EXPRESSION AND DISTRIBUTION OF THE COMPLEMENT RECEPTOR gC1qR IN BOVINE SPERM.

Expresión y distribución del receptor de complemento gC1qR en los espermatozoides bovinos.

Robert Valeris [1]***, Patricia Villamediana*** [1]***, Armando Quintero*** [2]***, Omaira Parra*** [3]***, Jorge Sandoval*** [3] ***and Berhane Ghebrehiwet*** [4]

[1] *Unidad de Investigación en Biotecnología Animal. Facultad de Ciencias Veterinarias y Facultad Experimental de Ciencias, Universidad del Zulia, Maracaibo, Venezuela.* [2] *Unidad de Investigación en Producción Animal (UNIPA). Facultad de Ciencias Veterinarias, Universidad del Zulia, Maracaibo, Venezuela.*
[3] *Unidad de Investigaciones Clínicas. Facultad de Ciencias Veterinarias, Universidad del Zulia, Maracaibo, Venezuela.*
[4] *State University of New York at Stony Brook, Department of Medicine, Stony Brook, NY 11794-8161, U.S.A.*
E-mail: robertvaleris@yahoo.com.mx

ABSTRACT

The complement receptor gC1qR/p33, which recognizes the globular heads of C1q, is a multicompartmental and multifunctional protein and has been shown to play a role in reproduction in humans. The objective of this research was to determine the gC1qR distribution and expression on plasma membrane in bovine sperm before and after heparin treatment. Thus, capacitation of sperm derived from frozen-thawed bovine semen was induced through heparin incubation and its effectiveness was assessed with a CTC stain. Subsequently, indirect immunofluorescence assays were conducted with mAb (60.11, anti-gC1qR) to assess gC1qR distribution and expression. Data analysis demonstrated that gC1qR is expressed on the plasma membrane of bovine sperm. gC1qR showed a capacitation-related redistribution, migrating to the acrosome region: 175 sperm in heparin-incubated aliquots vs. 109 in control ($P<0.05$, $n=300$) showed fluorescence over the acrosome region. This distribution is similar to that reported in humans. Similarly, either gC1qR expression or its accessibility to antibodies increased after capacitation. This also correlates with what has been observed in humans. These results suggest that gC1qR could participate in primary sperm-oocyte interaction in bovines.

Key words: gC1qR, bovines, complement, sperm.

RESUMEN

El gC1qR, un receptor para el dominio globular del C1q, es una proteína multicompartamental y multifuncional, cuya intervención en el proceso de fecundación en humanos se ha demostrado recientemente. Los objetivos de esta investigación fueron describir la distribución de gC1qR y valorar su expresión en la membrana plasmática de espermatozoides bovinos antes y después del tratamiento con heparina. La capacitación de espermatozoides provenientes de semen descongelado bovino se indujo con una incubación con heparina y su efectividad se evaluó con la tinción CTC. Posteriormente, se realizaron ensayos de inmunofluorescencia indirecta con el anticuerpo monoclonal 60.11 (anti-gC1qR). El análisis de los datos demostró que el gC1qR es una molécula ubicada en la membrana plasmática de los espermatozoides bovinos que presenta una redistribución tras la capacitación, concentrándose en la región acrosomal: 175 espermatozoides en las alícuotas incubadas con heparina vs. 109 en el control ($P<0,05$; $n=300$) presentaron fluorescencia en la región acrosomal; esta distribución es semejante a la documentada en humanos. Igualmente, el nivel de expresión o de accesibilidad de gC1qR en el espermatozoide bovino aumentó tras la capacitación, hecho que se ha observado también en humanos. Estos resultados sugieren que el gC1qR podría participar en la interacción primaria entre el espermatozoide y el ovocito en bovinos.

Palabras clave: gC1qR, bovinos, complemento, espermatozoide.

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INTRODUCTION

Fertilization is a programmed process through which two completely different cells, sperm and oocyte, fuse to form a zygote, a cell with a somatic number of chromosomes [24, 25]. Zygote development begins immediately after sperm and oocyte haploid pronuclei come closer to one another and combine their chromosomes to form one diploid nucleus, containing the parental genes. Fertilization in mammals is the ultimate outcome of a complex array of molecular events that allow the capacitated sperm to recognize and bind to oocyte extracellular coat, zona pellucida (ZP), to undergo acrosome reaction, and to fuse with oocyte plasma membrane [20, 23, 24, 25, 27].

