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Polymeric nanoparticle formulation of Octapeptide (NP-OP): *In vitro* release and *in vivo* effect in common marmosets, *Callithrix jacchus* Linn.

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Octapeptide (OP)/FSH-Receptor Binding Inhibitor-8 (FRBI-8), is a synthetic peptide corresponding to N-terminal sequence of purified fraction of Follicle Stimulating Hormone Binding-Inhibitor (FSHBI), isolated earlier from human ovarian follicular-fluid. In order to avoid the repeated drug-administration, OP-loaded, polymeric polylactide (PLA) nanoparticle formulation (NP-OP), was developed using multiple-emulsion technique. This yielded an average particle size of 120 nm with 70% encapsulation-efficiency. *In vitro* release profile of NP-OP showed sustained release of OP for 21 days. *In vivo* anti-fertility studies were conducted in marmosets. Results indicated that control animals conceived in the same cycle while two of three treated animals failed to conceive in treatment cycle. The *in vivo* studies thus corroborate with *in vitro* release of OP, demonstrating its anti-fertility activity in 66% of animals.

Keywords: Anti-fertility, Encapsulation efficiency, Follicle stimulating hormone binding-inhibitor, Polylactide, Synthetic peptides, TEM

Pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the key regulators of ovarian folliculogenesis. These are known to be directly or indirectly modulated by several intra-ovarian factors. An Octapeptide (OP), corresponding to the N-terminal sequence of FSH binding inhibitor (FSHBI), which was purified from human ovarian follicular fluid, has been shown to inhibit FSH binding to the granulosa cells *in vitro*[1]. Further, *in silico* studies have revealed that OP specifically inhibits the binding of FSH to its receptor[2]. It also induces follicular atresia in mice[3] and interferes with normal ovarian function in non-human primates like marmosets[4] and bonnet monkeys[5]. These studies have evinced the potential of Octapeptide as an anti–fertility agent/non-steroidal contraceptive, which specifically affects the action of FSH alone. Studies with OP in marmosets revealed that the peptide when administered on days 6–10 post-partum (dpp), exhibited 75% anti-fertility effect primarily due to luteal insufficiency[4]. However, this treatment presented with a drawback where the small size of this peptide mandated the requirement for multiple drug injections in follicular phase of their ovarian/menstrual cycle. To overcome this limitation, an attempt has been made to modify the drug delivery mechanism of OP by formulating polymeric nanoparticles with sustained drug release profile.

Drug delivery is an interdisciplinary and independent field of research and is gaining the attention of pharmaceutical researchers, medical doctors and industry. A safe and targeted drug delivery could improve the performance of some classic medicines already available in the market, and will also have implications for the development and success of new therapeutic strategies such as anticancer drug delivery, peptide and protein delivery and gene therapy. In the last decade, several drug-delivery technologies have emerged and a fascinating part of this field is the development of nano-scale drug delivery devices[6]. Nanoparticles (NPs) have been developed as an important strategy to deliver conventional drugs, recombinant proteins, vaccines and more recently, nucleotides[7]. NPs and other colloidal drug-delivery systems modify the kinetics, body distribution and drug release of an associated drug. Various nanoparticulate drug-delivery systems

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including polymeric NPs, liposomes, ceramic NPs, magnetic NPs, polymeric micelles and dendrimers as well as their applications have been reported in therapeutics, diagnostics and imaging[8].

Therapeutic outcome of potent macromolecules like peptides, proteins and nucleic acids (DNA, siRNA) strongly depend on development of appropriate carriers which would be suitable for controlled delivery of the intact actives to required tissues, cells and intracellular compartments. Success of therapy based on peptide molecules is severely limited by their *in vitro/in vivo* degradation issues, shorter half-life and inappropriate concentration required for activity. Loading of such therapeutics into polymeric nanoparticles can be an excellent strategy to provide sustained release which would avoid the need of multiple injections regimen for treatment. Development and application of synthetic polymer nanoparticulate carriers have been reported for oral, pulmonary, systemic and nasal delivery routes of oligopeptides, peptides and proteins[9].

The present work is aimed at developing a biodegradable polymeric nanoparticle formulation of OP as an anti-fertility agent sufficing its monthly intake during the follicular phase of an ovarian cycle.

