Microtechnology for Hormone Release in Programmed Animal Reproduction

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ABSTRACT

The control of the estrous cycle and ovulation in bovine breeders significantly improves the livestock cattle business. Controlled hormone release from biocompatible, biodegradable and injectable microbeads is a promising technology for drug intervention programs to control the estrous cycle of cattle. Microbeads loaded with progesterone were obtained using polyvinyl alcohol and dropping/gelling technique as polymer matrix and microencapsulation technology, respectively. The effects of chemical composition, release medium and the dissolution-diffusion processes of progesterone through the matrix were studied. In-vitro results for hormone release are presented.

Keywords: Microencapsulation, microtechnology, biomaterials, hormone release, animal production, Argentina.

1. INTRODUCTION

Hormones treatment is essential for the control of the estrous cycle in cattle when programmed animal production is performed (Rathbone et al., 2001). Progesterone (Pg) plays a prominent role in drug intervention programs to control the estrous cycle for fixed-timed artificial insemination in cattle without the need for estrous detection. Specifically, Pg inhibits the expression of estrus and prevents ovulation by suppressing LH release. Currently, exogenous Pg is administered by slow release from intravaginal devices of silicon, polyurethanes, ethylene-vinylacetate copolymers, among other polymers (Rathbone et al., 1998). Intravaginal devices are relatively cheap, biocompatible, and they have been approved by regulatory agencies and represent an important section of the World's market for veterinary products (Rathbone et al., 1999). However, their size and complex geometrical shape make difficult its storage, transport, insertion and extraction from the vaginal cavity. Its manipulation must be carried out under aseptic conditions and avoiding any accidental contact between the device and the skin of the operator. In addition, exhausted devices must be burned or buried to prevent future accidental contamination (Walsh et al., 2008; Rothen-Weinhold et al., 2000). Pg release from biocompatible-biodegradable, injectable microparticles is an attractive alternative to the widely used intravaginal devices.

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Injectable microparticles have found a number of veterinary applications as drug delivery vehicles. Enzymes (Scocca et al., 2007), antibiotics (Sun et al., 2004), DNA therapeutics (Van Drunen Littel, 2006; Alpar et al., 2005), vaccines (Bowersock et al., 1999) and other drugs (Gavini et al., 2005) have been released for different purposes from injectable polymeric microparticles.

Natural and synthetic polymers have been extensively studied for numerous microparticulated drug delivery systems (Winzenburg et al., 2004). Natural polymers such as collagen and hyaluronic acid based polymers are abundant, easy to isolate and process, with minimal foreign body reaction and its enzymatic degradation can provide appropriate degradation kinetics for exhaust microparticles. Challenges to using natural polymers includes high cost to purify, natural variability of isolated polymer, and variations in the enzymatic degradation depending on the injection site (Riddle et al., 2004). Synthetic degradable polymers such as poly(glycolide), poly(lactide) and its copolymers are widely used in human medicine and certainly showed good performance in animal studies. The main advantages of these polymers are their high biocompatibility with degradation products (basically, lactic and glycolic acids) easily cleared by the body. They are available is a range of molar masses, well defined molecular structures, purities and monomers ratios in case of copolymers. The main disadvantages are the high cost and rapid loss of mechanical properties due to the non-specific bulk degradation mechanism of the polymers (Riddle et al., 2004). Synthetic non-degradable polymers such as poly(ethylene oxide) and poly(ethylene glycol) are highly biocompatible, non-toxic and most importantly cheaper than the mentioned poly(hydroxy esters). Their limitations reside in the lack of degradation and difficulties for functionalization (Metters et al., 2000).