It is now well documented that complement components and related proteins play a relevant role in fertilization. These molecules have been linked to sperm-oocyte interaction and to the intracellular signaling brought about by this interaction, as well as to the protection of sperm from damage caused by the complement present in female reproductive tract [8, 9, 22].

In addition to their role in the induction of a wide range of biologic responses as well as activation of the classical pathway, C1q and its receptors (gC1qR and cC1qR) have been reported to participate in several cellular events, including gamete interaction leading to fertilization [8, 13]. In humans, exogenous C1q has been shown to enhance sperm binding to oolema of ZP-free hamster oocytes, in a concentration-dependent manner. However, this interaction seems to inhibit sperm penetration into the oocyte [8]. Also, C1q promotes agglutination in capacitated sperm, but not in freshly ejaculated sperm [8].

In humans, gC1qR was first characterized as a receptor for the globular heads of C1q [10, 11]. However, gC1qR has recently been considered as a multifunctional and multicompartmental protein, based on its ubiquitous expression, its ability to interact with various plasma proteins as well as microbial ligands, and its participation in several ligand-mediated cellular responses [2, 11].

Mature gC1qR is a highly acidic homotrimeric protein that migrates as a single 33-kDa chain under reducing conditions [13]. gC1qR does not have any conventional transmembrane domain in its sequence nor a glycosylphosphatidylinositol (GPI) anchor, suggesting that any role of this molecule in intracellular signaling should need an association with transmembrane proteins such as β_1-integrins [12, 14].

It has been reported that gC1qR is present in human sperm lysates, after a Triton X-114 phase separation, and that its expression increases in human sperm plasma membrane after capacitation [13].

Determining the identity of the molecules involved in sperm-oocyte interaction could lead to breakthroughs that will eventually allow the design of more efficient *in vitro* fertilization protocols in domestic species, especially in bovines. In addition, this could also serve as a basis for predicting sires' reproductive performance.

The objective of this research was therefore to determine gC1qR distribution and expression on plasma membrane in bovine sperm before and after heparin treatment.

MATERIALS AND METHODS

Frozen semen samples

Ejaculates from 5 fertile Holstein and Brahman bulls in regular service were obtained from the artificial insemination center of VIATECA® at Machiques, Zulia State, Venezuela. These bulls produced high quality semen with normal sperm morphology and good fertility. After semen collection by using an artificial vagina, sperm concentration and subjective scores of motility (wave motion) were evaluated. In addition, ejaculates were diluted and used to assess individual sperm motility. Only samples with an initial sperm motility greater than 70-75% were used for freezing.

Ejaculates were diluted with a skim milk-egg yolk (EY) diluent, containing 1% EY. In particular, the base solution (Solution A) containing skim milk (15%), EY (1%) and antibiotics (gentamicin, 50mg/Ltr). Sperm dilution was performed in a two-step procedure, first adding at 30°C the base extender up to two times the final desired sperm concentration and then a second extender (Solution B) at 5°C to achieve a final concentration ~ $30x10^6$ sperm/mL. The sperm samples in solution A were slowly cooled to 5°C. Cooling down to 5°C lasted for about 2 h. The second extender differed from the base diluent in the replacement of water (14%, v/v) with the same volume of glycerol (final concentration = 7%). Then, extended samples were held for equilibration at 5°C for 2 h more. After the equilibration of the diluted sperm samples, the extended sperm was loaded into 0.5 ml plastic straws. Immediately, the straws were frozen in nitrogen vapors, 4 cm above the surface of the liquid nitrogen, for 10 minutes and then plunged into liquid nitrogen.

Frozen semen samples processing

One straw from each sire (5 straws in total) was thawed (submersion in 37°C water for 20 seconds) using the artificial insemination protocol described by Brackett [1]: Once thawed, the contents of 5 straws were mixed to form trial semen stock, to reduce sire-associated variation. All trials described in this and further sections were done in triplicate.

In every trial, thawed semen was centrifuged (using a Hettich EBA 8S centrifuge, Germany.) in a 40/80% Percoll discontinuous density gradient (Sigma Aldrich, MO, USA, P-1644) at 800 *g* for 20 minutes. Sperm pellets at the bottom of the polypropylene tube were resuspended in 1 mL of mDM (modified defined medium) and were again centrifuged at 800 *g* for 5 minutes. Pellets were resuspended in 1 mL of mDM and sperm concentration was adjusted to 110 x 10^6 sperm/mL.

