

Expression of inhibin B in gametes by different Echinoderms: Sea urchins (*Lytechinus variegatus*) and Sand Dollars (*Dendraster excentrus*)

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Abstract

Background: Once environmental stressors enter an ecosystem, all levels can be affected and in a number of different ways, e.g., fecundity. A specialized bioassay, an ELISA detecting inhibin B, that helps measure primate fertility status appears to do the same in non-primates. In mammals, inhibin B is a protein hormone expressed by ovarian granulosa cells and is vital in the regulation of ovarian function, including folliculogenesis. In males, inhibin B is expressed by Sertoli cells and plays a role in testicular function, including spermatogenesis.

Methods: This study measured and compared inhibin B levels between different echinoderms, sea urchins (*Lytechinus variegatus*) and sand dollars (*Dendraster excentrus*), and contrasted levels between female and male gametes.

Results: In the sea urchin, inhibin B content was significantly higher in females (oocytes) than males (spermatozoa) with overall means of 6.361 ng/106 ova and 0.007 ng/106 spermatozoa, respectively. In the sand dollar, inhibin B content was also significantly ($P < 0.001$) higher in females (oocytes) than males (spermatozoa) with overall means of 3.366 ng/106 oocytes and 0.057 ng/106 spermatozoa, respectively. Of particular note, inhibin B levels were notably higher in both gametes of the sand dollar than the sea urchin.

Conclusions: Inhibin B levels can be detected in echinoderms using a commercially available ELISA kit. Inhibin B levels in the echinoderm are dependent on species as well as animal sex. Inhibin B levels appear to be a good candidate biomarker for reproductive monitoring in echinoderms. Inhibin B could dramatically affect what we know regarding effects of stressors, and the urgent actions for effective and cost-effective ocean water conservation. Further studies will elucidate the role of inhibin B on echinoderm reproduction.

Introduction

Marine invertebrates, such as the sea urchin, are a time honored model for investigational studies in developmental biology [1]. The effects of stressors, for example nutritional deficiencies, anthropogenic chemical contaminants, and biotoxins in marine animals have been very difficult to assess using traditional methods. The inability to demonstrate the magnitude and extent of effects of environmental stressors such as climate change, red tides and contaminants on marine animals represents a significant impediment to their conservation. The identification and subsequent detection of biomarkers allows scientists to correlate effects, such as impaired reproduction, with possible causes.

An enzyme-linked immunosorbent assay (ELISA) specific for inhibin B serves to detect this biomarker of fecundity potential in individual mammals in both sexes [2,3]. The inhibin B ELISA assay was developed to promote better understanding of reproductive potential and fitness of humans [4,5], and appears to function effectively for other mammals as well [6,7]. Inhibin B is a peptide hormone which belongs to the transforming growth factor- β (TGF- β) superfamily [8]. In the mammalian female, inhibin B is expressed by granulosa cells of the ovaries and has been shown to be vital in the regulation of ovarian function. As the ovarian follicle cohort size decreases, which is expected as the female ages, a decrease in inhibin B levels is observed. In the mammalian male, inhibin B is expressed by the Sertoli cells and plays a role in Leydig cell steroidogenesis (i.e., testosterone production)

and regulating follicle stimulating hormone (FSH) production by the anterior pituitary. Inhibin B has been shown to be an excellent marker of male reproductive health, i.e. testicular function.

An assay for specific markers of fertility can be used to facilitate investigations into gonadal dysfunction [9-11] and even help assess reproductive potential or status [12,13]. Environmental and human-caused stressors can affect wildlife populations in several ways, some more critical than others. One of the more serious effects can involve fecundity and reproductive success. Therefore, the study objective was to investigate inhibin B levels between different echinoderms, sea urchins [14] and sand dollars [15], and to also compare levels between female and male gametes. We also demonstrate the utility of the inhibin B ELISA assay to detect fecundity potential in echinoderms

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Key words: echinoderm, fecundity, gamete, inhibin b, oocytes, sand dollar, sea urchin, spermatozoa, TGF-beta

Received: January 22, 2019; **Accepted:** January 29, 2019; **Published:** February 04, 2018

Materials and Methods

Individual sea urchins (*L. variegatus*) and sand dollars (*D. excentrus*) were injected with 0.5 mL KCl (0.5M) to induce gamete shedding. Shed gametes were collected in sterile sea water and gamete concentrations were recorded via a Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel). Following centrifugation (400xg, 10 minutes), and discarding the supernatant, the gametes were resuspended in 1 mL cold lysis buffer (10% Triton-X-100 in sterile water) and stored at -70°C until assayed for inhibin B.

