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Preparation of canine relaxin by Fmoc-solid phase synthesis and regioselective disulfide bond formation within the A- and B-chains

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Abstract

Background: The chemical synthesis of multi-disulfide bonded heterodimeric peptides such as insulin has long been of significant scientific and commercial interest as well as a major challenge. The development of improved protocols which includes regioselective disulfide bond formation has greatly advanced the capacity to prepare and study insulin-like peptides including canine relaxin, an important regulator of parturition and indicator of canine maternal health.

Methods: Separate, efficient solid phase synthesis of the two constituent chains (24 residue A and 35 residue B) was followed by stepwise formation of each of the three disulfide bonds, one intra within the A-chain and two interchain, by oxidation, thiolysis and iodolysis respectively.

Results: Synthetic canine relaxin having a total of 59 residues was prepared in good overall yield and shown by several criteria to be highly purified. The peptide was shown to be less potent than human relaxin (H2 relaxin) in binding to and activating the human relaxin receptor, RXFP1, in transfected cells. A circular dichrosim spectroscopic analysis showed that the canine relaxin also possessed significantly less secondary structure compared to H2 relaxin which may account for its reduced activity.

Conclusions: The synthetic protocols developed in our laboratory enabled the successful preparation of the complex, small insulin-like protein, canine relaxin. This will, in turn, allow a detailed study of both the tertiary conformation of this peptide and its role in canine reproduction.

Keywords: Canine relaxin, H2 relaxin, circular dichroism spectroscopy, regioselective disulfide bond formation, RXFP1 receptor assay, solid phase peptide synthesis

Introduction

In the human, the insulin/relaxin superfamily comprises of ten members, one of which is relaxin-2 (also known as H2 relaxin). It consists of two peptide chains, A and B, and contains six conserved cysteine residues which form three disulfide bonds in a pattern that is unique to all the insulin/relaxin superfamily members, one within the A-chain and two others linking the A- and B-chains together [1,2]. With the exception of the invariant cysteine residues and a glycine within the B-chain, there are significant differences in primary structure between species and also in chain lengths. The A-chain typically has 24 residues but is as short as 20 residues (equine relaxin). The B-chain has greater variations of length and can be as short as 29 residues (also equine) and extending to 33 (guinea pig).

Relaxin is primarily produced by the corpus luteum of the ovary and/or placenta of pregnancy in most mammals where it exerts multiple essential actions on the female reproductive tract to support pregnancy, facilitate delivery and prepare the mammary glands for lactation [3]. More recently, our group and others have shown that locally produced relaxin has much wider actions, including roles in the cardiovascular [4,5] and central nervous systems [6,7] and an essential role in collagen turnover [8]. These actions of relaxin are mediated via a leucine-rich repeat-containing G-protein coupled receptor, known as LGR7 [9], which has more recently been re-named relaxin family peptide 1 (RXFP1) receptor [10]. H2 relaxin (also commercially named serelaxin) has recently passed a successful Phase III clinical trial for the treatment of acute heart failure due to its potent vasodilatory and cardioprotective properties [11].

Canine relaxin is of particular interest both structurally and biologically. Its isolation from pregnant dog ovaries and subsequent chemical characterization showed it to be one of the largest mammalian relaxins and has a 24 residue A-chain and 35 residue B-chain (**Figure 1**) [12]. It has been extensively studied for its roles in the dog, particularly during pregnancy due to it being a multiple birth mammal [13-15]. A homologous radioimmunoassay has been developed against synthetic canine relaxin and shown to be able to detect as little as 0.19 ng of relaxin [16]. It has formed the basis of a commercially available relaxin assay (ReproCHEK™) which provides a sensitive and accurate diagnosis of pregnancy in the domestic dog and which circumvents the need for specialized equipment and experienced personnel. The same assay has been shown to be effective in wild wolves, coyotes and, curiously, in domestic cats [17-19].

