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# Advances in Ovarian Superstimulation Protocols for Enhanced Oocyte Yield in Bovines

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## Introduction:

Buffaloes play a crucial role in the livestock economy, particularly in countries like India, where they contribute significantly to milk production and rural livelihoods. However, improving their reproductive efficiency has always been a challenge due to inherent biological limitations such as low follicular reserves, fewer recruitable follicles, and higher rates of follicular atresia. These factors ultimately result in reduced oocyte yield and limit the success of advanced reproductive technologies. In recent years, Ovum Pick-Up (OPU) combined with in vitro embryo production (IVEP) has emerged as a powerful tool for accelerating genetic improvement in buffaloes. This technique allows repeated, minimally invasive collection of oocytes from live donors, enabling the production of multiple embryos from genetically superior females. As a result, OPU-IVEP has opened new avenues for faster multiplication of elite germplasm and enhanced herd productivity. Despite its advantages, the efficiency of OPU-IVEP in buffaloes remains lower compared to cattle. The number of oocytes recovered per session is often limited, which directly affects embryo production rates. To overcome these constraints, researchers and practitioners have focused on strategies that can increase the number of available follicles and improve oocyte quality. One of the most promising approaches is superstimulation, which involves the use of hormonal treatments to stimulate the ovaries and promote the development of multiple follicles simultaneously. By increasing the pool of aspiratable follicles, superstimulation enhances oocyte recovery, improves embryo yield, and ultimately boosts the overall efficiency of OPU-IVEP programs.

## What is Superstimulation?

One of the potential strategies to improve the efficiency of Ovum Pick-Up (OPU) involves follicular wave synchronization and ovarian stimulation with exogenous gonadotropins. Follicular wave

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synchronization prior to OPU has been shown to enhance both embryo production rates and post-transfer conception rates in bovines. Hormonal treatments that stimulate ovarian activity and promote follicular development can significantly increase the number of oocytes retrieved, thereby optimizing in vitro embryo production (Vieira et al., 2014)

**Goal:** Increase number of follicles → more oocytes → more embryos

## Different Methods of Superstimulation prior Ovum-pick up in Bovines:

### 1. Multiple-Dose FSH Protocols:

The most commonly used approach involves administration of FSH in multiple injections at 12-hour intervals to mimic the natural follicular recruitment process and prevent premature follicular dominance.

### 2. Four-Dose (FSH4) Protocol:

In this regimen, a total dose of approximately 200 mg FSH is divided into four equal injections (50 mg each), administered at 12-hour intervals over two days. This protocol has been shown to effectively increase the number of medium-sized follicles and improve follicular uniformity. Higher initial doses may enhance early follicular recruitment due to stronger stimulation during the early phase of folliculogenesis (Fortune et al., 2001).

### 3. Six-Dose (FSH6) Protocol:

Alternatively, the same total dose of FSH may be divided into six smaller doses (~33 mg each), administered over three days. Although this protocol also promotes follicular growth, some studies suggest slightly lower efficiency in generating medium-sized follicles compared to the four-dose regimen. In a controlled study, da Silva et al. (2017) demonstrated that both FSH4 and FSH6 protocols significantly increased the number of medium follicles (6–10 mm) while reducing the proportion of small follicles (<6 mm). However, these improvements in follicular dynamics did not translate into significant differences in oocyte recovery rate, oocyte quality, or blastocyst production.

### 4. Synchronization-Based Superstimulation Protocol:

To optimize the response to FSH, superstimulation is commonly combined with synchronization of follicular wave emergence. A typical protocol includes:

- **Day 0:** Administration of progesterone device and estradiol benzoate
- **Day 3:** Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) for luteolysis
- **Day 3–5:** FSH administration (4 or 6 doses)
- **Coasting period:** 30–48 hours after last FSH injection
- **Day 7:** Ovum pick-up (OPU)

This approach ensures synchronized follicular development and improves the proportion of follicles at an optimal size during OPU (Sirard et al., 1999; Blondin et al., 2002).