Poly(vinyl alcohol) (PVA) is promising synthetic polymer combining most of the advantages of the previously mentioned polymers. It is biocompatible, easily to functionalize through the pendent alcohol groups, it is available in a wide range of molar masses, purities, with practically no variations between production batches, it has good mechanical strength and stability and most important it is not expensive (PVA Mowiol Brochure, 2008). Disadvantages related to lack of degradation may be partially solved by controlling the dissolution of the PVA matrix in the tissues surrounding the injection site. PVA injectable microparticles may be especially advantageous for the controlled release of hormones such as progesterone (Pg), estradiol and prostaglandine, which are highly soluble in alcohols but almost insoluble in water. Exemplary, 100 % ethanol (EtOH) medium can dissolve Pg up to 101.3 g / L, while pure water (H2O) dissolve less than 0.01 g / L (Bunt et al., 1997). Therefore, PVA matrices with excess of alcohol groups are expected to dissolve and release the hormone very fast. Conversely, highly swelled in H2O polymer matrices would dissolve and release the hormone very slow. Consequently, an optimal swelling degree which dissolve and release Pg at an appropriate kinetics, as required by drug intervention programs, should exist somewhere between the mentioned extremes. This work details the influence of the swelling degree on the dissolution-diffusion process of Pg crystals entrapped into PVA microbeads and immersed in different media. A protocol for the synthesis of Pg loaded PVA microbeads and in-vitro studies is presented.

2. EXPERIMENTAL PART

2.1 Materials

PVA (Mw = 205000 g/mol, 88% hydrolyzed; Mowiol 40-88, Kuraray, USA) was selected as base material for the preparation of polymer matrices. Micronized Pg USP30 quality >99.3% (Farmabase, Italy) was selected as case study for hormone release. Boric acid (BH), sodium hydroxide (NaOH), sodium chloride (NaCl) and EtOH (all of them with A.C.S. Pro-analysis quality >99.5%; Cicarelli, Argentina) were used as crosslinker of PVA, for pH and ionic strength adjustment of the gelling bath and preparation of release media for the in-vitro studies, respectively. The H2O was distilled prior to use.

2.2 Preparation and Characterization of PVA Microbeads

Stock aqueous solution was prepared containing 10 wt% of PVA functionalized with 0.3 wt% of BH at pH = 4.4. The molar ratio between BH to -OH groups in the PVA resulted $4.4\ 10^{-2}$. The PVA-BH stock solution was equally divided in five parts. Different amounts of Pg were added to each part loading the PVA-BH solution at 0, 5, 20, 33 and 50 wt%. A stock aqueous gelling solution was prepared containing of [NaOH] = $1.67\ 10^{-2}$ mol/L and [NaCl] = 0.50 mol/L. Microbeads were obtained by dropping the PVA-BH-Pg solution into the NaOH-NaCl gelling solution under moderate stirring and thermostatted at 40ºC according to a detailed procedure (Rintoul, 2009). The initially viscous PVA-BH-Pg liquid drops resulted instantaneously gelled preserving the spherical shape when immersed in the gelling solution. The gelling reaction was fast enough to entrap all Pg crystals in the polymer matrix avoiding any escape to the gelling solution which remains transparent and clear. After dropping, the beads were stabilized in the gelling solution during 30 min and recovered by filtration. Subsequently, the beads were dried at 80ºC during 72h.

2.3 Swelling and Mass Transfer Processes Studies

Microbeads samples were immersed in different media and left to swell, release Pg and dissolve the PVA polymer matrix. The release media were physiologic solution (0.9 wt% NaCl aqueous solution) and EtOH/H2O mixture 40/60 vol% (Bunt et al., 1997). All release studies were carried out at 38ºC. Swelling, hormone release, dissolution of polymer matrix and solvent transfer between the microbeads and the release medium are phenomena that may occur simultaneously and may have multiple and crossed interactions. Wet weights of swollen samples at different times were measured until equilibrium was reached. Afterwards, the samples were dried at 80ºC during 72h and reweighed to determine the total residual solid content after release. Subsequently, the microbeads were put into an excess of pure EtOH during 24h to remove Pg and determine the residual Pg. Finally, they were dried at 80ºC during 24h and reweighed to determine the residual PVA polymer matrix. All conditions are presented in Table 1.

