induces a reproducible hormonal response as seen previously (Bo *et al.*, 1994). The presence of SC fat tissue allows slower diffusion of pFSH from the injection site and therefore a better efficacy. SOV is influenced by the body condition score of the cow (BCS). The BCS is a rating of the body condition and is determined by visual and/or tactile analysis of fat deposits in the SC tissue. The range of the scale can be different from one country to another. (Bewley, *et al.*, 2010). Using a five points BCS (1: very thin, and 5: very fat), a single SC injection into the neck will induce SOV only in cross-breed cows with a BCS greater than 2 (Bo, *et al.*, 1994). A single SC injection in the neck in Japanese Black cows with a BCS of about 5 (1: very thin, and 9: very fat) showed a SOV induction similar to that of the multi-injection protocol. The results were similar by injecting the total dose in 10 or 50mL. The intermediate BCS indicates that fat of the SC tissue in the neck slows the release of pFSH (Hiraizumi, *et al.*, 2015).

Given the previous results (Table 2), SC injection of the total dose of Folltropin-V[®] or Antrin-R10[®] (saline solution) in one injection induces a SOV in cows in some specific cases being influenced by many parameters as the species of the cow (different sensitivity to pFSH), the percentage of SC fat tissue (BCS) and the SC injection site (neck versus shoulder). Treatment with 2 IM injections per day for 4 days decreases the variability of response and is, therefore, more universal. This explains why treatment in one SC injection of the total dose of commercial products only in saline as the vehicle is not present in the veterinary market.

Table 2 – Summary of SOV induction in cattle by modification of the multi-injection protocol of marketed products. Standard dose refers to the total dose used to induce SOV in cattle with the multi-injection protocol. (^a 1<BCS<5; ^b 1<BCS<9; ND: not determined)

Marketed Product	Standard dose	Solvent	Injected Volume	Route of administration	Injection number	Species	SOV	References
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: behind shoulder	x1	Rubia Gallega cows	Yes	(Fernández, <i>et al.</i> , 1993)
Folltropin-V [®]	1	NaCl 0.9%	ND	Subcutaneous: behind shoulder	x1	Fleckvieh cows	Yes	(Schallenberger, <i>et al.</i> , 1994)
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: behind shoulder	x1	Cross-breed beef cows (1<BCS<5) ^a	Yes	(Bo, <i>et al.</i> , 1994)
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: neck	x1	Cross-breed beef cows (BCS<2) ^a	No	(Bo, <i>et al.</i> , 1994)
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: neck	x1	Cross-breed beef cows (BCS>2) ^a	Yes	(Bo, <i>et al.</i> , 1994)
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: behind shoulder	x1	Holstein cows	No	(Lovie, <i>et al.</i> , 1994)
Folltropin-V [®]	1	NaCl 0.9%	20 mL	Subcutaneous: behind shoulder	x2: 15mL, day 0 5mL, day 2	Holstein cows	No: better than x1 injection	(Lovie, <i>et al.</i> , 1994)
Antrin-R10 [®]	1	NaCl 0.9%	10 mL	Intramuscular: ND	x1	Holstein cows	No	(Yamamoto, <i>et al.</i> , 1994)
Antrin-R10 [®]	1	NaCl 0.9%	5 mL	Subcutaneous: ND	x1	Holstein cows	No	(Takedomi, <i>et al.</i> , 1995)
Antrin-R10 [®]	1	NaCl 0.9%	10 mL	Subcutaneous: neck	x1	Japanese Black cows (BCS ≈ 5) ^b	Yes	(Hiraizumi, <i>et al.</i> , 2015)
Antrin-R10 [®]	1	NaCl 0.9%	50 mL	Subcutaneous: neck	x1	Japanese Black cows (BCS ≈ 5) ^b	Yes	(Hiraizumi, <i>et al.</i> , 2015)

5. Modification of the current multi-injection protocol by changing pFSH formulation

To decrease response variability while reducing the number of injections, studies were also carried out by changing pFSH formulation. The goal is to sustain the release of pFSH over 4 days either by modifying the solvent vial such as in the case of Antrin-R10 A1[®] (Table 3) or by modifying the vial containing pFSH powder.