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**A. Slow-Release FSH Formulations:**

Antrin-R10 Al® Antrin-R10 Al® is a third-generation ovarian superstimulation product developed to simplify gonadotropin administration in bovine reproductive programs. Unlike conventional porcine follicle-stimulating hormone (pFSH) preparations, which typically require 6–8 intramuscular injections over a period of 3–4 days, Antrin-R10 Al® achieves effective superstimulation with a single subcutaneous injection (Kimura, 2016). The formulation consists of pFSH dissolved in saline and combined with aluminum oxyhydroxide gel (Al-gel), a sustained-release carrier. Electrostatic interactions between the positively charged Al-gel and negatively charged pFSH molecules result in the adsorption of nearly all of the administered hormone, allowing its gradual release over approximately four days (Chang et al., 1997; Kimura et al., 2007). This sustained-release mechanism reduces the need for repeated handling of donor animals, thereby improving labor efficiency and animal welfare.

Aluminum-based gels have long been used as adjuvants in vaccine formulations because of their ability to prolong biological activity. Although aluminum salts may induce localized inflammatory reactions or granuloma formation, the low concentration of Al-gel used in Antrin-R10 Al® and its subcutaneous route of administration minimize these adverse effects (Baylor et al., 2002; Kimura, 2016). Currently, the product is commercially available in Japan, and a worldwide patent application has been filed to facilitate its future commercialization in Europe and the Americas (Kimura et al., 2016). The development of Antrin-R10 Al® represents a significant advancement in bovine ovarian superstimulation by providing a practical and efficient alternative to conventional multiple-injection FSH protocols.

**B. Modification of Solvent Vials Using Sustained-Release Excipients:**

To simplify conventional superovulation protocols that require multiple FSH injections, researchers have explored the incorporation of sustained-release excipients into the solvent vial of porcine follicle-stimulating hormone (pFSH). These formulations are designed to create a viscous depot following subcutaneous (SC) or intramuscular (IM) administration, thereby slowing hormone absorption and maintaining effective circulating FSH concentrations for several days. Various polymers, including hyaluronic acid (HA), polyvinylpyrrolidone (PVP), gelatin, and polyethylene glycol (PEG), have been successfully used to achieve superovulation with only one or two injections instead of the traditional 6–8 injection protocol (Hill et al., 1985; Takedomi et al., 1995; Yamamoto et al., 1994T; Tríbulo et al., 2012)

Among these excipients, HA (2%) and PVP (25–50%) have shown promising results by prolonging FSH release and producing ovarian responses comparable to conventional multiple-injection regimens. Similarly, PEG combined with novocaine has been reported to support sustained hormone delivery, where novocaine enhances early hormone absorption through local vasodilation, while PEG maintains prolonged release for approximately four days. These polymers are widely recognized as safe pharmaceutical excipients and are included in the FDA inactive ingredient database for injectable applications (FDA, 2020).

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Despite their effectiveness, formulation challenges remain. High concentrations of HA and PVP substantially increase solution viscosity, making hormone reconstitution and injection difficult (Kimura, 2016). This effect is largely attributed to polymer chain entanglement, which increases resistance to flow as polymer concentration and molecular weight rise. Attempts to reduce HA concentration improved injectability but compromised the sustained-release effect, resulting in inadequate ovarian stimulation when administered as a single injection (Tríbulo et al., 2011). However, dividing the dose into two administrations restored superovulatory responses comparable to conventional protocols (Tríbulo et al., 2012).

The molecular weight of PEG also plays a critical role in determining treatment success. PEG molecules that are too small release FSH too rapidly, whereas excessively large molecules delay hormone release beyond the optimal stimulation period. Consequently, only an intermediate molecular weight range provides the desired four-day release profile required for effective superovulation. Gelatin-based formulations have also shown potential, although their application remains limited due to concerns related to animal-derived raw materials and batch-to-batch variability, which may affect hormone release characteristics (Hill et al., 1985).