Materials and Methods

Formulation of nanoparticles—Nanoparticles (NPs) were prepared using double emulsion solvent evaporation method as reported previously[10]. For no-peptide nanoparticles, 100 µL of 1% w/v PVA solution was added to 1.0 mL of deionized water and for OP loaded nanoparticles, 50 mg OP was dissolved in 1.0 mL deionized water (W_1 phase) which were further added to 1 g PLA dissolved in 60 mL dichloromethane (DCM) (oil-O phase). The W_1 and O phases were vigorously mixed for 30 sec to form a simple W_1/O emulsion using a vortex at maximum speed (2800 rpm) followed by probe sonication (10 min duration with 15 sec pulse) to obtain a primary emulsion with fine droplets. This primary emulsion was then dispersed in 100 mL surfactant solution (W_2 phase) at 0.5% w/v concentration and subjected to probe sonication (10 min duration with 15 sec pulse) leading to formation of W_1/O/W_2 double emulsion. The double emulsion was later suspended in 300 mL dilute surfactant (0.5% PVA) solution and stirred overnight on a magnetic stirrer to effect the evaporation of solvent (DCM). Later the suspension was subjected to centrifugation at 13,000 g and pellet was collected, washed with distilled water. Cryoprotectant, mannitol, was added at a concentration of 10% w/v and freeze dried using Labconco Freezone 4.5®. The dried product was then stored in desiccator at -20 °C until further use.

Morphological characterization of nanoparticles—The size of nanoparticles in suspension and in freeze dried form (re-dispersed in deionized water) was determined by Dynamic Light Scattering (DLS) using Malvern Zetasizer, USA. The morphological characteristics of OP loaded and blank nanoparticles were determined using Transmission Electron Microscope (TEM). Primarily, freeze dried nanoparticles were re-dispersed in deionized water and allowed to get adsorbed onto the carbon-coated grid. Negative staining was carried out using ammonium molybdate and further evaluation of these samples using Philips Tecnai G^2 12 TEM at 80 KV. Photographs were captured using mega View III Sis CCD camera.

Percent drug loading (%DL) and percent encapsulation efficiency (%EE)—To determine the amount of drug carried by the nanoparticles, both the surface-bound and encapsulated drug quantities are estimated. The relative proportion of drug and polymer used in the process determines the efficiency of drug loading. For estimating this property, nanoparticles were divided into two sets A and B. In set A, the amount of drug adsorbed onto the surface of particle was estimated by accurately weighing out and suspending 4 mg of peptide-loaded nanoparticle formulation (NP-OP) into 1 mL distilled water. The suspension was then centrifuged at 13,000 rpm at 25 °C for 20 min. Following the centrifugation process, supernatant was separated and subjected to peptide analysis using Micro BCA (Bicinchonic acid) protein assay kit (Thermo-Scientific-Pierce, Rockford IL). For set B, the total amount of drug present in the nanoparticle formulation was estimated by dissolving 4 mg of nanoparticle in 2 mL of dichloromethane. The sample was sonicated for 2 min followed by extraction of the peptide using 1 mL of saline. 150 µL of this aqueous peptide extract was assayed in duplicates using MicroBCA kit. The plate was read at 562 nm on the ELISA reader along with standards. Blank nanoparticles prepared under the same conditions were also assayed as control. The drug loading (DL) and per cent encapsulation efficiency (EE) was determined using the following formulae:

$$DL\ (\%) = \frac{\text{Amount of drug in nanoparticles (NP)}}{\text{Mass of nano particle recovered}} \times 100$$

$$EE\ (\%) = \frac{\text{Amount of drug in (NP)}}{\text{Total amount of drug}} \times 100$$

In vitro release of OP from NP-OP—Blank and NP-OP (equivalent to 50 mg) were suspended in 2.0 mL of normal saline and incubated overnight at 37 °C at 200 rpm in a shaker incubator. The suspension was centrifuged at a specific time interval of 24 h and supernatant was removed and replaced with the same amount of fresh saline for 21 days. The peptide content of all the aliquots was determined using MicroBCA (Bicinchoninic acid) protein assay kit from Thermo Fisher Scientific Inc. (Rockford, IL, USA) as per Patel *et al.*[11]. The identity of Octapeptide was verified by RP-HPLC.