5. 1. Modification of the solvent vial by adding excipients

Several articles have been published on the development of a sustained-release dosage form of pFSH to allow SOV in cows after 1 or 2 injections just by adding different types of excipients in the solvent vial (Table 3). This research is based on the injection by SC/IM of a viscous solution containing pFSH to decrease the rate of diffusion towards the bloodstream. SOV in cows in only 1 injection was obtained by adding hyaluronic acid (HA, 2%w) (Tribulo *et al.*, 2011; 2012), polyvinylpyrrolidone (PVP, 25-50%w) (Takedomi *et al.*, 1995; Yamamoto *et al.*, 1995), gelatin (3.2%w) (Hill *et al.*, 1985) or polyethylene glycol (PEG, 3-140%w) supplemented with novocaine (Kosovskij *et al.*, 2016) (Table 3). Novocaine allows vessels vasodilatation and blood to flow to the injection site. These products are presented the same way to that of commercial products with the presence of a powder vial (natural pFSH) and a solvent vial (sustained-release solution) for the reconstitution before injection. PEG or PVP permits injection both in SC or IM, whereas HA shows satisfying results only following IM administration. Gelatin was only tested by SC in the neck. Interestingly, all these polymers are included in the FDA inactive ingredient database for each of the routes of administration tested (FDA, 2020). The use of injectable polymers improves the controlled release compared to the single SC injection of a saline solution of pFSH and therefore, a decrease of the variability of pharmacological response related to the species and BCS. Nevertheless, with this type of formulations, new limitations have been identified. At the concentrations used, the viscosity of PVP (25-50%w) and HA (2%w) solutions is too high to allow easy handling (Kimura, 2016). The homogenization of the pFSH powder and the injection of the solution become difficult. In most cases, flow resistance is due to entanglements of the polymer chains at rest or low shear rate and then to frictional forces between them in motion (high shear rate). Indeed, above the entanglement concentration, a polymer with a high molar mass promotes entanglements and increases the viscosity of the solution (Graessley, 1974). For PVP, even using a medium/low molar mass of 40,000g.mol⁻¹, handling remains difficult (Kimura, 2016). A decrease in PVP concentration would facilitate injection if the *in vivo* efficacy can be preserved.

Regarding HA, no information on the influence of the molar mass is available (Tríbulo *et al.*, 2011). To solve the viscosity problem, Tríbulo *et al.* (2011) used a lower concentration of HA (1%w) instead of decreasing the molar mass but the induction of a SOV was not observed. The decrease in viscosity, resulting in a faster release of pFSH, did not lead to a hormonal response in cows. Injection of the pFSH with HA (1%w) in two steps (75% of the total dose at day 0 and 25% at day 2) was studied and showed similar results to the multi-injection protocol (Tríbulo *et al.*, 2012).

Gelatin has been poorly detailed in its use to induce SOV and the viscosity under the tested conditions was not discussed in the literature (Hill *et al.*, 1985). In general, the limitation of gelatin is related to its animal origin. Minimal differences in the purification process and batch production of gelatin may also alter FSH-releasing properties (Olsen *et al.*, 2003) and cause variations in the hormonal response.

Regarding pFSH and PEG/novocaine formulation, the molar mass of PEG is reported as an important parameter. A PEG with a molar mass of less than $6,000\text{g}\cdot\text{mol}^{-1}$ or greater than $70,000\text{g}\cdot\text{mol}^{-1}$ does not induce SOV in cows (Kosovskij *et al.*, 2016). The use of an insufficiently viscous solution will result in a release in less than 4 days and on the contrary, an excessively viscous solution will result in a release of more than 4 days. In both cases, the SOV will not be induced or optimal (Kosovskij *et al.*, 2016). Novocaine, a local anesthetic, promotes the diffusion of pFSH to the blood in the early hours while PEG prolongs its release over 4 days (Kosovskij *et al.*, 2016).