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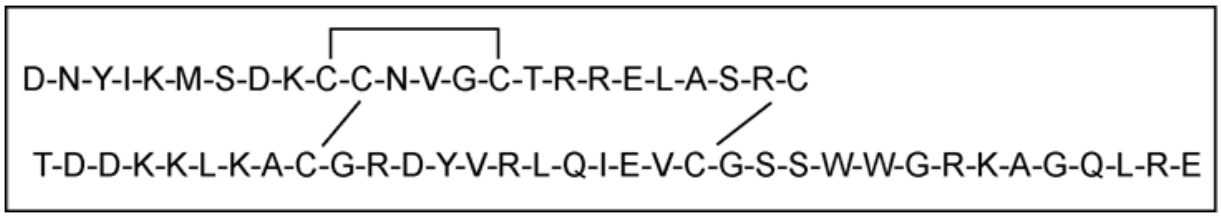

Figure 1. The primary structure of canine relaxin.

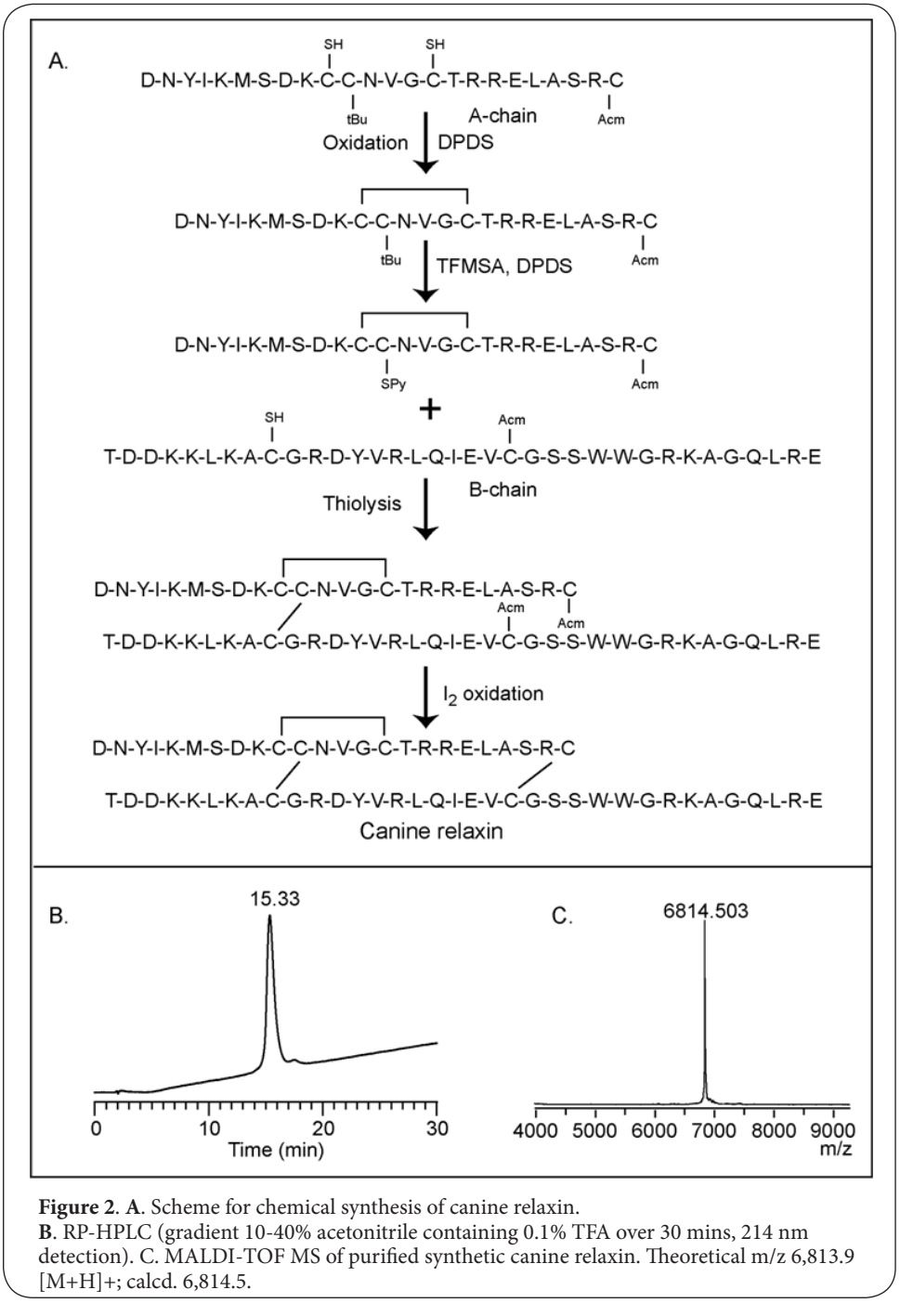

Figure 2. **A**. Scheme for chemical synthesis of canine relaxin.
B. RP-HPLC (gradient 10-40% acetonitrile containing 0.1% TFA over 30 mins, 214 nm detection). C. MALDI-TOF MS of purified synthetic canine relaxin. Theoretical m/z 6,813.9 [M+H]+; calcd. 6,814.5.

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The chemical synthesis of canine relaxin was previously reported nearly 20 years ago [16]. It used a combination of the older Boc- and now predominantly employed Fmoc-solid phase synthesis chemistry for the separate preparation of the two S-regioselectively protected chains followed by a four step process of sequential disulfide bond formation, chain linkage and selective *N*(indole)-deprotection [20]. Overall yield of purified canine relaxin was 5%. It was shown to be fully biologically active in inducing relaxation of the mouse interpubic ligaments *in vivo*. We sought to determine if the synthesis of canine relaxin could be improved using a simpler protocol that has been developed in our laboratory (**Figure 2A**) [21] and which wholly employs the more mild Fmoc-based synthesis chemistry without the need for additional post-combination deprotection. We also undertook to compare the secondary structure of the peptide with H2 relaxin as well as by the more sensitive *in vitro* relaxin receptor binding and activation assays.

Materials and methods

All amino acid derivatives were purchased from GL Biochem (Shanghai, China) and PEG-polystyrene solid supports from Applied Biosystems Inc (Melbourne, Australia). H2 relaxin was a gift from Corthera/Novartis Inc (San Francisco, USA).

Solid phase peptide synthesis