Treatment protocol in marmosets—Common marmosets (*Callithrix jacchus*) aged between 3-4 years and maintained at 22 ± 2 °C, 50 - 60% RH and a photoperiod of 14 : 10 h L : D schedule, provided water ad libitum were maintained at the Institutional Animal House facility. The protocol was approved by the Institution's Animal Ethics Committee (IAEC-NIRRH). Three animals were used in each group. Each female (control and treated groups) was cohabitated with a male marmoset throughout the experiment. Marmosets do not menstruate. Therefore, the blood samples were collected 2-3 times a week to monitor cyclicity, conception and pregnancy by estimating progesterone levels. As reported earlier[4], the progesterone levels are <10 ng/mL in follicular phase and are higher than 10 ng/mL in luteal phase. Normal cycles in marmosets are 28-30 days with the follicular phase ranging spanning first 9-10 days followed by luteal phase for the next 20 days while the gestation period averages 144 days. If the animal conceives, its progesterone levels continue to remain high after day 30 of the cycle.

Placebo nanoparticles (without OP) were administered subcutaneously in 0.2 mL of saline to control on day two post-partum (2 dpp). NP-OP equivalent to 2.5 mg of OP was injected on day 2 dpp in treatment group. Blood samples (0.4-0.6 mL) were collected every alternate day. The changes in the ovarian cyclicity were monitored by estimating circulating progesterone levels. Serum progesterone levels were assayed by ELISA as reported earlier[5], using a diagnostic test kit from Diagnostics Biochem, Canada Inc. (Dorchester, ON, Canada).

Results

Particle size is one the major driving factor in controlling the drug release in peptide loaded nanoparticles, the process for which demands stringent optimization to achieve desired particle size and release profile. Particle size of nanoparticle is governed by various factors including sonication energy, sonication time, surfactant concentration and aqueous to organic ratio etc. Based on earlier studies[11], Poly Lactide (PLA) was selected as biodegradable polymer for the preparation of nanoparticles. It is more hydrophobic compared to PLGA and so exhibit slower degradation *in vivo*, which is helpful in sustaining the drug release for prolong period. The average diameter by DLS was found to be 120 nm.

Ultra structure of NP-OP—The appearance of the NP-OP under transmission electron microscopy revealed that the particles were round and the size of these particles was 120.35 ± 19.57 nm (Fig.1). The nanoparticles ranged from 89 to 145 nm.

Drug loading capacity and encapsulation efficiency—Drug loading (DL) and encapsulation efficiency (EE) are crucial parameters for nano therapeutics. Drug loading indicates the amount of polymer required to accommodate specific amount of drug while EE reflect the efficiency of method to encapsulate the added drug. Nanoparticle formulation process parameters should be optimized so that higher drug loading with maximum encapsulation could be achieved. This will minimize the loss of drug and prevent burst release on administration. DL and EE of

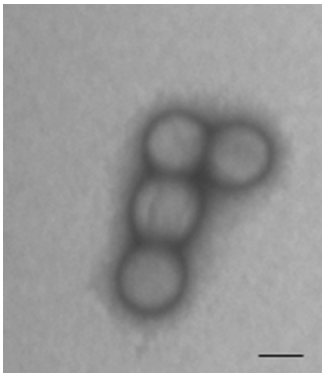

Fig. 1—Transmission electron microscopy (TEM) photograph of NP-OP showing rounded nanoparticles of sizes 120 nm; Bar = 100 nm.

nanoparticles were calculated as per the formulae mentioned in above section. The DL capacity was found to be 57 % while the EE of NP-OP was 70%.

In vitro release of OP from NP-OP for 21 days—A continuous release of the peptide was observed for all the 21 days. The cumulative data based on the protein content in the supernatants from day 1-21 is expressed in Fig. 2. OP loaded nanoparticles showed an increase in protein content from 0.1-0.7 mg/mL in released media over the period of 21 days. A linear regression was noted (R^2= 0.9916) in the release of the peptide from day 1 to day 21 without any initial burst release. Value of R^2 suggests the zero order release profile of OP from nanoparticulate system. Such type of release profile maintains steady state plasma concentration of drug and hence very ideal for treatment especially which require multiple dosing. Uniform release of drug over prolonged period of time was successfully achieved by formulating OP into polymeric nanoparticles.