Few papers have been published concerning the absence of SOV using viscous solutions, but in one example, 1% carboxymethylcellulose (CMC) and 30%w PVP did not show convincing results compared with the multi-injection protocol (Smith *et al.*, 1973). The molar mass or the grade of the polymers, which might affect the release was however not given (Smith *et al.*, 1973) and could help to explain those results compare to the induction of the SOV obtained with PVP as shown before (Takedomi *et al.*, 1995; Yamamoto *et al.*, 1995). Besides, the CMC solution (1%) used is probably not viscous enough and does not release pFSH over 4 days.

To summarize, the induction of SOV in a single SC/IM injection by adding a viscous agent in the solvent vial has been demonstrated. However, the viscosity must be low enough to provide easy handling of the pFSH formulation but high enough to have a release over 4 days. If this equilibrium cannot be achieved, an injection in two steps of the total dose can be used to induce a SOV similar to the multi-injection protocol.

To go further, the use of an *in situ* forming implant such as the FDA-approved Atrigel™ system could overcome the problem of viscosity (Matschke, 2002). Atrigel™ technology is based on biodegradable polymers, usually poly(lactic acid) (PLA) or poly(lactic-co-glycolic acid) (PLGA), soluble in an organic solvent but not in water. If the organic solvent used to solubilize these polymers is miscible with water, then the injection of the organic solution into an aqueous medium leads to the precipitation of the polymers. An API in solution or suspension into the organic phase would be trapped in the polymer matrix and would be released by diffusion or during the degradation process (Dunn *et al.*, 1990). Usually, the target release time is 1 to 12 months (Matschke, 2002). A patent application based on Atrigel™ has been published in 2008 on the sustained-release of proteins, including FSH. In the patent, the molar mass of PLGA is lower than $5,000\text{g}\cdot\text{mol}^{-1}$ and comes directly in liquid form without adding any organic solvent. Protein in powder form is mixed with the PLGA solution and then injected in animals by one SC or IM injection. Depending on PLGA concentration, *in vivo* release profiles can be around 4 days. FSH is listed as an example of API but no release profile and no induction of a SOV in cattle have been mentioned in the patent (Cherif-Cheikh, *et al.*, 2008).

Table 3 - Summary of the papers and patents evaluating induction of SOV in cattle by adding excipients in the solvent vial of marketed products. No modification of the powder vial. pFSH dose refers to the total dose used to induce SOV in cattle with the multi-injection protocol. (^a 1<BCS<5; ^b 1<BCS<10; MM: molar mass; ND: not determined)

