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RESEARCH ARTICLE

EXTRACTION OF TOTAL RNA FROM SPERM OF NEW ZEALAND WHITE RABBITS FOR DETECTION OF PHOSPHOLIPASE C ZETA (PLC ζ).

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ABSTRACT

Fertilization is defined as the fusion of a spermatozoon to an ovum. Upon fusion, a sperm specific factor Phospholipase C ζ was inserted triggering long lasting Ca^{2+} oscillations which drive egg activation and early embryonic development phase. Previous studies indicate that PLCζ homologues have been identified in humans, mice, pigs, monkeys and chickens. Although a preliminary study exhibits that PLCζ is present in rabbit testis, the sequence has not been published and the study on sperm has not been verified. Therefore, this study was conducted to isolate and detect PLCζ in rabbit sperm. Rabbit semen was collected using the artificial vagina. The semen was purified by using EquiPure Bottom Layer to remove bacteria and unwanted components in the fresh ejaculate. Purified semen was centrifuged at 4000 rpm for 10 minutes to separate sperm and seminal fluid. The sperm pellet on the bottom of the tube was subjected to RNA extraction by using Easy Blue Total RNA Extraction Kit. The purity and concentration of total RNA was quantified. Amplification reaction was performed by using One-Step RT-PCR system. The size of target gene was determined by conducting gel electrophoresis and eventually the band detected on the gel was viewed under Alpha Imager™ 2200. The visible band strongly demonstrated that PLCζ is also present in rabbit sperm which play an important role to trigger calcium oscillations during fertilization.

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INTRODUCTION

Fertilization between the spermatozoon and the ovum occurs in the ampulla of the uterine tube. In this sperm-oocyte membrane fusion system, phospholipase C (PLC) is an enzyme in the sperm that has a role to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Fukami *et al.*, 2010). Activation of IP_3 signaling pathway initiates intracellular Ca^{2+} to be released. Long lasting Ca^{2+} oscillations drive egg activation and early embryonic development phase. There is evidence that this Ca^{2+} wave or oscillations also has role in block to polyspermy (Allison *et al.*, 2007). Recently, this sperm-specific factor, Phospholipase C ζ is widely known to trigger repetitive calcium oscillations in the concentration of free calcium ($[Ca^{2+}]_i$) leading to oocyte activation and embryo development in all mammals (Cooney *et al.*, 2010) including rabbits. In fertilization, prior to block to polyspermy, capacitated sperm successfully penetrates zona pellucida and initiates acrosomal reaction.

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Previous study demonstrates that PLCζ resides in the acrosomal and post-acrosomal regions and undergoes dynamic changes during capacitation and acrosomal reaction. PLCζ homologues have been identified in humans, mice, pigs (Swann *et al.,* 2006), monkeys (Cox *et al.*, 2002) and chickens (Coward *et al.*, 2005). Meanwhile, when a male infertility-linked human PLCζ mutation is introduced into mouse PLCζ, it completely abolishes both in vitro PIP^2 hydrolysis activity and the ability to trigger in vivo Ca^{2+} oscillations in mouse eggs. This indicates that PLCζ plays a role in fertility (Nomikos *et al.*, 2011). Furthermore, both human and cynomolgus monkey are capable to elicit Ca^{2+} oscillations in mouse oocytes similar to normal fertilization in the mice. Hence, this exhibits that PLCζ is highly conserved across mammalian species (Cox *et al.*, 2002). We report here a study to detect the presence of PLCζ in the RNA of rabbit sperm.

MATERIALS AND METHODS

Sample Collection

Semen from seven New Zealand White Rabbits was collected by using the artificial vagina. The semen from each rabbit was transferred into 7 separate 1.5 ml micro centrifuge tubes.

The fresh ejaculates semen was evaluated for sperm concentration (x10^6/mL), sperm motility and morphological features by light microscopy (Pranab *et al.*, 2010). To obtain the purified sperm which is free from immature cells and spermatocytes, the semen was processed by gradient centrifugation with EquiPure Bottom Layer (Pranab *et al.*, 2010). Approximately 1 mL of semen was gently layered on top of a pre-warmed (37˚C) 3mL EquiPure Bottom Layer in a centrifuge tube. The mixture was centrifuged for 4000 rpm for 10 minutes. The sperm pellet at the bottom of the tube was collected.

RNA Extraction

The purified sperm was used for RNA extraction to obtain purification of high-quality total RNA of the tissue or cell samples. Easy-Blue Total RNA Extraction Kit was used.