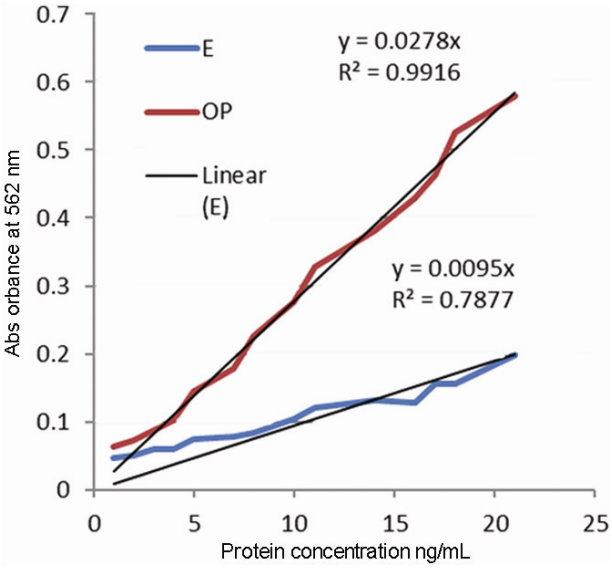

Fig. 2—Protein content in the supernatants collected daily to study *in vitro* release of OP from NP-OP (E = empty NP and OP = NP-OP).

The OP released from the NP formulation during the *in vitro* release studies was assessed for its purity by analytical RP-HPLC. Fig. 3 gives the retention time for the peaks of (a) synthetic OP (b) released OP from nanoformulation on day 8. Single peaks obtained for all the samples confirmed the purity of the released peptide. The OP peak was obtained at 2.95 min while the peak for released OP was obtained at 3.25 min on day 8 and (c) represents the peak obtained for mixture of synthetic and released OP. The single homogenous peak obtained at 3.125 min confirms that the OP is not degraded during the processes of formulation as well as release. Thus, the results confirmed that OP is released continuously without degradation.

In vivo effect of NP-OP in marmosets—*In vivo* effect of NP-OP on fertility of post-partum marmosets was studied. The ovarian cyclicity, conception and pregnancy in control and treated marmosets were monitored by estimating circulating levels of progesterone as earlier. Out of the three marmosets treated with NP-OP on day 2 dpp, one animal conceived (T_3) while two animals (T_1, T_2) demonstrated failure to conceive in the treated cycle (Fig. 4). All the three controls (C_1, C_2, C_3) treated with blank NP conceived in the same cycle as revealed by the plasma progesterone levels (Fig. 4) followed by full term pregnancy (~144 days) thereafter. The two treated animals (T_1 and T_2) conceived in the subsequent cycle followed by full

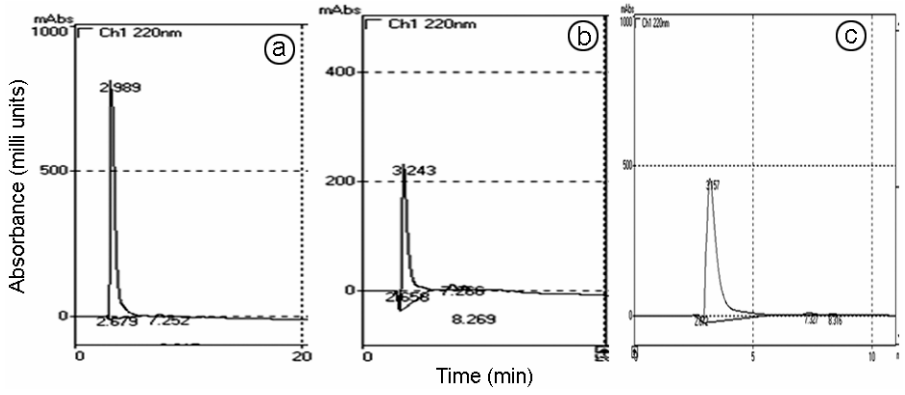

Fig. 3—HPLC profiles of (a) Synthetic OP (retention time: 2.989 min) (b) NP-OP released on day 8 (retention time: 3.243 min) (c) mixture of synthetic OP and NP-OP released on day 8 single peak obtained at 3.157 min at 220 nm.