	Products	FSH dose	Excipients / Solvent	[C]	Injected volume	Route of administration	Injection number	Species	SOV and limits	References
Papers	Antrin-R10 [®]	1	Polyvinylpyrrolindone (K-30, 40,000g.mol ⁻¹), water for injection	50%w	4 mL	Subcutaneous: ND	x1	Holstein cows	Yes. High viscosity. Difficult to use	(Takedomi <i>et al.</i> , 1995)
	Antrin-R10 [®]	1	Polyvinylpyrrolindone (K-90, 360,000g.mol ⁻¹), water for injection	25%w	4 mL	Subcutaneous: ND	x1	Holstein cows	Yes. High viscosity. Difficult to use	(Takedomi <i>et al.</i> , 1995)
	Antrin-R10 [®]	1	Polyvinylpyrrolindone (K-30, 40,000g.mol ⁻¹), water for injection	30%w	11 mL	Intramuscular: ND	x1	Holstein cows	Yes. High viscosity. Difficult to use	(Yamamoto <i>et al.</i> , 1995)
	Folltropin-V [®]	1	Hyaluronic acid (MM ND), water for injection	2%w	10 mL	Subcutaneous: base of the ear	x1	Red Angus cows	Yes. High viscosity. Difficult to use	(Tribulo <i>et al.</i> , 2011)
	Folltropin-V [®]	1	Hyaluronic acid (MM ND), water for injection	1%w	10 mL	Intramuscular: neck	x1	Red Angus cows	No	(Tribulo <i>et al.</i> , 2011)
	Folltropin-V [®]	1	Hyaluronic acid (map-5, MM ND), water for injection	0.5-1%w	10 mL	Intramuscular: ND	x2: 7.5mL, day 0 2.5mL, day 2	Cross-breed cows (<i>e.g.</i> Simmental, Angus) (BCS>3) ^a	Yes. 2 injections	(Tribulo <i>et al.</i> , 2012)
	FSH-P [®]	1	Carboxymethylcellulose (MM ND), water for injection	1%	5 mL	Subcutaneous: ND	x1	Cross-breed cows (<i>e.g.</i> Angus)	No	(Smith <i>et al.</i> , 1973)
FSH-P [®]	1	Polyvinylpyrrolindone (MM ND), water for injection	30%w	5 mL	Subcutaneous: ND	x1	Cross-breed cows (<i>e.g.</i> Angus)	No	(Smith <i>et al.</i> , 1973)	
FSH-P [®]	1	Gelatin (MM ND), NaCl, water for injection	3.2%w 0.9%	ND	Subcutaneous: Neck	x1	Cross-breed beef cows (BCS≈7) ^b	Yes	(Hill <i>et al.</i> , 1985)	
Patents	Folltropin-V [®]	1	Hyaluronic acid (high molecular weight), water for injection	0.5-1%w	10 mL	Intramuscular: ND	x2: 7.5mL, day 0 2.5mL, day 2	Cows (40-45 months, 500-550kg)	Yes. 2 injections	(Haisheng <i>et al.</i> , 2015)
	Commercial products (<i>e.g.</i> Pluset [®])	1	Polyethylene glycol (6,000-70,000g.mol ⁻¹), novocaïne, water for injection	3-140%w 0.25-1%	5-15 mL	Intramuscular: Subcutaneous: ND	x1	Breed cows (<i>e.g.</i> Holstein)	Yes	(Kosovskij <i>et al.</i> , 2016)

The strategy of modifying only the solvent vial composition has the advantage of keeping production costs under control, the main contributor being pFSH in powder form (Hesser, 2011). Alternative approaches have also been investigated by implementing controlled release technologies directly on pFSH formulations and also by using chemical modification or recombinant technology.

5. 2. Modification of the pFSH powder vial

The modification of the pFSH powder vial of marketed products has been studied in different ways. First, new formulations were developed without modification of pFSH structure (Table 4) and then, structural modification of pFSH was investigated. It should be noted that the induction of a SOV in cattle was not tested in all cases.

5. 2. 1. Formulation approach

Preformed implant/gel systems containing pFSH have been examined and 3 different systems have been described in the literature (Table 4). PLGA powder mixed with pFSH micronized powder and designed by extrusion as an injectable millimeter rod enables to induce SOV in Holstein cow following SC implantation in the neck (Gresser *et al.*, 1992, Jimoh *et al.*, 1995a; 1995b).

A multiphasic controlled release system was also developed to mimic the multi-injection protocol in a single injection in Holstein cows (Table 4). In this system, PLA-based tubular capsules are capped with a PLGA membrane (40 μ m) and filled with a mixture of glucose, citric acid, sodium bicarbonate, and pFSH. In aqueous medium, carbon dioxide gas produced from this mixture causes the separation of the membrane from the capsule, releasing rapidly pFSH. Depending on the ratio between glucose, citric acid, and sodium bicarbonate, the capsule opens at desired times (from 2 to 96 hours). Thus, 8 different capsules (1/8 pFSH dose each) were implanted at the same time to replace the 8 IM injections (Gresser *et al.*, 1992, Jimoh *et al.*, 1995a). Hormonal response in Holstein cows was demonstrated using PLGA millimeter rod and PLA/PLGA capsules. Unfortunately, these experiments were carried out only on one cow per group (Gresser *et al.*, 1992, Jimoh *et al.*, 1995a). In both cases, the injection was done in SC in the neck *via* a trochar after local anesthesia (Gresser *et al.*, 1992, Jimoh *et al.*, 1995a).