This was carried out as previously described [21,22]. For canine relaxin bearing C-terminal acid on its two chains, synthesis of the chains was carried out using appropriately Fmoc-amino acid-preloaded hydroxymethylphenoxyacetyl-derivatised PEG-polystyrene. HCTU-activated Fmoc-amino acids were used throughout. Amino acid side chain protection was afforded by the following: Arg, Pbf; Asn and Gln, Trt; Asp and Glu, O-Bu^t; His, Trt; Lys, Boc; Ser and Thr, Bu^t. For the A-chain peptide, S-protection was afforded by Trt (Cys^{10,15}), Acm (Cys^{24}) and Bu^t (Cys^{11}). For the B-chain, Trt (Cys^9) and Acm (Cys^{21}) were used. No repeat couplings were carried out. N^a-Fmoc deprotection was with 20% piperidine in DMF. Assembly of both the A- and B-chain peptides commenced on 0.2 mmol scales respectively using a 4-fold excess of activated amino acid and 30 min coupling times. After acylation and deprotection of the final residues, cleavage from the solid supports and side chain deprotection was achieved by a 2.5-h treatment of the two separate peptide-resins with trifluoroacetic acid (TFA) and, for the B-chain, in the presence of phenol, thioanisole, ethanedithiol and water (82.5/5/5/2.5/5, v/v) with a few drops triethylsilane (TES). For the A-chain, cleavage was effected with TFA in the presence of ethanedithiol, water and TES (95/2/2/1) and isolation of the crude peptide was as previously described [23]. The resulting crude peptides were subjected to preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 column (Hesperia, USA) using a 1%/min gradient of CH_3CN in 0.1% aqueous TFA.

A-chain intramolecular disulfide oxidation - Crude cleaved [Cys^{10,15}(S-thiol), Cys^{11}(But), Cys^{24}(Acm)] A-chain (658 mgs, 222 µmol) was dissolved in H_2O (800ml) and to this was added 1 mM 2-dipyridyl disulfide (DPDS) in MeOH (100 mL, 100 µmol) (**Figure 2A**). Oxidation was complete after 2 h as monitored by analytical RP-HPLC. The peptide isolated by preparative RP-HPLC and subsequent freeze drying to give 392.0 mgs (59.6%) of purified [Cys^{11}(Acm), Cys^{24}(But)]-A-chain.