Quantification

For quantifying the RNA obtained, spectrophotometer was used. The best concentration which ranges between 1500ng/µl to 2000ng/µl and a purity within 1.7 to 2.0 is considered highly pure.

of QIAGEN one-step RT-PCR Enzyme Mix, 1µl of template RNA. It was carried out in a thermal cycler for 35 cycles.

Primer

The primer used for both forward and reverse were according to Fujimoto *et al.,* (2004).

Agarose Gel Electrophoresis

Agarose Gel Electrophoresis was conducted to determine the size of the DNA fragments. The larger the fragment of DNA, the more easily it becomes entangled in the matrix. Hence, the more slowly it migrates through the gel and vice versa. Thus, 0.7% agarose gel is recommended for the large molecule size range from 5 to 10 kb while DNA fragments size range from 0.2 to 1 kb is suggested for 2% agarose gel. Preliminary studies have shown that PLC ζ gene fragment size is between 450 to 500 bp. Therefore, 1.5% agarose gel (0.6 agarose powders in 40mL TBE buffer) was used. The matrix can be adjusted by increasing the concentration of agarose (tighter matrix) or by decreasing it (looser matrix). Beta actin was used as a control.

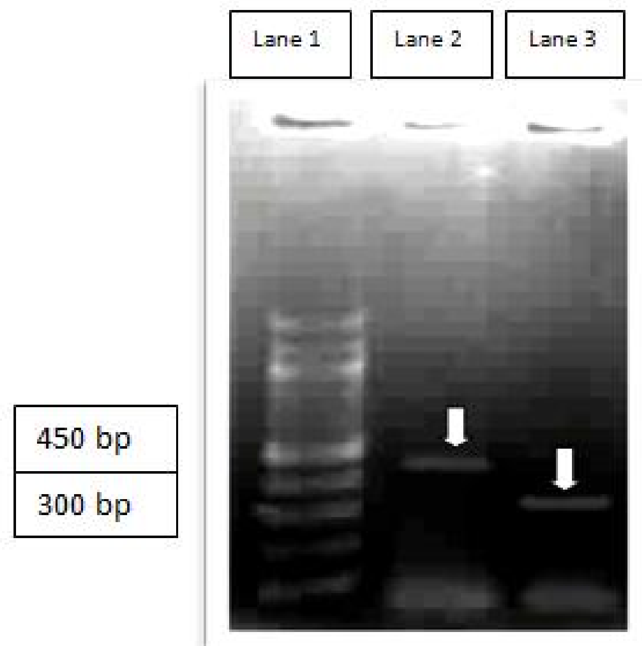

Figure 1. Bands (arrow) corresponding to target fragments of PLC ζ (450 bp) in rabbit sperm cDNA (lane 2) and PLC ζ (approximately 300 bp) in beta actin (lane 3) as control.

Table 1. Sequence of PLCζ primer from published journal

Primer	Sequence	Target	References
PLC f	5' – CATGTGAAACATATTTTTAAGGAAA – 3'	PLCζ	Fujimoto *et al.*, 2004
PLC r	5' – ATCCCCAAATGTCACTCGGTCC – 3'	PLCζ	Fujimoto *et al.*, 2004

One-Step RT PCR reactions

Amplification reactions was performed by using QIAGEN One-Step RT-PCT kit in 50µl volume containing 33 µl RNase-free water, 10.0µl 5x QIAGEN one-step RT-PCR buffer that contains 2.0 µl of dNTP mix (containing 10mM of each dNTP), 1 µl of forward primer, 1 µl of reverse primer, 2.0 µl

Gel Viewing

The gel was placed in the Alpha Imager™ 2200, at the center of the stage. The well-positioned gel was captured by the UV light and the data achieved was saved.

RESULTS AND DISCUSSION

In this study, the gel electrophoresis result in Figure 1 demonstrated that PLC ζ is present in rabbit sperm. The amplified band of PLC ζ was located approximately 400bp – 500bp. The size of PCR products obtained was similar to the results from previous study by Fujimoto *et al*. (2004). Low yield of semen obtained during semen collection may result in band fading in rabbit sperm DNA (lane 2) as shown in Figure 1. Furthermore, RNA is very unstable as compared to DNA. Therefore, possibility of RNA degradation might be one of the factors of fading. PLC ζ has been known to play important role in fertility in all mammals. Lacking of PLC ζ in sperm will lead to failure of embryonic development as the intracellular calcium was not initiated.

CONCLUSION

The successful detection of PLC ζ can serve as a platform for future studies to be conducted in other mammalian species. By obtaining the sequence of this enzyme, artificial PLC ζ might be synthesized to assist in infertility treatment in human and other animals in the future. The results obtained in this study strongly supported the previous findings which indicate that this sperm factor is present in all mammals including rabbits.

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