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Research paper

Evaluation of thermosensitive poloxamer 407 gel systems for the sustained release of estradiol in a fish model

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ABSTRACT

The purpose of this study was to develop and evaluate a delivery system comprising a thermosensitive gel for the sustained release of steroidal hormones in fish, over an extended period of time after a single intramuscular (*i.m.*) injection and for the improved reproductive performance in fish.

Controlled delivery systems based on thermosensitive gels are easy to prepare, low cost and high versatility dosage forms, which have been shown to be effective in several animal species for sustained release of hormones.

In this work, a thermosensitive gel system based on poloxamer 407 in water:ethanol medium, able to work as a prolonged release carrier for 17β-estradiol (E2), has been developed. Such a system was able to solubilize the lipophilic E2 and to gel at the required water temperature for fish rearing (20 °C). Moreover, the system exhibited the best injection condition at temperatures below 15 °C when the system behaved as a low viscosity Newtonian liquid.

The thermosensitive gel system was tested in vivo in the fish model, *Carassius auratus*, and the results compared with a single *i.m.* injection of E2 dissolved in corn-oil and other relevant control systems not containing E2. The results were particularly interesting, since fish injected with the E2 thermosensitive gel formulation, showed significantly higher levels of the circulating hormone than corn oil-E2 treated animals at 72 and 96 h after injection. In addition, the thermogel system was able to sustain the plasma level of E2 for about 11 days. The increased plasma levels of E2 were also accompanied by maintained higher values of plasma vitellogenin (VTG), thus suggesting that the thermosensitive polymer based delivery system could prevent rapid hepatic clearance of E2, resulting in prolonged stimulation of estrogen receptor-mediated pathways in goldfish.

1. Introduction

The use of pharmaceutical agents in aquaculture is a well-established practice for the treatment of fish diseases and for the modulation of fish growth and reproduction [1]. The latter point is particularly relevant for all the species reared in captivity inside commercial aquaculture facilities. In these conditions, many animals show evidence of some degree of reproductive dysfunction such as asynchrony in the time of ovulation, absence of volitional spawning, diminished sperm production or even the absolute failure of maturation and the presence of ovulation phases [2–5]. Although in some cases, these concerns can be overcome or at least mitigated by manipulation of various environmental parameters [4,6], in most cases hormonal treatments can be more effective means of controlling fish reproduction.

Abbreviations: EO, ethylene oxide; PO, propylene oxide; E2, 17β-estradiol; VTG, vitellogenin; SDS, sodium dodecyl sulfate; MS 222, 3-aminobenzoic acid ethyl ester methanesulfonate; EE2, 17-α-Ethynylestradiol; TEG, triethylene glycol; EIA, enzyme-immunoassay method; ELISA, enzyme-linked immunosorbent assay; BW, body weight.

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While in the last decades many efforts have been made to improve the efficacy of hormone drugs, leading to a wide variety of synthetic agonists of natural hormones, a lot of work still remains to be done in terms of development of appropriate and effective sustained delivery systems. In fact, to date most often multiple injections of hormones have to be given over the course of several days, or even over several weeks, in order to maintain elevated hormone plasma levels [3,4]. The multiple treatments require repetitive handling of brood stock and consequently labor, time and monitoring, moreover, this procedure is stressful to the fish and sometimes also results in adverse effects [5].

Despite the fact that sustained administration of hormones has been shown to be effective in aquaculture [7–9], at the present time, only minimal research is devoted to this issue and only a few sustained release systems have been developed and tested on fish breeding, such as cholesterol pellets implants [10,11], biodegradable microspheres [12,13], non-degradable monolithic implants [14,15] and more recently, osmotic pumps [16]. The sustained release cholesterol pellets implants are now actually marketed (Ovaplant™, Western Chemical Inc., USA) [17].

Although these systems have generally demonstrated a good performance when administrated to fish, the production and wider distribution on an industrial scale appears improbable due to the high production cost and to the difficulty of administering the delivery system.

The aim of this work was the development of a new sustained release system intended for the administration of hormones in aquaculture and characterized by low production cost, easy administration and high versatility. In particular, it was decided to study the possibility of using a thermogelling system, i.e. a system that is liquid at low temperature and gel at the body temperature of fish (20 °C). These particular characteristics should assure an easy administration of the system, as a liquid, and a prolonged drug release once the system has rapidly gelled inside the body of the fish. The use of a parenteral thermogelling systems has previously been shown to be effective for controlled release of hormones when applied in different animal species [18–20]. These formulations increased the relative bioavailability of steroidal hormones and resulted in the sustained release of testosterone after a single subcutaneous (*s.c.*) injection in rabbits [18]. Moreover there is evidence that thermogelling systems such as poloxamer can protect against local enzyme-mediated drug metabolism and stabilize peptide hormones [18,20,31].