To overcome viscosity-related limitations, newer drug delivery technologies such as in situ forming implants have been proposed. One example is the Atrigel™ system, which utilizes biodegradable polymers such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA). These polymers form a depot after injection, allowing gradual release of incorporated proteins over a controlled period. A patent describing a PLGA-based sustained-release formulation for proteins, including FSH, suggested that release profiles of approximately four days could be achieved following a single SC or IM injection. Although promising, the application of this technology for bovine superovulation remains experimental, and further studies are required to confirm its effectiveness under field conditions.

Overall, sustained-release excipient-based formulations represent a significant advancement in bovine ovarian superstimulation. By reducing the number of hormone injections while maintaining effective follicular stimulation, these systems have the potential to improve labor efficiency, animal welfare, and the consistency of superovulatory responses in embryo production programs.

**C. Superstimulation Protocols Using Pituitary-Derived FSH Preparations:**

Partially purified pituitary extracts containing follicle-stimulating hormone (FSH) have been widely used in bovine superovulation programs. Because FSH has a relatively short biological half-life in cattle, estimated to be approximately 5 hours or less, repeated hormone administration is necessary to maintain adequate circulating concentrations and achieve optimal follicular stimulation (Laster, 1972). Consequently, conventional superovulation protocols typically involve multiple FSH injections administered at 12-hour intervals over a period of 3–4 days (Demoustier et al., 1988; Looney et al., 1988).

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Studies have demonstrated that administering pituitary-derived FSH twice daily results in a superior superovulatory response compared with a single daily injection, highlighting the importance of maintaining consistent hormonal stimulation during follicular development (Looney et al., 1988; Walsh et al., 1993). Various dosage strategies have been adopted in the field, including both decreasing (tapering) and constant-dose regimens. However, comparative studies suggest that these approaches produce similar ovarian responses, with no clear advantage of one protocol over the other (Bó and Mapletoft, 2014; Bó et al., 2018).

In most conventional protocols, prostaglandin $F_2\alpha$ ($PGF_2\alpha$) is administered on the third or fourth day of FSH treatment to induce luteolysis and synchronize estrus. Some practitioners discontinue FSH administration following $PGF_2\alpha$ treatment, whereas others continue hormone administration until completion of the protocol (Bó and Mapletoft, 2014). Research has also shown that extending FSH treatment to six or seven days may improve follicular maturation and ovulation rates in certain donor animals (Bó et al., 2008).

Despite their effectiveness, conventional multiple-injection protocols present several practical limitations. The requirement for FSH administration every 12 hours increases labor demands and may reduce compliance, particularly under field conditions. Frequent handling and restraint of donor animals can also induce stress, which has been associated with reduced superovulatory responses and alterations in preovulatory luteinizing hormone (LH) secretion (Edwards et al., 1987). Therefore, the development of simplified superstimulation protocols that minimize animal handling while maintaining reproductive efficiency remains an important goal in bovine embryo transfer programs, especially for difficult-to-manage or stress-sensitive donors (Bó et al., 1991; Hockley et al., 1992; Biancucci et al., 2016).

**D. Use of Polymers in pFSH-Based Superstimulation Protocols:**

The incorporation of biodegradable polymers into porcine follicle-stimulating hormone (pFSH) formulations has emerged as an effective strategy to reduce the number of injections required during bovine superstimulation protocols. These polymers act as sustained-release carriers, gradually releasing FSH over several days after a single intramuscular administration. Because they are generally biocompatible, biodegradable, and non-reactive within biological tissues, polymers are well suited for veterinary reproductive applications (Sutherland, 1991).