Table 1. Conditions for swelling and mass transfer studies at 38ºC

	Physiologic solution (0.9 wt% NaCl)				EtOH/H2O solution (40/60 vol%)			
Pg wt%	Microbeads g	Solution mL	Total Pg g	Total PVA g	Microbeads g	Solution mL	Total Pg g	Total PVA g
0	0.209	100.04	0.000	0.209	0.202	100.02	0.000	0.202
5	0.201	100.06	0.010	0.191	0.201	100.01	0.010	0.191
20	0.200	100.05	0.040	0.160	0.207	100.02	0.041	0.166
33	0.201	100.06	0.066	0.135	0.206	100.01	0.068	0.138
50	0.206	100.04	0.103	0.103	0.209	100.03	0.104	0.105

2.4 Hormone Release Studies

A dissolution test station was used to determine the release profile of Pg through differentially Pg-loaded PVA microbeads (Table 2). Release experiments were set in order to evaluate the dissolution and diffusion of Pg through the swelled PVA matrix in EtOH/H2O solution. Hormone release studies were carried out keeping constant the total amount of Pg at 5 mg. Aliquots of the supernatant release media were withdrawn from the test station at different release times. The cumulative amount of Pg in the samples was determined using a UV-VIS spectrophotometer (Model: UV-2401PC, Shimadzu Japan) operating at 244 nm and previously calibrated with Pg standard solutions. Figure 1 presents the UV calibration for Pg at 244 nm.

Table 2. Conditions for hormone release studies at 38ºC

	EtOH/H2O solution (40/60 vol%)			
Pg wt%	Microbeads g	Solution mL	Total Pg mg	Total PVA g
0	0.102	140.22	0.00	0.102
5	0.102	140.10	5.10	0.097
20	0.025	140.03	5.00	0.020
33	0.015	140.03	4.95	0.010
50	0.010	140.16	5.00	0.005

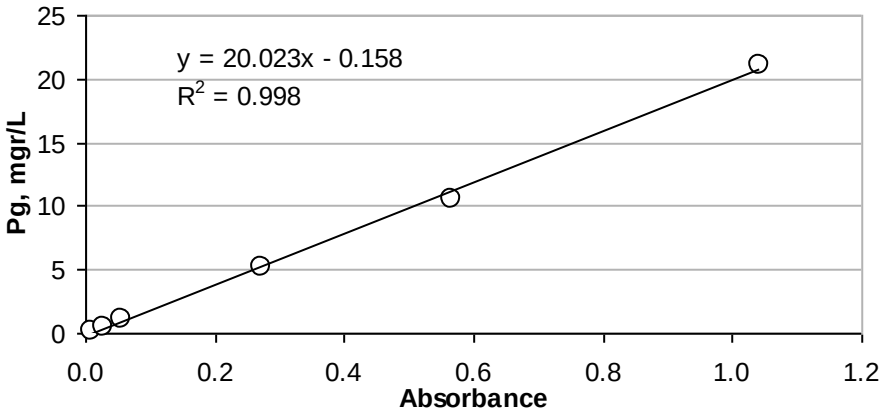

Figure 1. UV-VIS spectrophotometer calibration curve for Pg. Standard Pg concentrations were plotted as a function of the corresponding absorbance at 244 nm.

3. RESULTS

3.1 Microbeads Characterization

Spherical PVA microbeads with diameters between 1.30 and 2.40 mm were obtained. Figure 2 shows the appearance of the microbeads under magnification. Figure 3 shows the microbeads diameter as a function of their Pg load. Slight linear increment in diameter was observed when the Pg load in the microbeads was varied from 0 to 50 wt%.

Figure 2. Appearance of Pg loaded microbeads under 20X magnification.

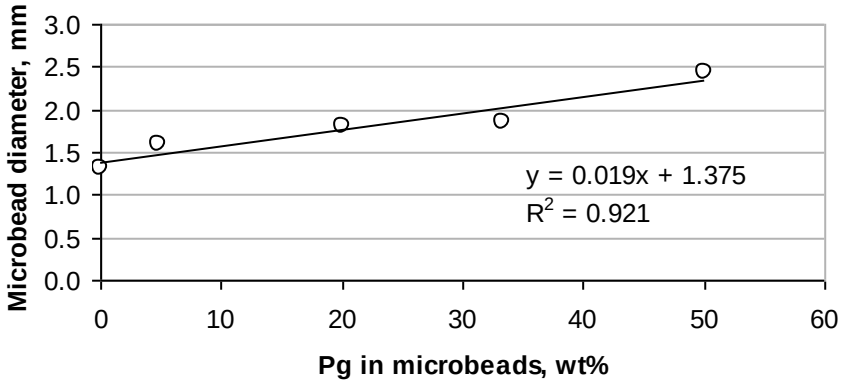

Figure 3. Average diameter of PVA microbeads as a function of the Pg loading.