[Cys^{11}(Pyr), Cys^{24}(Acm)]A-chain - Intramolecular disulfide bonded [Cys^{11}(But), Cys^{24}(Acm)]-A-chain (223.7mg, 76 µmol) was converted to the Cys^{11} S-pyridinyl form by treatment with DPDS (**Figure 2A**) in neat TFA (5.0 mL) containing thioanisole (0.5 ml) chilled to ≤0°C. 5.0 mL TFMSA/TFA (1:5 v/v) was added and stirred for 20-30 mins maintaining the temperature at or below 0°C. The peptide was then precipitated in ether and the pellet suspended in 6M GdHCl for purification. The target peptide was isolated by preparative RP-HPLC to give 164.2 mgs (72.5%).

Combination of [Cys^{11}(SPy), Cys^{24}(Acm)]A-chain with [Cys^9(S-thiol), Cys^{21}(Acm)]B-chain - A-chain peptide (164.0 mgs, 54.8 µmol) was dissolved in 8M GdHCl (8 mL) and added to purified B-chain (133.8 mg, 32.4 µmol) in the same buffer (8 mL) (**Figure 2A**). The mixture was stirred vigorously at room temperature or 37°C for each buffer respectively, and reaction monitored by analytical RP-HPLC. After 30 mins (or 24 h if using the GdHCl buffer), the reaction was terminated by addition of glacial acetic acid, and the target product isolated by preparative RP-HPLC to give 131.3 mg (58.3%).

The final disulfide bond formation - The [Cys^{24}(Acm)]A-chain/[Cys^{21}(Acm) B-chain] (131.0mg, 18.8 µmol) was dissolved in glacial acetic acid (136 mL) and 80mM HCl (13 mL) and to this was added dropwise 113 mL of 20 mM iodine/acetic acid (2.3 mol) (**Figure 2A**). After 1 h, the reaction was stopped by addition of 113 mL of 20 mM ascorbic acid. Preparative RP-HPLC, as described above, was then used to isolate and purify the product 13.8 mg (10.8%), yield 6.3% overall relative to starting purified B-chain.

Peptide characterization

The purity of the synthetic peptide was assessed by analytical RP-HPLC on a Vydac C4 column (pore size 300 Å, particle size 5 µm, 4.6 x 250 mm) using a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The product was confirmed by MALDI-TOF mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. The peptide was quantitated by amino acid analysis of a 24 h acid hydrolysate using a Shimadzu microbore RP-HPLC system. Circular dichroism (CD) spectroscopy was carried out on an Applied Photophysics Chirascan Plus instrument (Leatherhead, UK) using the following settings: wavelength range 195 to 250 nm, scanning speed 50 nm/min, bandwidth 0.1 nm, cell length 1 mm at room temperature. The peptide samples were prepared at 0.3 µg/µl in phosphate buffered saline (PBS: 20 mM potassium phosphate buffer with 137 mM NaCl pH 7.4). The raw data from the spectra in millidegree of ellipticity (θ)

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were converted to mean residual weight ellipticity (MRE) [24].

Ligand binding assay

Human embryonic kidney (HEK)-293T cells stably transfected with RXFP1 were grown in RPMI-1640 media (Sigma, Australia) supplemented with 10% fetal calf serum (FCS), 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and plated into 24 well poly-L-lysine coated plates for whole cell binding assays. Competition binding experiments were performed with europium-labelled H2 relaxin [25] in the absence or presence of increasing concentrations of unlabeled peptides. Non-specific binding was determined by addition of 500 nM H2 relaxin. All data are presented as the mean±S.E.M. of the percentage of the specific binding of triplicate wells, repeated in at least three separate experiments, and curves fitted using one-site binding curves in GraphPad Prism 5 (GraphPad Software). Statistical differences in pIC50 values were analysed using one-way ANOVA coupled to a Newman Keul's multiple comparison test for multiple group comparisons in GraphPad Prism 5.

