Improving Human and Animal Health with Protein Engineering

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Novel High Affinity and Long-Acting Recombinant Bovine FSH Analogs for Veterinary Superovulation

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Introduction

Bovine ovarian superovulation is the current state-of-the-art method employed worldwide to maintain and improve milk and beef production through genetic selection of the best quality donor cows and best quality transferable embryos. These embryos may either be transferred fresh or frozen both to local recipient cows as well as conveniently shipped to any international locale. The current international market for superovulation is about $20 million per year but with an increase in both population and worldwide food shortages, the growth of new markets is expected (2, 3).

There are many limitations of current bovine ovarian superovulation and wide variations of the number of transferable embryos recovered, as well as great inconvenience and labor cost associated with total eight twice daily injections of porcine pituitary FSH such as Folltropin-V® (Bioniche) and other similar products. Use of bovine pituitary-derived FSH has been banned by the FDA and other national regulatory agencies because of a large number of reported cases of infection with the prion causing fatal bovine spongiform encephalopathy which also causes a related fatal disease in humans after ingestion.

There are no approved recombinant veterinary FSH products, and currently used crude porcine pituitary extract preparations are not usually highly purified or prepared in compliance with human recombinant GMP standards and viral safety studies. Certain lots could already be or become infected with porcine prions, virulent viruses or other animal-derived pathogens. Thus, there are serious safety and regulatory concerns by the FDA and other national and international agencies for all animal non-recombinant veterinary FSH preparations.

Objective

To design, produce and characterize totally novel more potent and safe recombinant bovine FSH (bFSH) analogs with prolonged half-life and no attenuation of response in three successive monthly injections of the same cows and thus no immunogenicity.

Design & Methods

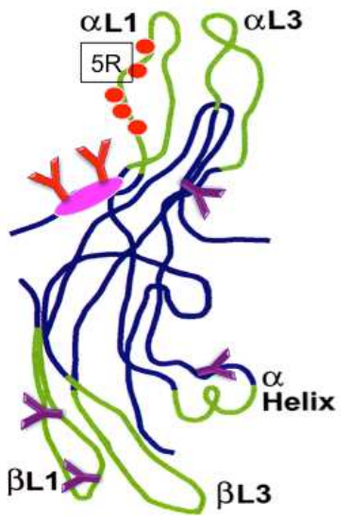

Fig. 1. Schematic illustration of modifications in bovine FSH analog TR55601. Substitutions to arginine (R) in 5 positions (5R) and 6 amino acid insert (pink oval) with two neo-glycosylation sites designed to mask any potential immunogenicity are marked in the αL1 peripheral loop and N-terminal fragment of the α-subunit. Two additional N-linked oligosaccharide chains are marked by "Y" in red. Previous studies with human glycoprotein hormone superagonists included our pioneering work - see Ref. 1.

Results

Table 1. Development of novel highly efficient production, purification and characterization methods for FSH analog TR55601 from transiently or stably transfected CHO cells. Comparison of 4 different production lots of the new TR55601 analog. In contrast to previous experiments with a human TR4401, we have found that TR55601 analog is more difficult to sialylate completely. Certain conditions and/or certain cells (Lot 2 and 3) resulted in a product with both poor terminal sialylation as confirmed by isoelectric focusing (IEF) and pharmacokinetic (PK) assay (Figs. 2 and 3)

Lot #	cDNA/ Vectors	Production (no O_2 control)	Feeding	Purity SDS-PAGE	Carbohydrates/ sialic acid/IEF	In Vitro Activity	PK	In Vivo Steelman-Pohley	Super-ovulation in cows
1	Trophogen/ Genscript	Transient, **adherent, roller bottles, pH control**	Optimal @ Trophogen	low	optimal	high	**optimal**	ND	**optimal**
2	Trophogen/ Catalent	Stables, suspension, no pH control	Suboptimal @Catalent	high	suboptimal	high	poor	poor	poor
3	Trophogen/ Catalent	Stables, suspension, pH control	Optimal @ Trophogen	very high	suboptimal	high	poor	ND	poor
4	Trophogen/ Genscript	Stables, **adherent roller bottles, pH control**	Optimal @ Trophogen	low	optimal	high	**optimal**	optimal	ND