3.2 Swelling and Mass Transfer Processes

The weight increment of microbeads swelled in physiologic and EtOH/H2O solutions is presented in Figures 4 and 5, respectively. The swelling path of microbeads immersed in physiologic solution showed three different stages. An initial fast swelling up to a weight increment of about 7 times after 10h of swelling followed by a slow weight decrease until 30h of swelling and, finally, a very slow but progressive weight decrease were observed. In general, the wt% of Pg seems to not affect significantly the swelling path. Slight deviation of the swelling path of microbeads loaded at 20, 33 and 50 wt% from those loaded at 0 and 5 wt% was observed. Higher Pg load in the microbeads enhanced the weight loss in the third stage of the swelling path. Ultimately, the weights decreased at the same rate in all cases. The swelling path of microbeads immersed in EtOH/H2O solution also presented three stages. The initial swelling stage occurred at the same rate than in physiologic solution. The weight increment reached a maximum between 9 and 10 times after 5h of swelling time. The weight decrease in the second stage was faster than in physiologic solution. And finally, the third stage presented the same trend as in the physiologic solution case.

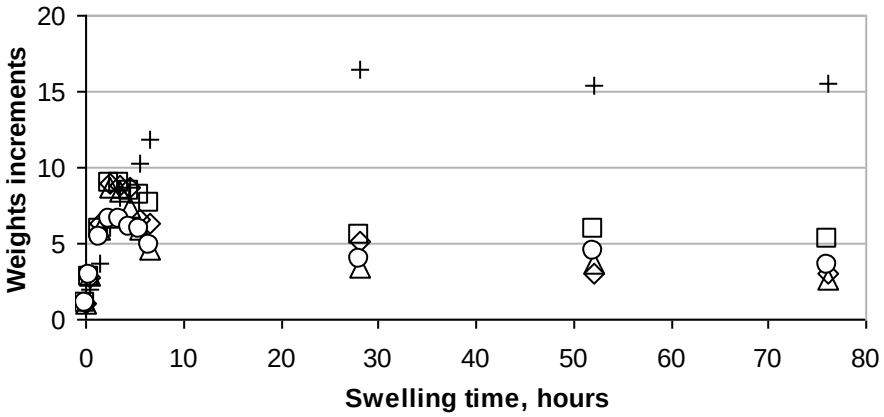

Figure 4. Weight increment of PVA microbeads during the swelling period. Swelling medium: Physiologic solution (0.9 wt% NaCl). T = 38ºC. Pg load in the microbeads: 0 (+), 5(□), 20(◊), 33(Δ) and 50(○) wt%.

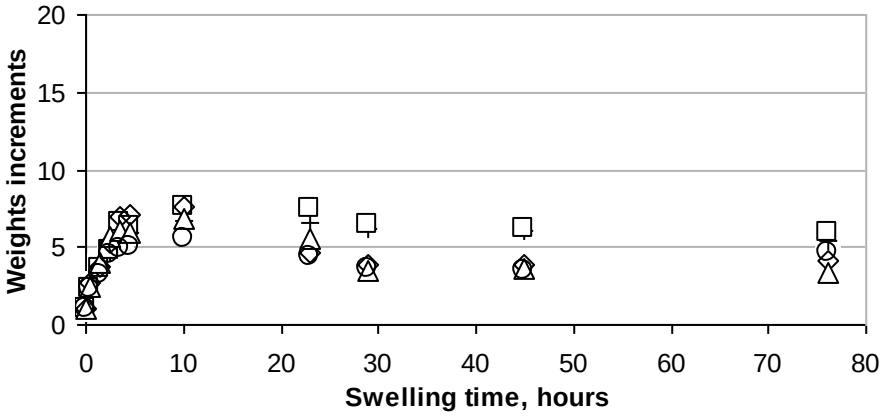

Figure 5. Weight increment of PVA microbeads during the swelling period. Swelling medium: EtOH/H2O solution (40/60 vol%). T = 38ºC. Pg load in the microbeads: 0 (+), 5(□), 20(◊), 33(Δ) and 50(○) wt%.