Functional cAMP assay

The influence of the various ligands on cAMP signalling in cells expressing RXFP receptors was assessed using a cAMP reporter gene assay as previously described [26]. Briefly, HEK-293T cells in 96 well plates were co-transfected with either RXFP1 and a pCRE-β-galactosidase reporter plasmid. 24 hours later co-transfected cells were treated with increasing concentrations of canine relaxin in parallel to 10 nM of H2 relaxin for RXFP1 transfected cells respectively. After 6 hours, the cell media was aspirated and the cells frozen at -80°C overnight. The amount of cAMP-driven β-galactosidase expression in each well was determined as previously described [26]. Ligand induced stimulation of cAMP was expressed as a percentage of the maximum H2 relaxin response. Data points were measured in triplicate and each experiment was repeated at least three times. Statistical differences in pEC50 values were analysed using one-way ANOVA coupled to a Newman Keul's multiple comparison test for multiple group comparisons in GraphPad Prism 5.

Results and discussion

The chemical synthesis of a heterodimeric two-chain, three disulfide linked peptide remains a formidable undertaking and has been the subject of many attempts to both optimize and simplify, most recently by way of one-pot processes [27,28]. Our approach of using efficient Fmoc-solid phase synthesis of suitably S-protected A- and B-chains followed by stepwise formation of the three disulfides has proven to be highly effective and robust with many assemblies of insulin-like peptides successfully accomplished [21,22,29-33]. In order to acquire sufficient quantities of canine relaxin to undertake detailed structural and biological studies, we employed our approach (**Figure 2A**) and subsequently obtained highly purified peptide (**Figures 2B** and **2C**) in overall yield of more than 6% relative to the starting crude B-chain. An early assembly reported by others using both Boc- and Fmoc-chemistry, regioselective disulfide bond formation and one additional work-up step provided the peptide in an also good 5% yield [16]. In our case, despite the length and complexity of the two chains (in particular, the B-chain), use of low-loading hydrophilic solid supports led to their straight forward assemblies [34]. Our experience is that the greatest losses stemmed not from the syntheses of the respective chains or the individual disulfide bond forming steps but from the intermediate HPLC purifications, particularly of the B-chain alone which is poorly soluble.

The synthetic canine relaxin was evaluated for its propensity to bind to and activate human RXFP1 receptor that was over-expressed in HEK cells. As shown in **Figure 3**, the peptide both strongly binds to (**Figure 3A**) and activates (**Figure 3B**) the receptor although the affinity (pIC50=8.31±0.03; $p<0.01$ vs H2 relaxin, pIC50=9.08±0.07) and activity (pEC50=9.61±0.03; $p<0.05$ vs H2 relaxin, pEC50=10.30±0.04) of the peptide is significantly lower compared with H2 relaxin. Importantly, the activity decrease matched the decreased affinity and is likely the result of species differences in the ligand receptor pairing. Notably, mouse relaxin has a lower affinity for, and activity at, human RXFP1 than its native mouse RXFP1 receptor [22]. The peptide was then assessed for secondary structural conformation using CD spectroscopy (**Figure 4**). H2 relaxin exhibits a CD spectrum typical of predominantly α-helical peptides in aqueous solutions characterized by a strong negative band at 208 nm and a weaker negative band at 222 nm. The calculated helix content is around 39% (based on the 222 nm band [35]). In contrast, the canine analogue shows a significantly reduced α-helix structure. The dominant band is blueshifted to 204 nm and the curve intensity is dramatically reduced. The spectrum resembles those of type C CD curves, representing a series of type I (III) β-turns or 3-10 helices. The calculated α-helix content drops below 20%. This significantly reduced secondary structure may also be a contributory factor towards the reduced bioactivity results. It is noteworthy, however, that synthetic equine relaxin, which contains just 48 residues, possesses a similarly reduced overall α-helical content whilst being equipotent to H2 relaxin in binding to human RXFP1 [21]. It is important to note that the sequence difference in overlapping primary structures between the canine and H2 relaxin is about 55%, sufficient perhaps to also contribute to the observed difference in activity. Nevertheless, the successful acquisition of the complex canine peptide will now allow us to both obtain its tertiary structure via NMR spectroscopy and more thoroughly study its biochemistry and physiology in the canine.

