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Research Article

The Purified ~55 Kda Uterovaginal Lizard Protein Retards Sperm Motility In A Diverse Group Of Vertebrates

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Abstract

Sperm storage in female invertebrates and vertebrates is a fascinating aspect of reproductive success across animal phyla, yet the underlying mechanisms remain largely elusive. Using the Indian Garden lizard *(Calotes versicolor)* as a model organism, we previously identified and sequenced an abundant yet unique ~55 kDa protein from uterovaginal junction (UVJ) flushings without any homology to protein databases. Characterizing this protein exhibited retardation of lizard sperm motility in a concentration- and time-dependent manner [1]. The present study demonstrates that this protein exhibits similar inhibitory effects on sperm from mice, rats, and humans without compromising viability. These results suggest that the ~55 kDa protein has significant potential for applications in prolonging sperm survival even in heterologous sperm and has the potential to assist endangered species conservation.

Keywords: Female sperm storage; Spermatozoa; Reptiles; Oviduct; Reproduction; Sperm storage tubules.

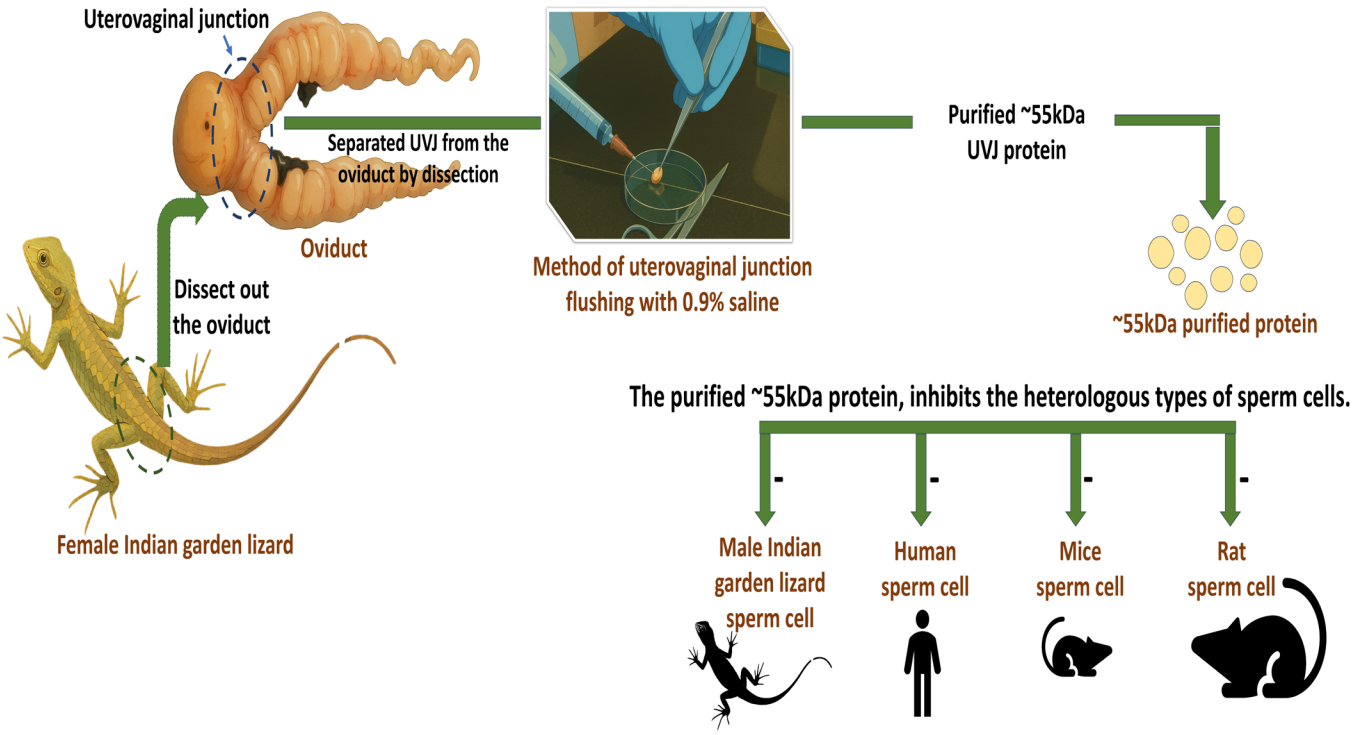
Highlights:

• The purified ~55 kDa protein from uterovaginal junction (UVJ) flushings significantly retarded lizard sperm motility in a concentration- and time-dependent manner.