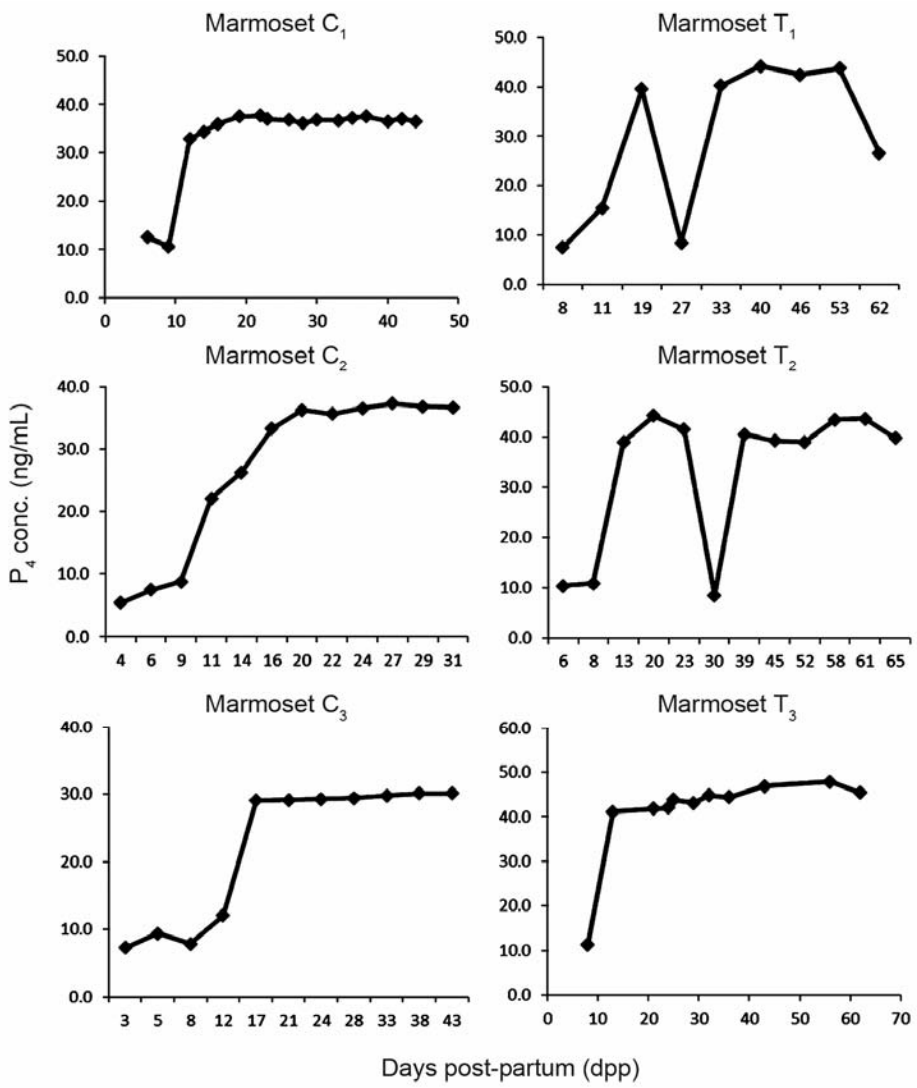

Fig. 4—Plasma progesterone profile of post-partum marmosets control group (left panel) and treated group (right panel). Control animals were injected with 2.5 mg blank nanoparticles while treated were injected with 2.5 mg NP-OP on day 2 post-partum.

term pregnancy with normal pups, suggesting recovery and no harmful effects of NP-OP treatment.

Discussion

Nanotechnology based drug delivery system combines polymer science, pharmaceutics, and cell biology. It aims for better controlled drug delivery, improved efficacy, prevention of harmful side effects and increased availability of the drug.

Encapsulation of protein and peptide based therapeutics into nanoparticles offer better control over their pharmacokinetic of the drug. A precise control over the release of these potent macromolecules can significantly potentiate their therapeutic efficacy. OP loaded nanoparticles were successfully developed using double emulsion evaporation method. HPLC studies suggest that organic solvent and process parameters did not affect its structure. Denaturation in the presence of organic solvent or by sonication is the major problem in encapsulation of higher molecular weight proteins. As OP is only 8 amino-acid containing peptide, such a possibility of denaturation is reduced. However, the retention of protein activity during manufacturing processes and assessment of released protein from nanoparticle is a challenging task. This nanoparticulate system was formulated using PLA as the polymer and PVA as stabilizer. The formulations of these nanoparticles involve exhaustive screening based on the physical, chemical and biological

properties like particle size, encapsulation efficiency, drug loading, *in vitro* release and biological activity.