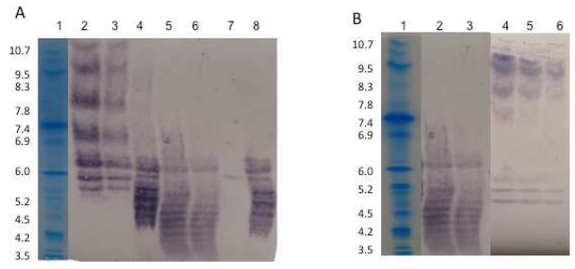

Fig. 2. A. Charge heterogeneity analysis using IEF followed by Western blot. Suboptimal sialylation of Lot 3 (Lanes 2 and 3) is in sharp contrast with optimal highly acidic isoforms detected in Lot 4 (Lanes 5 and 6). Lane 1, IEF 3-10 marker; Lane 2, TR55601/Lot 3(8μg); Lane 3, TR55601/Lot 3(4μg); Lane 4 and 8, TR4401 (1μg); Lane 5, TR55601/Lot 4 (8μg); Lane 6, TR55601/Lot 4 (4μg); Lane 7, IEF 3-10 marker.

B. Analysis of charged isoforms using neuraminidase (Vibrio cholerae), IEF and Western blot. Untreated TR55601/Lot 4 sample (Lanes 2 and 3) and TR55601/Lot 4 sample treated with neuraminidase before applying to 3-10 IEF gel (Lanes 4-6) . Lane 1, IEF 3-10 marker; Lane 2, untreated TR55601/Lot 4 (8μg); Lane 3, untreated TR55601/Lot 4 (4μg); Lane 4, treated TR55601/Lot 4(4μg); Lane 5, treated TR55601/Lot 4 (2μg); Lane 6, treated TR55601/Lot 4 (1μg). The IEF profile for the neuraminidase digested isoforms has shifted to the pI range from 7.8 to 10.0. The average shift of pI is about 5 pH units and multiple bands (close to 10 bands) transformed into one major band (pI ~9.5) and three minor bands (pI 7.8-10.0), indicating that the majority of the observed charge heterogeneity (Fig. 2A – Lot 4) is dependent on terminal sialic acid residues with a minor component of other modifications such as deamidation and/or proteolytic degradation. The residual basic bands (pI 4.8-5.5) shown in B are nonspecific, derived from neuraminidase preparation.

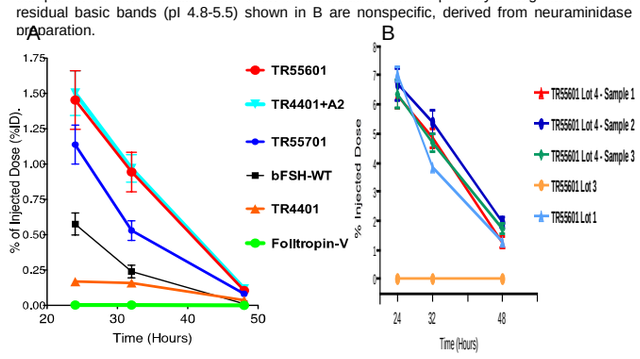

Fig. 3. A. Pharmacokinetic (PK) screening assay of selected bFSH analogs after single subcutaneous injection in mice. Long-acting TR55601 (5R+Insert 1) was selected as the lead analog based on several criteria including PK properties. In each experiment 5 mice were used for each preparation. Blood samples were taken at 24, 32 and 48 h after injection, plasma was isolated and analyzed using bFSH ELISA. Endogenous mouse FSH levels were deducted and the data were expressed as % of injected dose (%ID). **B. PK screening assay of various TR55601 lots after single subcutaneous injection in mice.** In each experiment 5 mice were used for each preparation. Previously discussed major differences between Lot 3 and Lot 4 in IEF (Fig.2) are confirmed by these PK profiles.

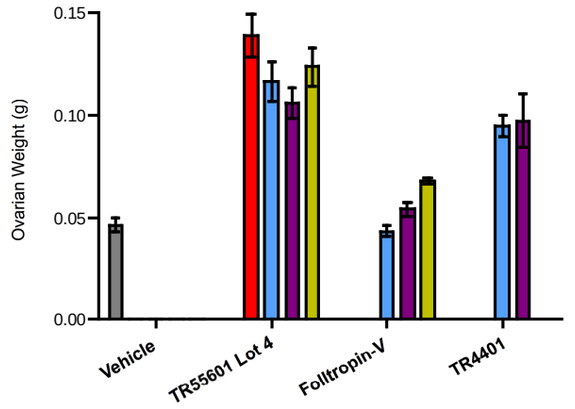

Fig. 4. Classic Steelman-Pohley bioassay with hCG augmentation of ovarian weight in immature (22 days old) Sprague-Dawley female rats. Ovarian weights were measured 72 hours after dosing. Data are presented as average total ovarian weight of two ovaries ± SEM (n=5 per dose, per group). Rats were stimulated with one single injection of test article or vehicle, supplemented with 40 IU of hCG. The following dosage groups were used: Group 1 was receiving hCG only (no FSH), Groups 2-5 were receiving TR55601 Lot 4 (0.33 μg, 1.0 μg, 3.33 μg, and 10 μg, respectively from left to right), Groups 6-8 were receiving Folltropin-V® (3,333 μg, 10,000 μg, and 30,000 μg respectively from left to right), and Group 9-10 was receiving TR4401 (1.0 μg and 3.33 μg).