• This motility-retarding effect is not limited to homologous (Indian garden lizard) sperm but also affects heterologous sperm (human, mouse, and rat) in a reversible manner, dependent on concentration and time.

• Heterologous species sperm motility inhibited more sensitively compared to lizard sperm, indicating that this ~55 kDa protein may function as a universal sperm motility retarding factor.

Graphical abstract



Introduction

In various animal species, females have evolved a remarkable adaptation: the ability to store sperm in a viable state for extended periods within their reproductive tracts and utilize them when favourable conditions arise, until their eggs are ready for fertilization [2-4]. This phenomenon has been observed across a wide range of taxa, from insects to mammals, including *Drosophila melanogaster* (fruit fly), *Pelodiscus sinensis* (Chinese soft-shelled turtle), *Calotes versicolor* (Indian garden lizard), *Coturnix japonica* (Japanese quail), and *Myotis velifer* (Vesper bat) [5]. Notable strategies employed by these species include synchronizing male and female reproductive cycles and laying cleidoic eggs [6-8]. Despite the prevalence of sperm storage among female animals, the underlying molecular mechanisms remain largely unknown.

The Indian garden lizard, *Calotes versicolor*, is an agamid lizard widely distributed throughout India [9]. Previous studies from our laboratory have investigated the histological and histochemical characteristics of the sperm storage tubules (SSTs) located at the uterovaginal junction (UVJ) of the oviduct [10-12]. Furthermore, an earlier investigation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude UVJ flushing revealed the presence of approximately 30-50 additional proteins, including an abundant protein with a molecular weight of ~55. Interestingly, this ~55 kDa protein was found to reversibly and concentration-dependently inhibit the motility of washed epididymal sperm, and its expression was observed throughout the year, regardless of the stage of the reproductive cycle [1].

The primary objective of the current study was to purify and further characterize the ~55 kDa UVJ protein. Additionally, we investigated the effects of this purified protein on heterologous cell types, including mouse, rat, and human sperm cells, in vitro.

Materials and Methods

Animals

Indian Garden Lizards *(C. versicolor)*

During the reproductive phase (May to July), we captured 4 healthy female and 2 male *C. versicolor* individuals per month from the wild in the surrounding areas of Mysuru, India. The lizards were selected based on a snout-vent length greater than 9 cm. We obtained permission to capture and use these animals from the Chief Conservator of Forests

(Wildlife) and Chief Wildlife Warden, Bengaluru, Karnataka (Letter No. PCCF(WL)/E2/CR-03/2020-21).

Rodents

We also used Wistar albino mice (male, 6 - 8 weeks old; weighing 20 - 25 g) and Wistar rats (male, 6–8 weeks old; weighing 200–250 g) obtained from the Central Animal Facility, University of Mysore, Mysuru, India (Approval No.: UOM/IAEC/02-2024-2025). All animals were housed with adequate ventilation, food, and water under 12-hour light and dark cycles ad libitum and were monitored throughout the experiment.

Purification of ~55 kDa protein sperm motility inhibitory factor from female *Calotes versicolor*

Building upon our previous study [1] , we isolated and purified the ~55 kDa protein sperm motility inhibitory factor from female garden lizards during their reproductive season. Three to four female garden lizards were anesthetized within 24 hours of capture and autopsied. The isolated utero vaginal junction (UVJ) was flushed with physiological saline and centrifuged at 10,000 × g for 10 minutes at 4 °C. The pellet was discarded, and the supernatant was concentrated using Centricon tubes (cut-off > 3 kDa) at 4000 × g for 45 minutes at 4 °C.