The particle size depends upon the type and concentration of surfactant used in the preparation process. The average size of NP-OP as revealed by TEM was 120 nm (range 88-149) in the present study. Interestingly, the earlier reports using polymers for the formulation of nanoparticles have also reported range of 100-200 nm[12-18]. Encapsulation efficiency makes up a crucial parameter, especially for a hydrophilic drug, which mostly undergoes poor encapsulation due to diffusion of drug into aqueous phase. In the present study, 70% EE was observed for NP-OP. This was comparable to the earlier reports showing 48-85% EE[12,18-20]. Further, sustained release of OP was achieved without any burst release effect for 21 days from PLA polymeric nanoparticles in this study. Initial burst of the drug/peptide within 24 h from nano-carriers has been reported[12,15,21]. Burst release of drug molecules is one of the major problems associated with polymeric nanoparticles. Hydrophilic drugs are more prone to burst release due to poor encapsulation in the core of nanoparticles. Protein-polymer association, protein:polymer ratio, particle size and sonication time and intensity have been shown to be important criteria in release of the drug[22]. Results of *in vitro* release depicted the zero order release profile with negligible burst release, indicating the even distribution of OP in polymeric matrix. Similar observations for the negligible burst release of encapsulated drug from PLA nanoparticles have been reported earlier[14]. It is possible that the high concentration of PLA facilitates for proper accommodation of OP into polymeric matrix instead of surface localization. The purity of the released OP was assessed by RP-HPLC which exhibited single homogenous peak at comparatively same retention times as for the synthetic OP. It suggests that the structural integrity is unaffected by nano processing and within the release medium.

Granulosa cells are devoid of vasculature and these along with theca cells in ovarian follicles are separated by basement membrane which allows only small particles to pass through it. OP being a small peptide can be permeated through basement membrane and can induce apoptosis of granulosa cells[5]. The NP-OP could also cause apoptosis in granulosa cells[11]. Recently, Chitosan nanoparticles incorporating considerable amount of peptide could also be effectively transported through blood-brain-barrier[23]. Thus, enhanced permeability and retention of nanoparticles has been reported[17] and is advantageous in reaching the target site. Enhanced biological activity of the drug with nano-carrier *in vivo* has been reported[20, 24, 25].

Elazar *et al.*[26] used polymeric nanoparticles containing osteopontin and bone sialoprotein antisenses (AS) in rat model for bone metastasis. They indicated that AS delivery by NP showed better therapeutic efficacy than osmotic mini-pumps. Golub *et al.*[27] reported 2-4 days *in vitro* release of NP-VEGF encapsulated by PLGA. They further conducted *in vivo* study and observed more potent increase in total vessel volume by NP-VEGF compared to the naked VEGF. *In vitro* and *in vivo* evaluation of salmon calcitonin (sCT) loaded polymeric nanoparticles has been reported by Glowka *et al.*[20]. The release profile can be fast, within few hours, or as slow as for 4 weeks depending upon the quality of PLGA used for formulating nanoparticles. *In vivo* studies conducted by them in rats showed elevated levels of sCT for 3 days and increased bioavailability compared to sCT solution. In the present study, *in vitro* release of OP has been shown from NP-OP for 21 days. *In vivo* effect of NP-OP was similar to that of OP reported earlier in marmosets[28]. *In vivo* efficiency of NP-OP can possibly increase if a second dose is administered on day 8 of the cycle, in the late follicular phase. One of the treated animals conceived in the treatment cycle. It is possible that the dose may not have been sufficient to cause anti-fertility effect. Thus, a second injection of NP-OP on day 8 may reveal 100% anti-fertility effect.

Kumar *et al.*[24] developed ethionamide loaded PLGA NPs to achieve prolonged drug release for the treatment of multidrug resistant tuberculosis. The *in vivo* studies revealed no toxic effect of the treatment in mice. In the present study out of the 3-treated marmosets, the animal which conceived delivered normally and those 2 animals which did not conceive, mated in the next recovery cycle suggesting no toxic effect of NP-OP.

Mahapatro and Singh[29] have discussed various surface modifications of nanoparticles to enhance in vivo efficacy along with their specific applications. Drug loaded PEGylated PLGA has been shown to be a promising formulation for cancer therapy by *in vitro* and *in vivo* release of the drug[30-33].

Zhou *et al.*[34] reported synthesis of degradable RNA-loaded NPs. Thus along with NPs of synthetic

peptides (in the present study), synthetic RNAs are also being studied for their *in vivo* effects. OP alters the normal ovarian function, inducing luteal insufficiency in marmosets and amenorrhea in bonnet monkeys thereby altering the fertility of the animals however the post treatment cycles were normal. The major drawback faced was the requirement of the multiple injections in follicular phase. Biological activity of nanoparticles of the peptide has been reported earlier[11]. The present study also clearly reveals the potential of nanotechnology in eliminating the multiple dose regimens. A single dose administration of NP-OP suffices to produce desired pharmacological action instead of everyday injections of naked OP in monkeys during follicular phase[4,5]. Various researchers over the globe have been working on the gonadotropin analogues, antagonists and agonists for its use as a contraceptive. In conclusion, it may be possible to develop once a month biodegradable nanoparticles of OP as an anti-fertility agent.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

1 Navalakhe RM, Jagtap DD, Nayak SU, Nandedkar TD & Mahale SD, Effect of FSH Receptor Binding Inhibitor-8 (FRBI-8) on FSH Mediated Granulosa Cell Signaling and Proliferation, *Chem Biol Drug Des,* (2013) 177-188.