The inhibin B enzyme-linked immunosorbent assay (ELISA) was conducted per the manufacturers' (Beckman Coulter, Inc., Chaska, Minnesota, USA) instructions and processed on an automated plate processor (Dynex DSX Automated Plate Processor, Dynex technologies, Chantilly, Virginia, USA). Inhibin B standards, inhibin B controls, and duplicate gamete samples were incubated in microtitration wells which had been coated with the inhibin B capture antibody. After incubation and washing, the wells were exposed to a biotin-labelled antibody for detection. After a second incubation and washing step, the wells were incubated with streptavidin-horseradish peroxidase (HRP). After a third incubation and washing step, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured is directly proportional to the concentration of inhibin B present. The set of inhibin-B standards was used to plot standard curves of absorbance vs. inhibin B concentration. Based on the standard curves, the concentration of inhibin-B in the gamete samples was calculated (pg/106 cells) for each specimen collected.

Statistical calculations were performed with SigmaPlot for Windows, version 13.0.0.83 (Systat Software, Inc, San Jose, California, USA). Student's t-test analysis was used to investigate the relationships between the measures of the study.

Results

Inhibin B levels between *D. excentrus* oocytes and *L. variegatus* oocytes were while not significantly different may still be of biological significance ($P = 0.09$, Figure 1), which may be a factor of small sample size ($n=18$ total). Inhibin B levels were significantly ($P < 0.01$, Figure 2) higher in *D. excentrus* spermatozoa than *L. variegatus* spermatozoa.

Inhibin B levels in *D. excentrus* ranged from a low of 0.001 pg/106 spermatozoa to a high of 0.014 pg/106 spermatozoa (mean = 0.0568 ± 0.014 pg/106 spermatozoa) in males. In female *D. excentrus*, inhibin B levels ranged from a low of 0.357 pg/106 oocytes to a high of 9.166 pg/106 oocytes (mean = 3.364 ± 1.121 pg/106 oocytes). Inhibin B levels were significantly ($P < 0.01$, Figure 3) higher in *D. excentrus* spermatozoa compared to *D. excentrus* oocytes.

Inhibin B levels in *L. variegatus* spermatozoa ranged from a low of 0.005 pg/106 spermatozoa to a high of 0.236 pg/106 spermatozoa (mean = 0.007 ± 0.001 pg/106 spermatozoa). In *L. variegatus* oocytes, inhibin B levels ranged from a low of 0.242 pg/106 oocytes to a high of 15.791 pg/106 oocytes (mean = 6.361 ± 1.797 pg/106 oocytes). Inhibin B levels were significantly ($P < 0.001$, Figure 4) higher in *L. variegatus* spermatozoa compared to *L. variegatus* oocytes.

Discussion

The transforming growth factor-beta (TGF-beta) superfamily is a multifunctional group of cytokines. Members of this superfamily

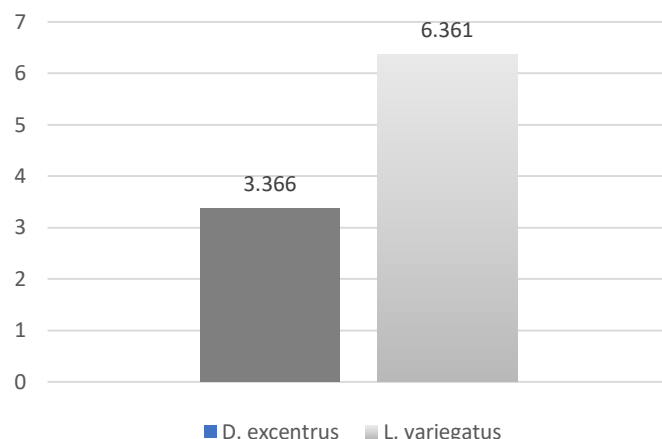


Figure 1. Inhibin B levels between *D. excentrus* oocytes and *L. variegatus* oocytes ($P = 0.09$)

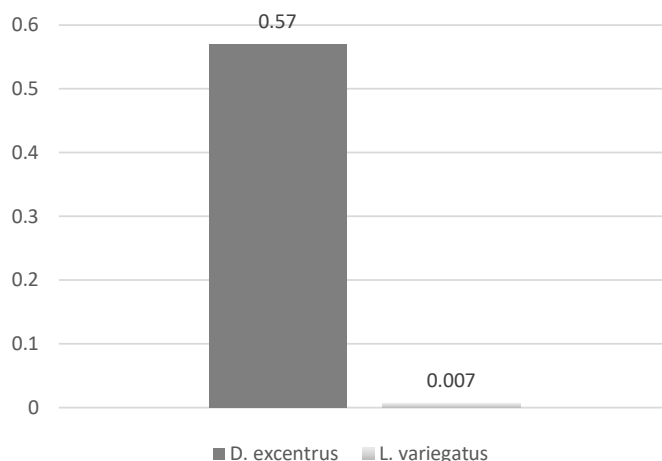


Figure 2. Inhibin B levels were significantly ($P < 0.01$) higher in *D. excentrus* spermatozoa than *L. variegatus* spermatozoa