The concentrated protein sample was estimated using Bradford's method [13]. The sample was then subjected to protein purification by chromatographic techniques using a Cibacron blue 3G-A pseudo-affinity column (0.5 cm × 10 cm) packed and pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.4. The ~55 kDa protein and other minor proteins were eluted in the void volume, as previously described [1], and were analyzed by SDS-PAGE and silver staining. The void volume fraction was pooled and concentrated using Centricon tubes (cut-off > 3 kDa). The concentrated sample was passed onto a DEAE-cellulose anion exchange column (0.5 cm × 10 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.4. The fractions were eluted with an increasing concentration of NaCl (10 mM - 1 M). The NaCl concentration, ranging from 200 to 250 mM, was used to elute the purified ~55 kDa protein.

Two-Dimensional Electrophoresis for ~55 kDa Protein

The concentrated ~55 kDa protein (100 µg) was prepared in a final volume of 125 µL using urea buffer (9 M urea, 2 M thiourea, 4% (w/v) CHAPS). The sample was mixed with 0.8% (w/v) DTT, 0.2% (v/v) ampholytes pH 3-10, and 0.002% bromophenol blue. The gel was stained with Coomassie brilliant blue (CBB) and photographed.

Isolation of Sperm Cells

Spermatozoa were isolated from male Indian garden lizard, mouse, rat, and human semen samples. Epididymides from respective animals were dissected and cut into 3-4 different sites. The tissues were incubated in 1 mL of Earle's balanced salt solution (EBSS) supplemented with 25 mM HEPES, 10% FBS, containing 1% Pen-Strep for 30 minutes at 37 °C. Motile sperm cells were collected and washed twice with EBSS media.

The processed healthy human semen sample was taken from the In Vitro Fertilization Centre (IVF)(Siri Caree IVF Centre, Mysuru). The sample was incubated in EBSS media for 30 minutes at 37 °C. Sperm cells were collected and washed twice with EBSS media. The final pellet was overlaid with 200 µL of EBSS and incubated at 37 °C for 1 hour. Following these tubes were kept angled at 45 degrees and incubated for 1 hr at 37 °C for direct swim up of spermatozoa. Viable spermatozoa were collected from the direct swim-up method and were used for further experimentation [1].

Sperm Motility and Viability Assay

The total number of cells was counted using a Mekler's chamber under a phase contrast microscope at x 400 magnification. Washed spermatozoa (2 x 105) were resuspended in 200 µL of EBSS media and treated with crude UVJ proteins or purified ~55 kDa protein for 0-180 minutes at 37 °C. Motility of spermatozoa was evaluated using Computer-Aided Sperm Analysis (CASA) at 3 different time intervals (0-180 minutes). Sperm viability was assessed using the dye exclusion method [1].

Results

Purification and Characterization of ~55 kDa Sperm Inhibitory Protein from UVJ

The concentrated sperm-free UVJ flushings were subjected to 10% SDS-PAGE examination, revealing the presence of 20-30 additional proteins. A major abundant protein band measuring around ~55 kDa was detected, regardless of the stage of the reproductive cycle (Fig. 1C). We purified the major protein using conventional chromatographic techniques on a Cibacron blue 3G-A column, followed by DEAE-Cellulose column chromatography as reported earlier [1].

When the Cibacron blue pseudo-affinity column was performed, the ~55 kDa protein was eluted in the void volume fraction (Fig. 1A) with other proteins (Fig. 1C). As we reported earlier, the ~55 kDa protein was further purified with DEAE-Cellulose anion-exchange column chromatography and was eluted at a fraction number of 100-120 in 10 mM phosphate buffer containing 200 mM NaCl, pH 7.4 (Fig. 1B). The protein corresponding to ~55 kDa following the DEAE-Cellulose column showed apparent homogeneity, as evidenced by 12.5% SDS-PAGE analysis using silver staining (Fig. 1D). To check the purity of the ~55 kDa protein, we conducted 2-D electrophoresis, and it appeared that a single protein spot was well-resolved upon CBB staining, with its pI identified between 4 and 5 (Fig. 1E).