2 Chitnis SS, Selvaakumar C, Jagtap DD, Barnwal RP, Chary KV, Mahale SD & Nandedkar TD, Interaction of follicle-stimulating hormone (FSH) receptor binding inhibitor-8: a novel FSH-binding inhibitor, with FSH and its receptor, *ChemBiol Drug Des,* 73 (2009) 637.

3 Chitnis SS, Navlakhe RM, Shinde GC, Barve SJ, D'Souza S, Mahale SD & Nandedkar TD, Granulosa cell apoptosis induced by a novel FSH binding inhibitory peptide from human ovarian follicular fluid, *J HistochemCytochem*, 56 (2008) 961.

4 Wadia P, Kholkute S & Nandedkar T, Antifertility effect of an octapeptide, a fragment of FSH binding inhibitor, in the common marmoset (*Callithrix jacchus*), *Contraception,* 67 (2003) 151.

5 Kulkarni-Chitnis S & Nandedkar T, Effect of octapeptide: FSH binding inhibitor on cyclicity of bonnet monkeys (*Macacaradiata*), *Contraception,* 76 (2007) 467.

6 Navalakhe RM & Nandedkar TD, Application of nanotechnology in biomedicine, *Indian J ExpBiol,* 45 (2007) 160.

7 Nandedkar TD, Nanovaccines: recent developments in vaccination, *J Biosci,* 34 (2009) 995.

8 Parveen S, Misra R & Sahoo SK, Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging, *Nanomedicine,* 8 (2012) 147.

9 Slomkowski S & Gosecki M, Progress in nanoparticulate systems for peptide, proteins and nucleic acid drug delivery, *Curr Pharm Biotechnol,* 12 (2011) 1823.

10 Bilati U, Allemann E & Doelker E, Poly(D,L-lactide-co-glycolide) protein-loaded nanoparticles prepared by the double emulsion method--processing and formulation issues for enhanced entrapment efficiency, *J Microencapsul,* 22 (2005) 205.

11 Patel AR, Kulkarni S, Nandekar TD & Vavia PR, Evaluation of alkyl polyglucoside as an alternative surfactant in the preparation of peptide-loaded nanoparticles, *JMicroencapsul*, 25 (2008) 531.

12 Li Y, Pei Y, Zhang X, Gu Z, Zhou Z, Yuan W, Zhou J, Zhu J & Gao X, PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats*, J Control Release,* 71 (2001) 203.

13 Wang Z, Chui WK & Ho PC, Design of a multifunctional PLGA nanoparticulate drug delivery system: evaluation of its physicochemical properties and anticancer activity to malignant cancer cells, *Pharm Res,* 26 (2009) 1162.

14 Azzi J, Tang L, Moore R, Tong R, El Haddad N, Akiyoshi T, Mfarrej B, Yang S, Jurewicz M, Ichimura T, Lindeman N, Cheng J & Abdi R, Polylactide-cyclosporin A nanoparticles for targeted immunosuppression, *FASEB J,* 24 (2010) 3927.

15 Li Z, Chen Z & Chang R, PLA-O-CMC nanoparticles: HGF loading and delivery behaviors *in vitro*, *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi,* 28 (2011) 314.

16 Wang B, Jiang W, Yan H, Zhang X, Yang L, Deng L, Singh GK & Pan J, Novel PEG-graft-PLA nanoparticles with the potential for encapsulation and controlled release of hydrophobic and hydrophilic medications in aqueous medium, *Int J Nanomedicine,* 6 (2011) 1443.

17 Dubey N, Varshney R, Shukla J, Ganeshpurkar A, Hazari PP, Bandopadhaya GP, Mishra AK & Trivedi P, Synthesis and evaluation of biodegradable PCL/PEG nanoparticles for neuroendocrine tumor targeted delivery of somatostatin analog, *Drug Deliv,* 19 (2012) 132.