Superovulation in Beef Cows

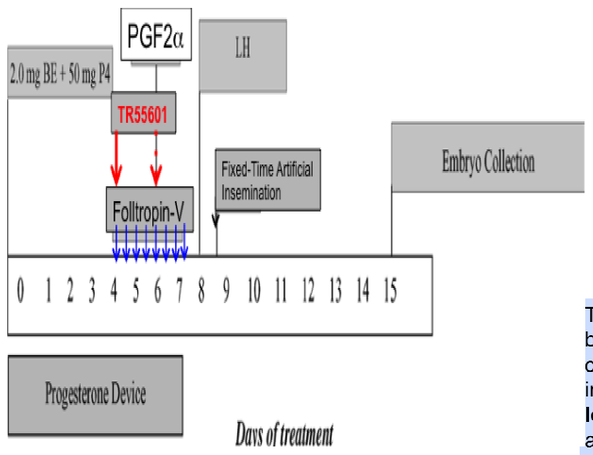

Fig. 5. Follicular wave synchronizing protocol for superovulation, induction of ovulation and fixed-time artificial insemination. Replacement of 8 injections of Folltropin-V® (Bioniche) with a single or double injection of TR55601. Treatment consisted of insertion of progesterone (P4) releasing intravaginal device and administration of benzoate estradiol (BE) on Day 0. Superovulatory treatments were initiated on Day 4 with TR55601 given as a single or double injection. Second split-dose injection was coinciding with $PGF_{2\alpha}$ treatments on Day 6. Progesterone device was removed with the last FSH injection on Day 7. On day 8 donors were receiving porcine LH and were inseminated without estrus detection 12 and 24h later, or once on Day 8 (16h after pLH). Ova/embryos were collected non-surgically on Day 15 (2, 3). Folltropin-V® was used as a control; total 300 mg* was given in 8 intramuscular (IM) injections twice daily over 4 days (mg* - based on highly impure NIH-FSH-P1 Reference Standard).

Results in Beef Cows

Table 2. Superovulation with single or split-single doses of TR55601 (rFSH). Mean (±SEM) number of corpora lutea (CL), follicles >10mm in diameter, number of cows with ≤ 2 CL at the time of ova/embryo collection, number of ova/embryos, fertilized ova and grades 1, 2, and 3 embryos (transferable embryos) in beef cows treated with a single (60μg) or split-single (40-20μg) I.M. injections of rFSH (TR55601) or 300 mg Folltropin-V® (Control) given in twice daily IM injections over 4 days.

Treatment	N	CL	Follicle >10 mm	Cows with ≤2 CL on Day 15	Total ova/embryos	Fertilized ova	Grade 1 embryos	Grades 1&2 embryos	Grades 1, 2,&3 embryos	No. "0" emb
Control	10	14.1 ± 2.1	3.2 ± 1.1a	0	12.7 ± 2.4	10.5 ± 1.6	7.5 ± 1.4	8.1 ± 1.4	9.0 ± 1.5	0
rFSH 60 μg	10	12.7 ± 2.8	4.6 ± 1.1ab	2	11.6 ± 3.0	9.1 ± 2.2	5.4 ± 1.2	6.6 ± 1.5	6.6 ± 1.5	2
rFSH 40-20 μg	10	13.9 ± 1.5	8.5 ± 1.9b	0	11.2 ± 1.9	9.9 ± 1.8	6.2 ± 1.4	7.4 ± 1.7	7.9 ± 1.7	0
P-value		0.6407	0.0262	0.1173	0.7317	0.6008	0.4339	0.6035	0.4462	0.1173

Table 3. Superovulation with single dose of TR55601 (rFSH). Mean (± SEM) number of ova/embryos, fertilized ova and grades 1, 2, and 3 embryos (transferable embryos) in beef cows treated with a single (60μg) I.M. injection of rFSH (TR55601) or 300mg Folltropin-V® (Control) given in twice daily IM injections over 4 days.