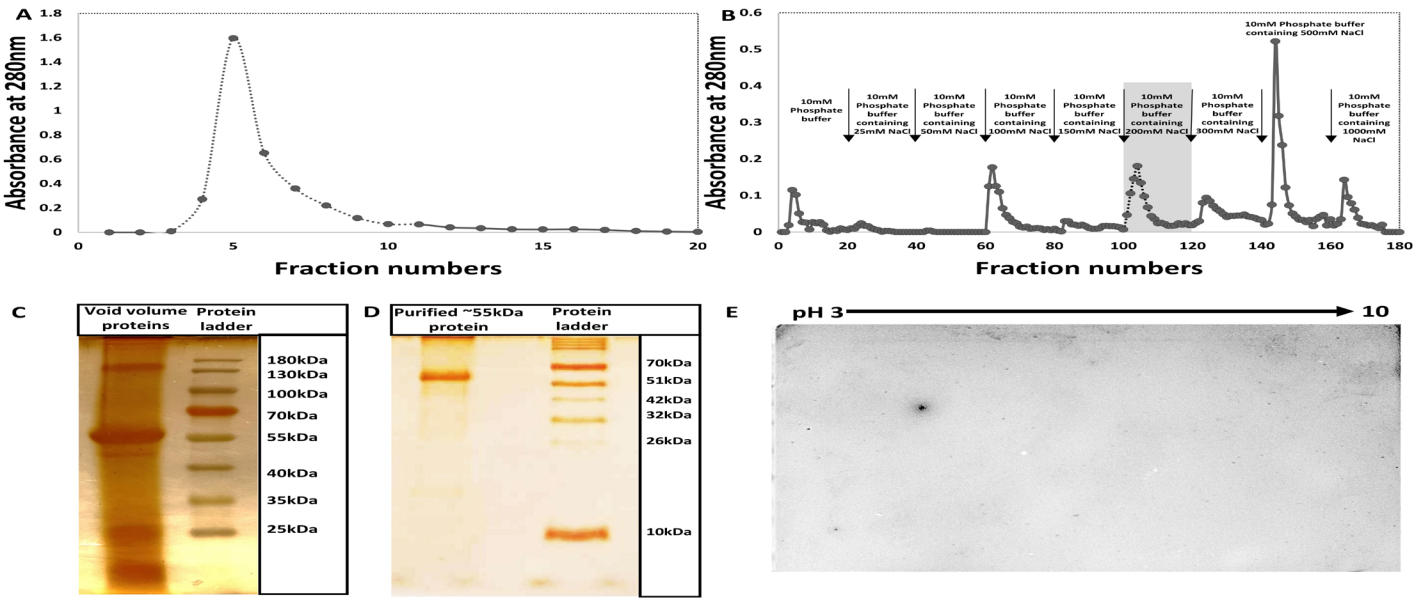


Figure 1: Purification of ~55kDa lizard protein and its analysis by electrophoretic techniques

Elution profile of UVJ flushing proteins on Cibacron blue column chromatography (0.5 cm × 10 cm) void volume fraction (A), elution profile of Cibacron blue unbound proteins on DEAE-Cellulose column chromatography (0.5 cm × 10 cm) at different NaCl concentration (0 – 1000 mM) (B), SDS-PAGE analysis showing UVJ flushing Cibacron blue unbound protein banding pattern respectively (C), SDS-PAGE (12.5%) analysis showing protein banding pattern of ~55 kDa protein eluted from DEAE-Cellulose at the concentration of 200 mM NaCl (D). Two-dimensional gel electrophoresis showing purified ~55 kDa UVJ protein (100 µg) was separated using linear 7 cm IPG strips, pH range 3 - 10 in the first dimension and 12% SDS-PAGE in the second dimension, showing a single protein spot (E).

Effect of Purified ~55 kDa Protein on Motility of Homologous and Heterologous Sperm Cells

In our previous study, we showed that the purified ~55 kDa protein inhibited the sperm motility of the Indian garden lizard *C. versicolor* reversibly (Lizard: L) (Shankar et al., 2018). 1 In this current study, we aim to understand the same inhibitory role of the purified ~55 kDa protein on heterologous species (, Human: H, Mouse: M, and Rat: R).