In the present study, 17β-estradiol (E2) was selected as the model hormone [21,22]. The sustained release system was prepared using poloxamer 407, a block copolymer of ethylene oxide (EO) and propylene oxide (PO) showing a thermogelling behavior which is dependent on its concentration and on the presence of other excipients. Suitable systems were prepared after a preliminary rheological characterization intended to define the correct concentration of the polymer and other excipients (i.e. sodium chloride and ethanol), necessary to obtain a thermosensitive gel with an appropriate gelation temperature for the administration in juvenile goldfish (*Carassius auratus*). The efficiency of the hormone release system was evaluated by monitoring both plasma E2 and vitellogenin (VTG) concentrations over a period of 18 days.

2. Materials and methods

2.1. Materials

Poloxamer 407 (LUTROL F-127, BASF, Burgbernheim DE), sodium dodecyl sulfate (Bio-Rad laboratories, USA), 17β-estradiol and 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Aldrich, St. Louis USA) were used as supplied. Ethanol (Sigma Aldrich, St. Louis USA) and sodium chloride (Carlo Erba, Rodano, IT) were standard reagents grade, at places in the text ethanol has been described as EtOH. Ultrapure water was produced with a laboratory deionizer (Osmo Lab UPW 2, Gamma 3, IT).

2.2. Methods

2.2.1. Formulation of the thermosensitive gelling system

The thermosensitive gelling formulations were prepared following the "cold" procedure [23]. Briefly, poloxamer powder was slowly dispersed in the required amount of degassed and deionized water under magnetic stirring while keeping the dispersion on an ice bath. Ethanol and NaCl were added at the end of the preparation procedure after the poloxamer was completely solubilized. E2 was dissolved in the ethanol present in the formulation before it was added to the poloxamer water dispersion. The compositions of the different sample preparations are given in Table 1. As can be seen the samples comprised poloxamer 407 in concentrations ranging from 20% to 22% w/v, NaCl from 0.9% to 3.5% w/v and ethanol from 25% to 50% w/v, while the E2 concentration was kept constant at 1.5 mg/mL. All samples were left at 5 °C for 24 h before being analyzed.

2.2.1.1. Rheological analysis. Rheological analysis was performed on the developed systems in order to define the optimal composition of the thermosensitive gel in terms of the concentrations of poloxamer 407, ethanol and NaCl for the preparation of a system characterized by a gelation temperature of 20 °C and a stable appropriate gel structure with controlled release characteristics.

Rheological analyses were performed in triplicate using a stress control rheometer (Stress-Tech, Reologica Instruments, Lund, Sweden) equipped with a cone-plate geometry (4/40) operating in the oscillation mode. The gap was 150 μm. The samples were studied by using temperature sweep tests in the temperature range 0°–50 °C at the rate of 1 °C/min, applying a stress of 10 Pa and a frequency of 1 Hz. The cross-over point between the elastic and viscous modulus was considered as the gel point of the different samples. This parameter, also corresponds to the temperature at which the phase angle is characterized by a value equal to 45° [24].

Formulations with thermogelling characteristics suitable for the aim of the work were further characterized using a viscometry test from 5° to 25 °C (Stress-Tech Reologica Instruments). For each temperature, an appropriate amount of the sample was analyzed, increasing the shear stress (σ) from 0.1 to 150 Pa and the corresponding shear rate (D) measured. Since the samples showed non-Newtonian behavior, at least in the gel form, all the flow curves obtained were analyzed using the 'power law model':

$$\sigma = k \cdot D^n + \sigma_0 \quad (1)$$

where k is the consistency index, n the power law index and σ_0 the yield stress. The consistency index k, also called 'power law viscosity', is related to the viscosity of the system, the 'power law index', n is related to the flow patterns (Newtonian or non-Newtonian) while the 'yield stress' represents the critical stress values necessary for the flow of the sample.

2.2.2. In vitro release of drug

Dissolution studies were performed following the previously described 'paddle over extraction cell method' [25–27]. All the experiments were carried out at 20 °C using an USP dissolution apparatus 2 (AT7 smart, Sotax, CH) equipped with Teflon Enhancer Cells (Agilent, USA) having a surface area of 4 cm^2 and mounted with an 11 μm pore size filter paper (grade 1, Whatman, UK) as a membrane, since a membrane with large pores allows the sample to remain in the enhancer cell during the experiments without interfering with the drug release.