The latter suspension was distributed in four 100-µL aliquots: 2 aliquots were incubated with heparin and constituted the treatment groups in chlortetracycline stain (CTC) and indirect immunofluorescence (IIF) trials; the other two were not incubated with heparin and constituted the respective control groups.

Sperm capacitation

Capacitation of treatment groups was induced by incubating the corresponding aliquots with 100 µL of heparin (Sigma Aldrich, MO, USA, H-9399; 50µg/mL of mDM) for 30 minutes at 37°C in 5% CO_2 in air. To assess the treatment effect on acrosome status and sperm capacitation dynamics, CTC stain was performed to the corresponding treatment group along with its control. To this end, 100 µL of sperm solution were mixed with 100 µL of CTC stain (750 µM CTC, 130 mM NaCl, 5 mM cysteine, and 20 mM Tris-HCl, pH 7.8) and immediately fixed by addition of 16 µL of 12.5% paraformaldehyde in 0.5 M Tris-HCl.

To observe CTC patterns, a 20 µL drop of sperm solution was mixed with a 20 µL drop of glycerol: PBS (2 mM KH_2PO_4, 6 mM Na_2HPO_4, 2 mM KCl, and 136 mM NaCL.) solution (1:1) over a slide, then a cover slip was applied and gently compressed so that spermatozoa were kept in a horizontal position. Observation took place within the hour by means of a fluorescence microscope (Axioskop 20, Zeiss, Germany.) and the number of sperm showing a homogeneous fluorescence all over the head (F pattern: uncapacitated sperm), fluorescence only over the acrosome area (B pattern: capacitated sperm) and of those showing dull or no fluorescence over the head (AR pattern: acrosome reacted sperm) was determined [7].

Complement receptor gC1qR expression and distribution on bovine sperm plasma membrane

Complement receptor gC1qR expression and distribution on bovine sperm plasma membrane were determined by indirect immunofluorescence, using the treatment group (50 µg/ml heparin for 30 minutes, see above) with its corresponding control. The technique used was the following:

Sperm were fixed in 1 mL of 3% paraformaldehyde in PBS, for 24 hours minimum at 4°C. Subsequently, two wash centrifugations with 500 µL of PBS (all centrifugations were at 800 *g* for 10 minutes, supernatants were discarded in each centrifugation). Pellets were resuspended in 500 µL of blocking solution (0.5 g of powdered nonfat milk; 25 µL of Triton X-100; 1mL of heat inactivated rabbit serum; 100 mL of pH 7.2 PBS) and were incubated for 30 minutes at 37°C, sheltered from light. After incubation, a wash centrifugation with 500 µL of PBS was performed. Pellets were resuspended in 1 mL of PBS and divided in two 475-µL aliquots. Then, 25 µL of mAb (60.11, anti-gC1qR) was added to one aliquot, to attain a 1:20 working dilution. 25 µL of PBS was added to the other aliquot (internal control). Both aliquots were incubated for 2 hours and 30 minutes at 37°C, sheltered from light and with occasional shaking. After incubation, the sperm solutions were washed, pellets resuspended in 500 µL of fluoresceine isotiocianate-conjugated goat anti-mouse IgG antibody (Sigma Aldrich, MO, USA, F-4018) in PBS (1:65), and further incubated in the dark (37°C, 1 h). The sperm were then washed, 2x 500 µL and the pellet resuspended in 500 µL of PBS.

One (20-µL) drop of glycerine-PBS (1:1) was mixed with another 20 µL drop of sperm solution on a slide; then, a cover slip and even pressure were applied so that spermatozoa were kept in a flat position. Fluorescence patterns and intensity were examined on the same day using the fluorescence microscope cited before. Triple observations were performed for each aliquot. Results were expressed as the cellular location in which fluorescence was observed for each experimental group: acrosome (A), equatorial band (E), postacrosome (P) and all over the head (H); and as a three-category ordinal scale for fluorescence intensity (low, medium and high intensity).