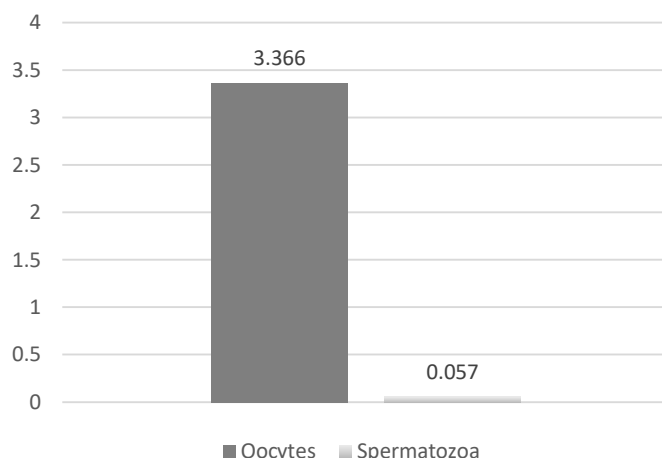


Figure 3. Inhibin B levels were significantly ($P < 0.01$) higher in *D. excentrus* spermatozoa compared to *D. excentrus* oocytes

are secreted proteins involved in many cellular functions including apoptosis, cell differentiation, cell growth and cell proliferation. Members of the TGF-beta family of genes reported to be present in echinoderms include, activin B [16], bone morphogenetic protein

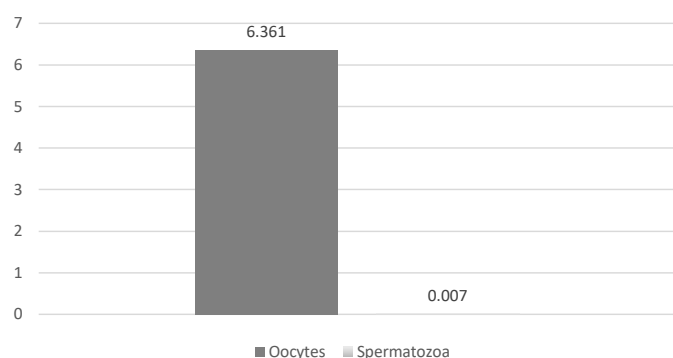


Figure 4. Inhibin B levels were significantly ($P < 0.001$) higher in *L. variegatus* spermatozoa compared to *L. variegatus* oocytes

(BMP-4), myostatin [17], and nodal [18], which all have roles in supporting development.

We have demonstrated, for the first time, that inhibin B is present in the gametes of an echinoderm, but the question remains, “what is the biological significance of inhibins in the echinoderm?” In mammals, the primary role of inhibins is to help regulate the negative feedback system in the hypothalamus-pituitary-gonad axis. Since echinoderms lack this regulatory system, this role of inhibins is highly unlikely. Since, inhibin B content in oocytes was found to be vastly higher than in spermatozoa, one functional possibility is that inhibin B serves as a chemoattractant to facilitate spermatozoa-oocyte interactions. However, TGF-beta family members are known to play fundamental roles in regulation of basic biological process, e.g., growth, development, and homeostasis [19], inhibins most likely facilitate gamete function by a yet unreported mechanism. Note, another member of the TGF-beta superfamily (anti-müllerian hormone, AMH) found to be of great importance in mammalian gonadal function was undetectable in echinoderm gametes (unpublished observations). This observation is not surprising for AMH, while it is essential during embryonic development for the correct formation of male and female structures in mammals, AMH is most likely not required in echinoderms in this capacity.

We evaluated the use of a protein biomarker developed to assess fertility potential of male and female humans and determined that it can also be used to assess fertility potential in echinoderms. Our tests of echinoderm gametes demonstrate that the ELISA specific for inhibin B works for these represented animals of the class Echinoidea. Appropriate validation will allow us to assess fecundity potential as a function of environmental parameters including, but not limited to geographic location, physiochemical nature, and, or exposures to toxins or contaminants. Environmental factors such as stress directly affect reproduction and survival [20]. One such environmental factor is the quality of our seawater. The physiochemical nature (e.g., pH and salinity) of the seawater into which gametes are released plays a key role in fertilization and early embryonic development and survival.

Rising carbon dioxide (CO_2) levels in our atmosphere are a key driver of environmental change. As CO_2 levels rise, seawater absorbs it which results in a lowering of mean pH, referred to as ocean acidification. Once we have demonstrated the relationship between inhibin B content in gametes and fecundity, we can observe the impact of ocean acidification on echinoderm fecundity potential.

Detection of inhibin B or similar TGF-beta superfamily members could dramatically affect both what we know regarding effects of

stressors, and the urgency of mitigation actions for effective and cost-effective conservation. Additionally, inhibin B detection has huge implications for evaluating environmental impact, mostly in assessing biodiversity hotspots [21]. Echinoderms are excellent monitors of the ocean habitat and may help in assessments of global ocean hotspots [1]. We believe that determining the fecundity/fertility potential via the use of a simple ELISA specific for a protein biomarker, such as inhibin B, in any animal (invertebrate or vertebrate) will aid in furthering our understanding of the regulatory events during fertilization and early development.

Additionally, these biomarkers have the potential for us to learn more about the reproductive capacity of invasive species which could result in programs to help eliminate their populations.

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