18 Guerreiro LH, Da Silva D, Ricci-Junior E, Girard-Dias W, Mascarenhas CM, Sola-Penna M, Miranda K & Lima LM, Polymeric particles for the controlled release of human amylin*, Colloids Surf B Biointerfaces,* 94 (2012) 101.

19 Nanjwade BK, Singh J, Parikh KA & Manvi FV, Preparation and evaluation of carboplatin biodegradable polymeric nanoparticles, *Int J Pharm,* 385 (2010) 176.

20 Glowka E, Sapin-Minet A, Leroy P & Lulek J & Maincent P, Preparation and *in vitro-in vivo* evaluation of salmon calcitonin-loaded polymeric nanoparticles, *J Microencapsul,* 27 (2010) 25.

21 Shi K, Cui F, Yamamoto H & Kawashima Y, Investigation of drug loading and *in vitro* release mechanisms of insulin-lauryl sulfate complex loaded PLGA nanoparticles, *Pharmazie,* 63 (2008) 866.

22 Mukherjee B, Santra K, Pattnaik G & Ghosh S, Preparation, characterization and *in-vitro* evaluation of sustained release protein-loaded nanoparticles based on biodegradable polymers, *Int J Nanomedicine,* 3 (2008) 487.

23 Yemisci M, Gursoy-Ozdemir Y, Caban S, Bodur E, Capan Y & Dalkara T, Transport of a caspase inhibitor across the blood-brain barrier by chitosan nanoparticles, *Methods Enzymol,* 508 (2012) 253.

24 Kumar PS, Ramakrishna S, Saini TR & Diwan PV, Influence of microencapsulation method and peptide loading on formulation of poly(lactide-co-glycolide) insulin nanoparticles, *Pharmazie,* 61 (2006) 613.

25 Kakizawa Y, Nishio R, Hirano T, Koshi Y, Nukiwa M, Koiwa M, Michizoe J & Ida N, Controlled release of protein drugs from newly developed amphiphilic polymer-based microparticles composed of nanoparticles, *J Control Release,* 142 (2010) 8.

26 Elazar V, Adwan H, Bäuerle T, Rohekar K, Golomb G & Berger MR, Sustained delivery and efficacy of polymeric nanoparticles containing osteopontin and bone sialoprotein antisenses in rats with breast cancer bone metastasis, *Int J Cancer,* 126 (2010) 1749.

27 Golub JS, Kim YT, Duvall CL, Bellamkonda RV, Gupta D, Lin AS, Weiss D, Robert Taylor W & Guldberg RE, Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth, *Am J Physiol Heart Circ Physiol,* 298 (2010) H1959.

28 Wadia P, *Studies on human ovarian follicular fluid peptide*: *Characterization and biological action*, Ph.D Thesis, University of Mumbai, India, 2002

29 Mahapatro A & Singh DK, Biodegradable nanoparticles are excellent vehicle for site directed *in-vivo* delivery of drugs and vaccines, *J Nanobiotechnology*, 9 (2011) 55.

30 Hu S & Zhang Y, Endostar-loaded PEG-PLGA nanoparticles: *in vitro* and *in vivo* evaluation, *Int J Nanomedicine,* 5 (2010) 1039.

31 Dhar S, Kolishetti N, Lippard SJ & Farokhzad OC, Targeted delivery of a cisplatin prodrug for safer and more effective prostate cancer therapy *in vivo*, *Proc Natl Acad Sci U S A*, 108 (2011) 1850.

32 Rojnik M, Kocbek P, Moret F, Compagnin C, Celotti L, Bovis MJ, Woodhams JH, Macrobert AJ, Scheglmann D, Helfrich W, Verkaik MJ, Papini E, Reddi E & Kos J, *In vitro* and *in vivo* characterization of temoporfin-loaded PEGylated PLGA nanoparticles for use in photodynamic therapy, *Nanomedicine (Lond),* 7 (2012) 663.

33 Zhao P, Wang H, Yu M, Liao Z, Wang X, Zhang F, Ji W, Wu B, Han J, Zhang H, Wang H, Chang J & Niu R, Paclitaxel loaded folic acid targeted nanoparticles of mixed lipid-shell and polymer-core*: in vitro* and *in vivo* evaluation, *Eur J Pharm Biopharm,* 81 (2012) 248.

34 Zhou J, Patel TR, Fu M, Bertram JP & Saltzman WM, Octa-functional PLGA nanoparticles for targeted and efficient siRNA delivery to tumors, *Biomaterials*, 33 (2012) 583.