The Pg free microbeads did not present a second stage that resulted characteristic in microbeads loaded at 5, 20, 33 and 50 wt%. Instead, the weigh increased continuously during 10h of swelling to enter directly in the third stage. The third stage presented a slight and progressive weight decrease as the other Pg loaded microbeads.

The residual values for PVA and Pg in exhausted microbeads are presented in Table 3. The residual PVA resulted significantly higher (near 15 wt%) in Pg free microbeads than in Pg loaded microbeads (around 3 wt%) for both, physiologic and EtOH/H2O release media. Conversely, the residual Pg resulted one order of magnitude higher in studies carried out in physiologic solution (40,1-76,8 wt%) than in EtOH/H2O solution (0.1-7.5 wt%). Interestingly, the residual Pg decreased with the increase of the Pg load when physiologic solution was selected as release medium. The residual Pg remained almost unchanged with the increase of the Pg load when EtOH/H2O solution was used.

Table 3. Residual Pg and residual PVA in exhausted microbeads.

Pg wt%	Physiologic solution (0.9 wt% NaCl)				EtOH/H2O solution (40/60 vol%)			
	Initial microbeads g	Residual weight g	Residual Pg wt%	Residual PVA wt%	Initial microbeads g	Residual weight g	Residual Pg wt%	Residual PVA wt%
0	0.209	0.034	0.0	16.2	0.202	0.029	0.0	14.5
5	0.201	0.014	76.8	3.1	0.201	0.014	0.1	2.8
20	0.200	0.029	62.0	2.9	0.207	0.025	2.1	2.8
33	0.201	0.029	39.1	2.5	0.206	0.031	5.6	3.6
50	0.206	0.045	40.1	3.3	0.209	0.069	7.5	3.2

3.3 Hormone Release

Figure 6 presents the cumulative hormone concentration in the EtOH/H2O release medium vs the release time. Two stages in the curves can be easily visualized. Firstly, the cumulative concentration of Pg increased linearly with release time. Secondly, the cumulative concentration reached a plateau indicating the end of the release process.

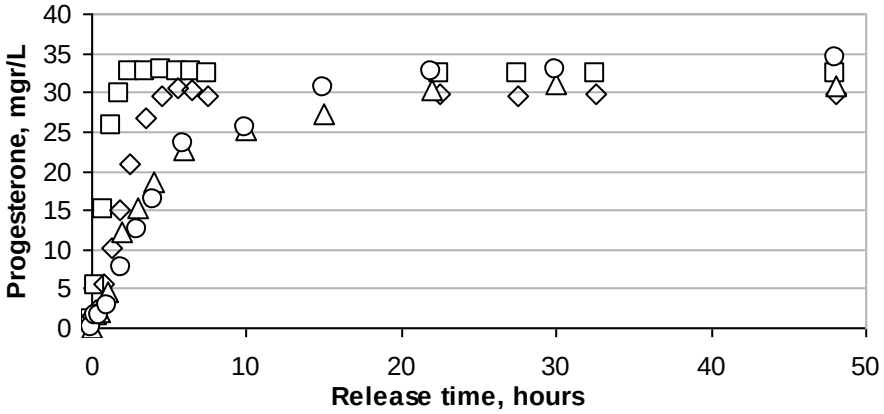

Figure 6. Cumulative hormone concentration vs. release time. Release medium: EtOH/H2O solution (40/60 vol%). T = 38ºC. Pg load in the microbeads: 5(□), 20(◊), 33(Δ) and 50(○) wt%.