We treated 100 µL of sperm cell volume with L: 100 µg, H: 40 µg, M and R: 30 µg of crude and different concentrations of purified ~55 kDa protein L: 50-75 µg, H: 10-20 µg, M and R: 5-10 µg. After three hours of incubation, untreated spermatozoa did not exhibit any changes in motility. On the other hand, sperm motility gradually decreased when treated with crude protein to L: 89.66±0.88%, H: 94.3±1.7%, M: 94.66±0.88%, and R: 95±0.5% at time 0 min to L: 54.66±1.45%, H: 67.6±3.7%, M: 53.33±0.88%, and R: 75.33±0.6% after 180 minutes of incubation period.

Additionally, in a concentration- and time-dependent way, the purified ~55 kDa protein reduced sperm motility. After treating the purified ~55 kDa protein to L: 50 µg, H: 10 µg, M and R: 5 µg, the sperm motility decreased from L: 90.33±0.33%, H: 91±1.15%, M: 92.66±1.20%, and R: 92.33±0.33% at the time 0 min to L: 47.66±1.45%, H: 46±2.08%, M: 45.33±1.76% and R: 45.33±2.02% after at 180 min of incubation period. Further increasing the purified ~55 kDa protein concentration to L: 75 µg, H: 20 µg, M and R: 10 µg further decreased the sperm motility within 20 min L: 88.66±0.88%, H: 92.3±1.8%, M: 92±1% and R: 93.66±0.88% at the time 0 min to L: 8.33±1.20%, H: 17.6±1.45%, M: 22.66±2.02% and R: 23±3.05 after at 180 min of incubation period. By using the eosin-nigrosin staining method, the viability study was done for all sperm cells (Fig. B, D, F, and H) and confirmed that the purified ~55 kDa protein's inhibitory action was not caused by a decrease in sperm viability. Surprisingly, after 180 min followed by the removal of various concentrations of crude uterovaginal protein (100 µg) and purified ~55 kDa protein (0–75 µg) by EBSS media, sperm motility was regained in all species (Fig. 2, A, C, E, and G). As a result, this purified protein influences the motility of sperm from both homologous and heterologous species is reversible. All the data shown above represent the mean s.d. of three independent experiments. However, the mechanism of this purified ~55 kDa protein and how this protein inhibits homologous and heterologous sperm cells is still not clear.