Several polymers have been evaluated for this purpose. Early studies demonstrated that pFSH dissolved in a 30% polyvinylpyrrolidone (PVP) solution and administered as a single intramuscular injection produced superovulatory responses comparable to conventional twice-daily FSH treatments (Yamamoto et al., 1994). Similar positive results were later reported by Takedomi et al. (1995) and Chasombat et al. (2013). However, other investigations observed reduced ovarian responses following single-dose PVP-based treatments, indicating variability in efficacy among different protocols and animal

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populations (Callejas et al., 2002). Likewise, polyethylene glycol (PEG)-based formulations have shown promising results, with single injections inducing satisfactory superovulatory responses in cattle (Choi et al., 2002). Another sustained-release approach involved the use of aluminum hydroxide gel, which successfully supported superovulation after a single administration of pFSH; however, its widespread use may be limited because aluminum hydroxide is primarily utilized as a vaccine adjuvant and may raise concerns regarding local tissue reactions (Kimura et al., 2007).

Among the various polymers investigated, hyaluronan (hyaluronic acid) has received considerable attention. Hyaluronan is a naturally occurring glycosaminoglycan widely distributed throughout animal tissues, particularly within the reproductive tract. Due to its excellent biocompatibility and ability to prolong drug release, it has become one of the most promising carriers for long-acting FSH formulations (Oh et al., 2010). Studies in beef cattle demonstrated that a single intramuscular injection of pFSH diluted in 2% hyaluronan produced superovulatory responses and numbers of transferable embryos comparable to those obtained using conventional twice-daily injection protocols (Bó et al., 2010; Tríbulo et al., 2011).Although the 2% hyaluronan formulation proved highly effective, its practical application under field conditions was challenging because of its high viscosity, which made mixing with pFSH difficult (Tríbulo et al., 2011). Reducing the concentration to 1% improved handling and injectability; however, the resulting faster hormone absorption led to reduced follicular development and a lower superovulatory response. Many follicles failed to reach ovulatory size by the time of estrus, suggesting that circulating FSH concentrations were not maintained long enough to support continued follicular growth (Tríbulo et al., 2011). To address this limitation, researchers evaluated protocols involving two injections of pFSH diluted in 1% hyaluronan administered 48 hours apart. These studies demonstrated ovarian responses and embryo yields similar to those achieved with the conventional multiple-injection regimen (Sugawara and Nikaido, 2014; Tríbulo et al., 2012). Additional research showed that even more dilute formulations, such as 0.5% hyaluronan, could achieve comparable results when administered twice during the treatment period, while offering improved ease of preparation and administration (Rogan et al., 2010; Tríbulo et al., 2012). Field trials conducted in Bos taurus beef cattle, particularly Angus donors, further confirmed the effectiveness of the two-injection protocol using 0.5% hyaluronan. Similar findings were reported in Marchigiana heifers, where reduced handling requirements were associated with improved animal welfare and, in some cases, enhanced embryo production (Biancucci et al., 2016). Notably, cattle receiving only two injections exhibited lower cortisol concentrations than those subjected to conventional twice-daily treatments, indicating reduced stress levels. This finding suggests that simplified superstimulation protocols may be particularly advantageous in animals that are difficult to handle or highly sensitive to repeated restraint (Biancucci et al., 2016). The effectiveness of hyaluronan-based formulations has also been investigated in Bos indicus cattle. In Nelore cows, a protocol consisting of two injections of pFSH in

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0.5% hyaluronan produced numbers of transferable embryos comparable to those obtained with conventional treatments, although the total number of recovered ova and embryos was lower (Bó et al., 2010). Similar studies in Brahman cattle found that two injections of 200 mg pFSH in 0.5% hyaluronan yielded embryo production results equivalent to the standard protocol. However, reducing the pFSH dose to 150 mg resulted in fewer transferable embryos, suggesting that inadequate gonadotropin support may limit the growth and maturation of all FSH-responsive follicles. Overall, polymer-based sustained-release formulations represent an important advancement in bovine superstimulation protocols. These systems reduce the need for repeated hormone administration, lower handling stress, and improve the practicality of embryo transfer programs. Nevertheless, achieving an optimal balance between hormone release duration and circulating FSH concentrations remains critical, particularly in Bos indicus breeds, where long-acting formulations may not always provide sufficient hormonal support for maximal follicular development.

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