Polyacrylamide gel (15%w) containing pFSH was tested in heifers (Table 4). In this case, pFSH powder is dissolved in an acrylamide solution before the polymerization step. This mixture forms a solid gel that releases pFSH over 4 days *in vitro*. Although plasmatic pFSH

concentration was similar to that of the control group using the multi-injection protocol, once implanted in the ear by surgery, the polyacrylamide gel did not induce SOV (Floyd, 2007). Bioactivity of the pFSH has not been evaluated in the formulation. pFSH could be partially denatured by the polymerization step or in the gel matrix after implantation (Floyd, 2007). Moreover, polyacrylamide is not listed in the FDA inactive ingredient database.

Another strategy reported is the continuous infusion of pFSH previously dissolved in saline using an implanted osmotic pump at a flow of 1/5 of the total dose per day. Hormonal response after a treatment of 5 days was demonstrated in Holstein cows but the SOV was less effective than when applying standard protocol. Only 2/3 of the total dose injected in the positive control group (multi-injection) was administered since a continuous infusion was used. The lower dose administered could explain this less effective hormonal response (Wubishet *et al.*, 1986). The need for implantation and removal of the osmotic pump from the SC tissue at the end of the treatment may not be optimal to decrease the labor.

The last formulation strategy concerns the use of pFSH-loaded PLA or PLGA microparticles (Takada *et al.*, 1997; Tracy *et al.*, 2004; Curtis, 2019) (Table 4). Unfortunately, only patent applications have been found and microparticles manufacturing is not always documented. Even if a veterinary application is promoted, the induction of a SOV in cattle is not performed. Microparticles of pFSH could be an interesting approach to sustain the release of pFSH. For example, Bydureon[®] (exenatide-loaded PLGA microspheres), enables to release this oligopeptide over one week. However, the manufacturing process is expensive and time-consuming. Besides, pFSH stability needs to be monitored during all the manufacturing steps.

Table 4 - Summary of SOV induction in cattle by modifying pFSH formulation of marketed products or pituitary extracts. No modification of the pFSH structure. pFSH dose refers to the total dose used to induce SOV in cattle with the multi-injection protocol (control group). (MM: Molar mass; ND: not determined)

	Composition	FSH dose	Preparation, manufacturing and injection process	Route of administration	SOV: species and limits	References
Preformed implants/gels	pFSH (pituitary extract) + PLGA (75/25, 125,000g.mol ⁻¹). PLGA/FSH: 70/30%w	1	Millimeter rod by mixing the PLGA and pFSH powder. Implanted <i>via</i> a trochar	x1 injection Subcutaneous: neck	Yes: Holstein cows	(Gresser <i>et al.</i> , 1992; Jimoh <i>et al.</i> , 1995a; 1995b)
	pFSH (pituitary extract) + Glucose, citric acid and sodium bicarbonate. In PLA (50,000g.mol ⁻¹) millimeter capsules + 40µm PLGA (75/25, 125,000g.mol ⁻¹) membranes	1	1/8 FSH dose in each capsules. Capsules up to 30mg of powder. 8 different cores of glucose, citric acid and sodium bicarbonate in 8 PLA capsules. 8 different <i>in vitro</i> burst times: 2/4/8/13/21/33/53/96 hours. Implanted <i>via</i> a trochar	x1 injection Subcutaneous: neck	Yes: Holstein cows	(Gresser <i>et al.</i> , 1992; Jimoh <i>et al.</i> , 1995b)
	pFSH (pituitary extract) In polyacrylamide gel (15%w)	1/2	Equivalent to 1mL of solid gel implanted by an injection gun	x1 injection Subcutaneous: ear	No: Heifers (ND)	(Floyd, 2007)
Osmotic pump	FSH-P® + NaCl 0.9%, water for injection. In osmotic pump (model : 2ML1)	2/3	Volume in osmotic pump: 2mL. Treatment over 5 days. Release rate: 1/5 of the dose per day. Implanted by surgery	x1 injection Subcutaneous: scapula	Yes: Holstein cows. Lower than the control group	(Wubishet <i>et al.</i> , 1986)
Microparticles	FSH (pituitary extract, specie ND) + PLA (3,000-30,000g.mol ⁻¹) or PLGA (3,000-30,000g.mol ⁻¹) + Surfactant (<i>e.g.</i> Tween 80)	1	Spray-drying of water in oil or solvent in oil solution/suspension to prepare the microparticles	x1 injection Intramuscular: ND Subcutaneous: ND	ND	(Takada <i>et al.</i> , 1997)
	FSH (pituitary extract, specie ND) + PLA (5,000-40,000g.mol ⁻¹) or PLGA (5,000-40,000g.mol ⁻¹) + Stabilizer (<i>e.g.</i> Trehalose) + Surfactant (<i>e.g.</i> Tween 80)	1	Manufacturing process: ND	x1 injection Intramuscular: ND Subcutaneous: ND	ND	(Tracy <i>et al.</i> , 2004)
	FSH (pituitary extract, specie ND) + PLGA (ratio ND, molar mass ND)	1	Manufacturing process: ND	x1 injection Intramuscular: ND Subcutaneous: ND	ND	(Curtis, 2019)