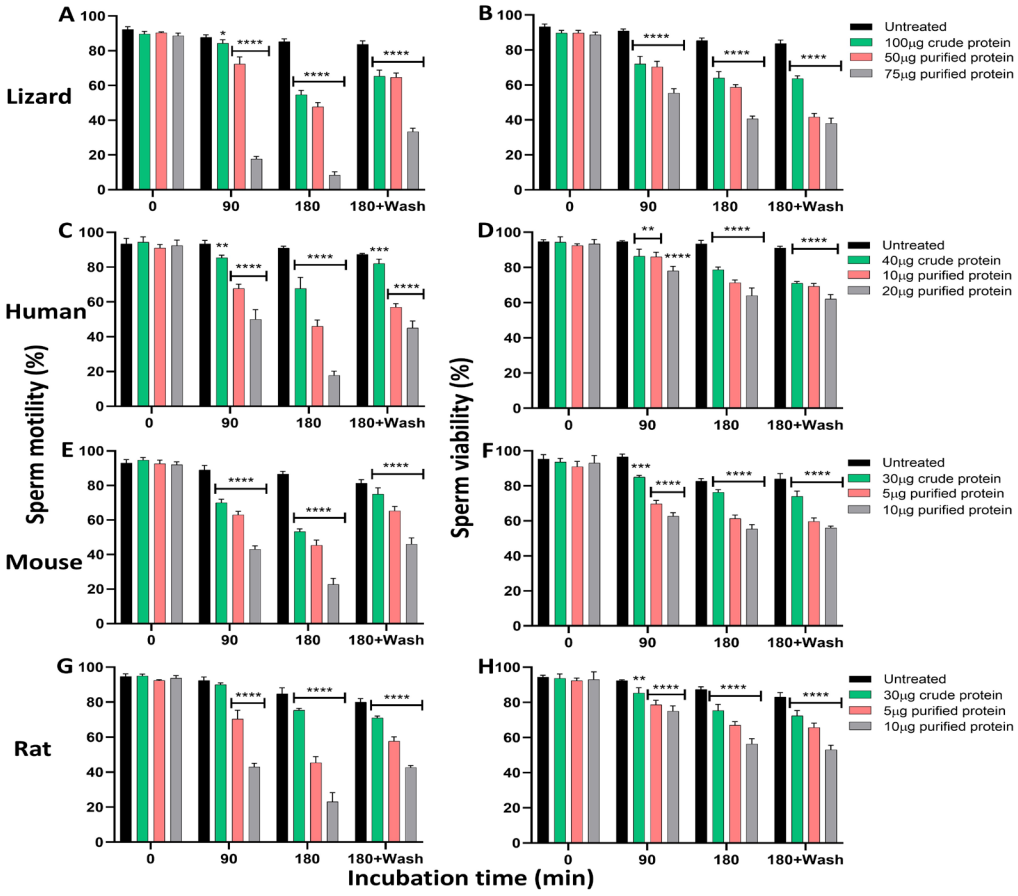


Figure 2: Sperm motility and viability assay

Effects of crude and purified ~55 kDa UVJ protein on motility (A, C, E and G) and viability (B, D, F and H) of homologous [Lizard (L): A, B] and heterologous [Human (H): C, D Mouse (M): E, F and Rat (R): G, H] species sperm cells over 180 min incubation period and after washing out UVJ protein. Concentration and time-dependent effects of crude UVJ protein (L: 100 µg, H: 40 µg, M and R: 30 µg) and purified ~55 kDa protein (L: 50, 75 µg, H: 10, 20 µg, M and R: 5, 10 µg). Data are the mean ± s.d. of three independent experiments. *P,0.01, **P,0.001, ****P,0.0001.

Discussion

Although female sperm storage is recognized in many animal phyla, following ejaculation from male species, sperm are moved into the vagina, where they become motile and few of them reach the SSTs [13]. Upon sperm entering the SSTs, the cells become quiescent, but little is known about the mechanism behind it [14-15]. In addition, compared to mammals, nothing is known about the long-term sperm sustenance in the reptilian oviduct.

The SDS-PAGE analysis of the Indian garden lizard uterovaginal flushings identified an abundant protein corresponding to ~55 kDa irrespective of the phases of the reproductive cycle and exogenous addition of this purified protein to lizard epididymal sperm, reversibly inhibited the motility in a time and concentration-dependent manner as reported previously by [1].

In the present study, we purified the ~55 kDa UVJ protein using previously described methods in our laboratory. We hypothesized that the motility-retarding effect observed might not be unique to lizards but could also be seen in other animals. To test this hypothesis, we treated the purified ~55 kDa UVJ protein with heterologous sperm, those from human, mouse, and rat. The heterologous species sperm motility was retarded in a much more sensitive fashion compared to lizard sperm suggesting that this ~55 kDa protein may be universal sperm motility retarding factor. Although the ~55 kDa protein inhibited the motility in all the animal species tested, all the sperm did not regained motility upon washing. If this protein is capable of inhibiting the sperm from heterologous species, this may have far-reaching applications in prolonging sperm storage, which is crucial for preserving the

sperm of endangered animal species, and in In-vitro fertilization (IVF) technology.

Declaration of competing interest

The authors declare that they have no competing financial interests.

Data availability

All data underlying the results are available as part of the article and supplementary materials.

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Conflict Of Interest

The authors declare no potential conflict of interest.

Author contributions

Gopal Kedihithlu Marathe: Supervision, review & editing, Conceptualization, Methodology, Validation, Project administration. Thumbala Andanaiah Gagan: Original draft, Investigation, Methodology, Visualization, Software, Data curation, Formal analysis. Anilkumar Chaluvadasi: Methodology and Data curation. Gururaj Siddaramegowda: Methodology and Data curation. Goutham Shankar: Methodology and Data curation. Shilpa Chengari Venkategowda: Resources. Titus Ruth Shantha Kumari: Project administration, Resources, Conceptualization, Validation.

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