Conclusions

Canine relaxin, one of the largest native relaxins with 59 residues, was successfully chemically prepared in good overall

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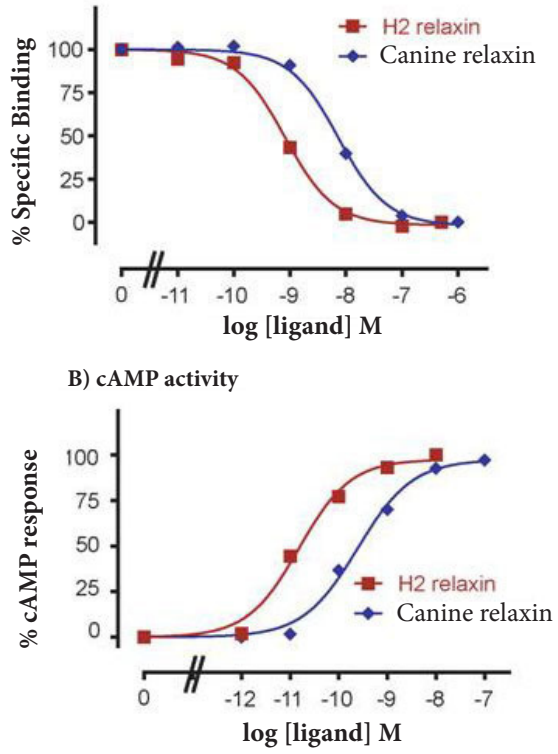

Figure 3. Activity of canine relaxin at RXFP1.
A. Competition binding of canine relaxin or H2 relaxin with europium-labeled H2 relaxin on HEK293T cells stably expressing RXFP1. Data are expressed as percentage of specific binding and are pooled data from at least three experiments performed in triplicate.
B. cAMP activity in HEK293T cells stably expressing RXFP1 using a pCRE-β-galactosidase reporter gene system. Data are expressed as percentage of maximum H2 relaxin stimulated response and are pooled data from at least three experiments performed in triplicate.

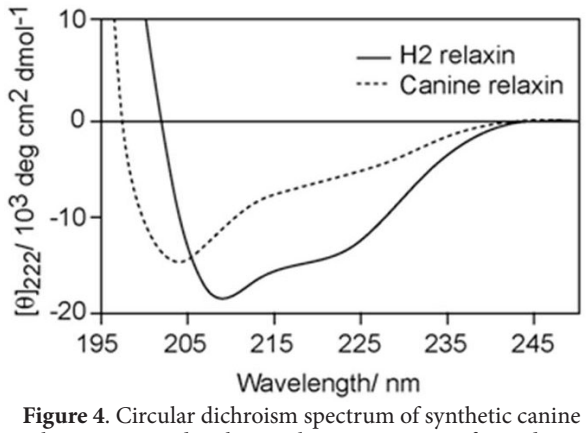

Figure 4. Circular dichroism spectrum of synthetic canine relaxin compared with H2 relaxin. CD was performed in 20 mM phosphate buffer at pH 7.4.

yield by a combination of efficient solid phase synthesis of the individual A- and B-chains followed by regioselective disulfide bond formation. The resulting complex peptide that contains three insulin-like disulfide bonds was shown by MALDI-TOF MS and RP-HPLC to be highly purified and to possess modest α-helical structure as assessed by CD spectroscopy. In *in vitro* activity assays using cells expressing the human relaxin RXFP1 receptor and CD study, synthetic canine relaxin was shown to be less potent and less structured compared with H2 relaxin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	JW	MAH	FL	LJC	JT	RADB
Research concept and design	√	√	--	--	--	--
Collection and/or assembly of data	√	√	--	--	--	--
Data analysis and interpretation	√	√	√	√	--	--
Writing the article	√	√	--	--	√	√
Critical revision of the article	--	--	--	--	--	--
Final approval of article	√	√	√	√	√	√
Statistical analysis	--	--	--	--	--	--
CD spectral studies	--	--	--	--	√	--

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