The modification of the powder vial containing the natural pFSH to develop a new formulation requires a complete modification of the manufacturing process. In addition to the high cost, the stability of the pFSH is a critical parameter that must be monitored throughout the process.

5. 2. 2. Structural modification approach

A completely different approach consists of changing the structure of the protein. Indeed, chemical modification of proteins can affect strong and beneficial pharmacokinetic changes (Zaman *et al.*, 2019). In the context of bovine SOV, the addition of PEG chains from 31,000 to 115,000g.mol⁻¹ on natural pFSH (Antrin-R10[®]) was achieved (Uchiyama *et al.*, 2010). In most cases, adding PEG increases the *in vivo* half-life of proteins by reducing the clearance rate from the body (Zaman *et al.*, 2019). The *in vitro* activity of pFSH with PEG chains decreases compared to free pFSH but is still significant. Unfortunately, the use of pFSH-PEG as an expected prolong-acting pFSH analog to induce SOV in cattle by a single injection has not been reported yet.

A new generation of products could be based on recombinant FSH (rec-FSH). Despite a high production cost, the use of rec-FSH has the advantage of avoiding pituitary impurities related to the extraction/purification process and thus the transmission of diseases between animals (Hesser, 2011). SOV was observed in Holstein cows by a bovine rec-FSH (rec-bFSH) with a multi-injection protocol (Looney *et al.*, 1988). A sustained-release solution mixed with a rec-FSH powder or a complete new sustained-release formulation could be developed to induce SOV in cattle by a single injection. The rec-bFSH mentioned above is manufactured from the unmodified DNA sequence and has the same properties as the natural FSH (*e.g.* half-life of a few hours). Recombinant technology is also able to produce FSH with a modified structure and new biological properties. Adding some glycosylation sites (CTP) and/or a covalent bond between the FSH α and the FSH β to modify pharmacokinetics parameters lead to prolong-acting FSH analogs (Hesser, 2011; Gifre *et al.*, 2017; Ben-Menahem, 2019). In Angus beef cows, SOV was demonstrated by a single injection of BoviPure-FSH[™] containing a recombinant hyper-glycosylated FSH $\alpha\beta$ single chain (rec-bFSH β CTP α) (Colgin, 2008).

6. Conclusions and perspectives

In the veterinary field, embryo transfer in cattle is essential. In the context of *in vivo* production, optimizing the number of transferable embryos by SOV is an important process. Treatment to induce SOV in cattle by several IM injections of pFSH in saline over 4 days prospered in the 1970s by placing on the market FSH powder extracted from pig pituitary gland (1st generation). In the 1990s, optimization of products from the 1st generation was performed by purifying the pFSH powder against pLH leading to a better hormonal response. Nevertheless, the use of a saline solution as a vehicle and the twice-daily IM injection protocol during 4 days remained similar to the 2nd generation.