Treatment	N	Total ova/ embryos	Fertilized ova	Grade 1 embryos	Grades 1 & 2 embryos	Grades 1, 2 &3 embryos (transferable)	Cows with "0" transf. emb,
Control	10	11.9 ± 2.5	10.5 ± 2.2	3.2 ± 0.8	4.7 ± 1.1	4.9 ± 1.2	2
rFSH 60 μg	14	13.4 ± 3.3	11.6 ± 3.0	3.5 ± 1.0	5.1 ± 1.5	6.1 ± 1.8	4
P- value		0.8263	0.7958	0.8903	0.8608	0.9992	0.6326

Table 4. Three superovulations with TR55601 (rFSH) at 30 day intervals. Mean (±SEM) number of CL, follicles >10mm in diameter, number of cows with ≤ 2 CL at the time of ova/embryo collection, number of ova/embryos, fertilized ova and grades 1, 2, and 3 embryos (transferable embryos) in beef cows treated with a single (60μg) or split-single (40-20μg) I.M. injections of rFSH (TR55601) or 300 mg Folltropin-V® (Control) given twice daily IM injections over 4 days. Cows were treated three consecutive times at ~30 day intervals (3 experiments combined).

Main Effects Experiments	N	CL	Follicles > 10mm	Cows with ≤ 2 CL on Day 15	Total ova/embryos	Fertilized ova	Grade 1 embryos	Grades 1 & 2 embryos	Grades 1, 2 & 3 embryos (transferable)	Cows with "0" transf. emb
Experiment 1	24	14.1 ± 1.4	5.0 ± 1.0	1	13.0 ± 1.6	11.0 ± 1.1	7.3 ± 0.8^a	8.4 ± 0.9^a	8.9 ± 1.0^a	1
Experiment 2	24	13.7 ± 1.5	4.7 ± 0.9	1	10.7 ± 1.8	8.9 ± 1.4	4.5 ± 1.0^b	5.6 ± 1.1^{ab}	6.6 ± 1.3^{ab}	2
Experiment 3	24	15.5 ± 1.9	5.3 ± 1.0	3	12.8 ± 2.2	11.1 ± 1.9	3.4 ± 0.7^b	5.0 ± 1.0^b	5.6 ± 1.2^b	6
P-value		0.9426	0.9195	0.4233	0.5881	0.5169	0.0031	0.0219	0.0480	0.0695
Treatments										
Control	30	13.9 ± 1.2	3.7 ± 0.6^b	0	11.3 ± 1.5	9.7 ± 1.2	4.9 ± 0.8	6.0 ± 0.8	6.7 ± 1.0	2
rFSH 60 μg	42	14.8 ± 1.3	5.9 ± 0.6^a	5	12.8 ± 1.5	10.8 ± 1.3	5.2 ± 0.7	6.6 ± 0.8	7.3 ± 0.9	7
P-value		0.9684	0.0224	0.0501	0.7893	0.9184	0.9361	0.9571	0.9840	0.2059
Experiment* treatment interaction		0.8644	0.7250		0.7720	0.8597	0.9385	0.9790	0.9769	

Summary & Conclusion

There was significant 3-10 fold increase in the in vitro potency of bFSH analog with five different substitutions (5R) in comparison to controls (*data not shown*). This in combination with the amino acid insert containing two carbohydrate chains resulted in **the first ever long-acting bFSH superagonist** with no attenuation of its intrinsic activity. Two days PK screening assay in mice indicated largely prolonged plasma half-life dependent on the number, the site of neo-glycosylation insert and the degree of terminal sialylation.

The long-acting lead bFSH superagonist TR55601 showed ~170x higher activity than control (Folltropin-V®, quantitated by bFSH ELISA) in rat bioassay as well as highly promising results in the initial studies in beef cow superovulation indicating that such analog could in a single or dual injection, match or exceed production of mature follicles and the number of transferable embryos compared to widely used Folltropin-V®.

This long-acting bovine analog TR55601 showed no significant attenuation of response in 3 successive monthly injections of the same cows and thus no immunogenicity.

This completely novel proprietary long-acting recombinant bFSH superagonist with two additional N-linked complex carbohydrate chains should increase the efficacy, convenience and safety of cow superovulation at largely reduced cost.

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Further Information

For more information about these studies, please contact Dr. M. W. Szkudlinski at mszkudlinski@trophogen.com

Trophogen is currently recruiting additional Veterinary Centers to participate in Clinical Trials, as well as new partners and veterinary products distributors - please contact Dr. V. Wolf at vwolf@trophogen.com

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