The initial rate of hormone release decreased with the increment of the hormone load in the microbeads. Figure 7 shows the rate of increment of the cumulative hormone concentration (d[Pg]/dt) as a function of the wt% of Pg in the microbeads. A power decay functionality between the releasing rate and the Pg load could be fitted with excellent correlation. See equation and correlation in Figure 7. Exemplary, the cumulative concentration of Pg increased at 19.7 mg/(L h) and 4.2 mg/(L h) for microbeads loaded at 5 and 50 wt%, respectively. The initial total interface area between the microbeads and the release medium was calculated assuming perfect spherical shape and an average diameter according to Figure 2 for all microbeads. The resulting

area values are plotted in Figure 7. Evidently, the decrease in the release rate of Pg has the same trend as the decrease of the total area of microbeads with the increase of the Pg load.

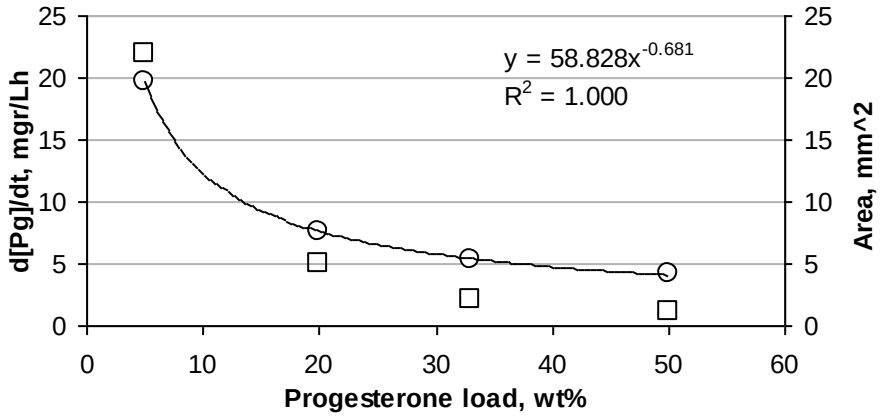

Figure 7. Rate of increment of cumulative Pg (○) and total microbeads area (□) as a function of the Pg load in the microbeads. Release medium: EtOH/H2O solution (40/60 vol%). T = 38ºC.

4. DISCUSSION

The transformation of the liquid PVA-BH-Pg solution into gel particles resulted by crosslinking of PVA through transformation of PVA-BH monodiol complex to PVA-BH didiol complexes. This crosslink bonding is naturally reversible. Therefore, the formed gel can be slowly dissolved under appropriate conditions. Pg crystals were trap into the PVA matrix during the gelation process. The poor solubility of Pg in aqueous systems, its affinity to alcoholic groups and the relatively rapid monodiol-didiol transformation may justify the total entrapment of Pg crystals in the gelled PVA microbeads.

Figure 2 shows the uniformity of the size distribution of microbeads. This is a consequence of the selected dropping/gelling technique as microencapsulation technology. This technique ensures the formation of very homogeneous microbeads by the formation of equally sized individual drops. The increment of the Pg load in the microbeads increased the final size of microbeads (Figure 3). Such increment may be related to an increment of the viscosity of the PVA-BH-Pg solution with the increment of the Pg load. Higher viscosity of the dropping solution could induce bigger drops. Thus, higher particle size of final microbeads is obtained. The selected size range of microbeads is the result of a compromise. On the one hand, they must be small enough to pass through veterinary needles. On the other hand, they must be big enough to minimize their displacement from the injection site.

Ideally, the hormone release shall occur much faster than the dissolution of the PVA matrix to avoid coupled effects between these two processes. More specific, Pg must be released during a period of several days while the polymer matrix must be dissolved in terms of weeks or months to assume unmodified properties of the microbeads during the release time. The kinetics of hormone release and dissolution of the polymer matrix depends on the characteristics of the microbeads but also on the conditions and properties of the release medium. Swelling, hormone release and dissolution of the PVA matrix were characterized in physiologic and EtOH/H2O solutions. Physiologic solution and soft tissues such as muscle and subcutaneous regions have