To reduce workload, technical errors in the injected dose and animal stress, different axes of formulation have been tested to allow a SOV in only 1 or 2 injections. The 3rd generation of products containing a sustained-release solution to solubilize the pFSH powder emerged recently on the Japanese veterinary market. Viscous solutions are the most described in the literature and a good efficacy was demonstrated. Preformed implants, poorly evaluated on cows, are more complex to set-up, but promising results were shown. Future formulations could be based on *in situ* forming implant, microparticles, chemical modification of pFSH or directly through the manufacture of rec-FSH with optimized pharmacokinetics parameters. Usually, the choice to develop a veterinary product is made according to the complexity of the manufacturing process, the cost and the easy handling of the final product.

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Declaration of interests

The authors declare that Vetoquinol is a veterinary company and is one of the financial support. This financial support may be considered as a potential conflict of interest.

CRedit authorship contribution statement

Quentin DEGUETTES: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **Elias FATTAL:** Supervision, Writing - review & editing, Funding acquisition. **Marinette MOREAU:** Supervision, Writing - review & editing, Project administration. **Elodie LEGO:** Supervision, Writing - review & editing. **Amélie BOCHOT:**

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① **Goal** **Superovulation**
Follicle-stimulating hormone
+ Sustained-release over 4 days

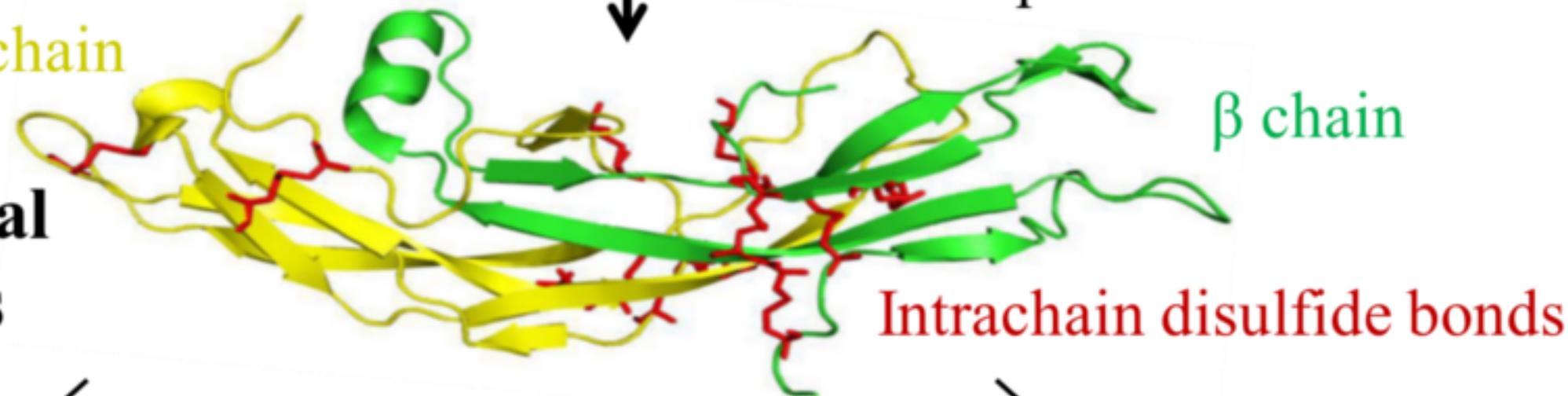


② **API^a** **Follicle-stimulating hormone (porcine)**
Glycoprotein hormone: heterodimer $\alpha\beta$
Molar mass $\approx 34,000\text{Da}$
Isoelectric point ≈ 5



③ **Formulation or structural modification approaches**

α chain



β chain

Intrachain disulfide bonds

Viscous
solutions

Follicle-stimulating hormone^b

Recombinant
technology

Implants
(performed, *in situ*)

Microparticles

Chemical
modifications

(performed, *in situ*)

Chemical
modifications

^a Active Pharmaceutical Ingredient

^b Follicle-stimulating hormone (humaine), protein data bank : 1fl7