the same ionic strength. On the one hand, this solution may simulate quite appropriately the interactions between the hydrophilic polymer matrix with the tissues surrounding the injection site. On the other hand, the results corresponding to in-vitro release studies may present a poor correlation with in-vivo studies due to the very low solubility of the hormone in physiologic solution. In-vivo studies may overcome this inconvenient by rapid metabolization of the hormone released from the microbeads. This condition is not easy to achieve in in-vitro studies using physiologic solution as release medium. EtOH/H2O mixtures are able to admit large amounts of Pg in solution. In such a case, a hormone drain with infinite capacity can be assumed. The weight increment curves in Figures 4 and 5 present three stages. Swelling, hormone release and dissolution of the polymer matrix processes may occur simultaneously in all stages. However, swelling, hormone release and dissolution of the polymer matrix are the dominant processes in the first, the second and the third stage, respectively. The swelling and matrix dissolution rates of microbeads occur similarly in physiologic and EtOH/H2O solutions. Therefore, they are exclusively dependent on the physical-chemical properties of microbeads. Contrarily, the weight decrease after swelling occurs much slower in physiologic solution than in EtOH/H2O solution. The result confirms the analysis of Pg solution properties in hydro-alcoholic media. The higher is the EtOH vol% in the release medium, the higher is the solubility and faster is the release of Pg in such medium. Here, variations in the quality of the release medium by selection of different injection sites may modify significantly the release kinetics of Pg loaded microbeads.

The residual PVA resulted significantly higher in Pg free than in Pg loaded microbeads. Less mechanical stability and cohesion of loaded particles as consequence of the pores generated by the dissolution of Pg crystals is suggested as explanation. The important differences observed in the residual Pg in microbeads immersed in physiologic and EtOH/H2O solutions may be a consequence of the solubility differences of Pg in such media. Physiologic solution can admit small amounts of Pg in solution. Therefore, when loaded microbeads are immersed in this medium a small amount of Pg tend to be released. In fact, the swelled PVA matrix is a hydro-alcoholic medium which can admit a large amount of Pg in solution. This amount of Pg can be assumed in direct relation to the PVA concentration in the swelled microbeads. The PVA concentration decreases with the increase of the Pg load in the microbeads. Thus, residual Pg remains higher in less loaded microbeads. The EtOH/H2O 40/60 vol% solution can dissolve more than 10 times the amount of Pg loaded in the microbeads. This prevented to reach the saturation limit of the solution. The residual Pg amount corresponds to the equilibrium between the swelled PVA matrix and the EtOH/H2O hydroalcoholic media.

The Pg releasing rate decreased with the increase of the Pg load in the microbeads (Figure 6 and 7). The phenomenon can be explained in terms of the reduction area for hormone release with the increment of the Pg load in the microbeads. Clearly, the number of microbeads necessary to accumulate a dose of Pg would be lower for the 50 wt% loaded microbeads than for the 5 wt% loaded microbeads. Moreover, the reduction in the number of microbeads is accompanied with a reduction of the total area for the transfer of Pg from the interior of microbeads to the release medium. Clearly, the reduction of release area and the decrease of release rate follow the same trend.

5. CONCLUSIONS

The hormone release kinetics depends on multiple factors related to the characteristics of the microbeads and the release medium. The control of physical-chemical properties of microbeads and the well understanding of the behavior of such microbeads immersed in the release medium are crucial for the correct design and development of injectable microbeads useful for drug intervention programs to control the estrous cycle for fixed-timed artificial insemination in cattle without the need for estrous detection. The swelling degree, the Pg load and the mass transfer area between the microbeads and the release medium are key parameters to control the dissolution-diffusion of Pg in the microbeads and further release to the medium. The swelling degree controls the dissolution of Pg crystals in the microbeads. The Pg load has influence on the size and mechanical stability of microbeads as well as in the residual weight of the exhausted microbeads. The area for mass exchange between the microbeads and the release medium is intimately related to the Pg load in the microbeads. In addition, it appears to have an important role in the swelling velocity and the rate for Pg release. In-vitro studies in different media are necessary for the comprehension of Pg release and for the establishment of starting points for in-vivo studies. Advanced in-vivo studies are recommended to be carried out under the following conditions: microbeads loaded at 50 wt% and subcutaneous injection sites.

6. ACKNOWLEDGMENTS

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