Statistical analysis

Data collected in every trial with the exception of fluorescence intensity determination in IIF trials, were nominal variables and due to that fact, they were analysed through a χ^2 test; by contrast, fluorescence intensity data were ordinal variables, and owing to that fact and data distribution, they were analysed through a Fisher's exact test. In every trial, statistically significant differences were those with a P value of less than 0.05. Statistical analysis was performed using SAS program® (SAS/STAC Software: SAS Inst. Inc.; Carry, NC. USA. 1996).

RESULTS AND DISCUSSION

All results mentioned henceforth constitute the sum of the results obtained from each aliquot (and their replicates, if any) by experimental group, since there were no intra-group statistically significant differences (P>0.05).

Sperm capacitation

Data obtained in CTC stain are displayed in TABLE I.

In CTC stain, there were statistically significant differences (P<0.05) between the experimental group incubated with heparin (50 µg/mL mDM), and the control group. Differences were found in the uncapacitated and capacitated sperm categories. In control group, the highest frequency was found in the uncapacitated sperm category (177/300 sperm), whereas in treatment group, the highest frequency was observed in the capacitated sperm category (163/300 sperm). In the acrosome reacted sperm category, there were no statistically significant differences between the experimental groups (P>0.05) (TABLE I).

Heparin is considered to be one of the best in vitro capacitating agents due to its potency, the difficulty of inducing the acrosome reaction even with high doses, and the fact that

TABLE I
CHLORTETRACYCLINE PATTERNS/
PATRONES DE CLORTETRACICLINA

	n	Uncapacitated sperm (F pattern)	Capacitated sperm (B pattern)	Acrosome reacted sperm (AR pattern)
without heparin	300	177[a]	98[a]	25
with heparin*	300	105[b]	163[b]	32

Values with different superscripts differ with a statistical significance of P<0.05 within the same category. * Heparin 50 µg/mL mDM.

it is naturally present in the female genital tract. Incubation in heparin 50 mg/mL mDM for 30 minutes was sufficient to induce the capacitation of cryopreserved bovine sperm (TABLE I). However, it was found that 32.6% of the cryopreserved sperm were already in the capacitated state. This probably is due to the freezing-thawing process, which has been shown to induce the capacitation of a sperm subpopulation [3, 7, 17] as well as other structural alterations [3, 18]. The data obtained in this study correlate well with those obtained by Cormier and Bailey [3], in that 35% of the sperm are capacitated before a heparin incubation and 50% thereafter.

Complement receptor gC1qR distribution on bovine sperm plasma membrane

Distribution data obtained in IIF trials are shown in TABLE II.

In Indirect Immunofluorescence (IIF) trials, there were statistically significant differences (P<0.05) between treatment and control groups in every fluorescence distribution category, except in equatorial region category. In control group, the decreasing order of categories according to their frequency was: H > A > P > E; whereas in treatment group, it was: A > H > P > E. Frequencies in treatment group were statistically higher (P<0.05) than those of control group in the following categories: acrosomal region (175/300 sperm vs. 109/300) and postacrosomal region (37/300 sperm vs. 19/300); by contrast, control group had a higher frequency in all-over-the-head category (172/300 sperm vs. 84/300) (TABLE II, FIG. 1-3).

A tendency of gC1qR to redistribute after capacitation was observed: it concentrated in the acrosome region on the sperm plasma membrane, similar to what has been reported in humans [13]. This distribution is frequently observed in molecules involved in ZP recognition during acrosome reaction, such as PH20 [16] and in proteins related to acrosome exocytosis bearing phosphorylated tyrosine residues [3]. By contrast, other molecules migrate to postacrosome region, where they could participate in sperm-oocyte fusion, such as FA-1 in bovines and humans [5] and DE in rats [15]. Alternatively, they could migrate to the inner acrosome membrane and be lost after acrosome reaction, such as CD46, CD55 and CD59 [6].

TABLE II
gC1qR DISTRIBUTION ON BOVINE SPERM PLASMA MEMBRANE/DISTRIBUCIÓN DEL gC1qR EN LA MEMBRANA PLASMÁTICA DE LOS ESPERMATOZOIDES BOVINOS

	n	Acrosome (A)	Equatorial (E)	Postacrosome (P)	All over the head (H)
Without heparin	300	109[a]	0	19[a]	172[a]
With heparin*	300	175[b]	4	37[b]	84[b]

Values with different superscripts differ with a statistical significance of P<0.05 within the same category. * Heparin 50 µg/mL mDM.

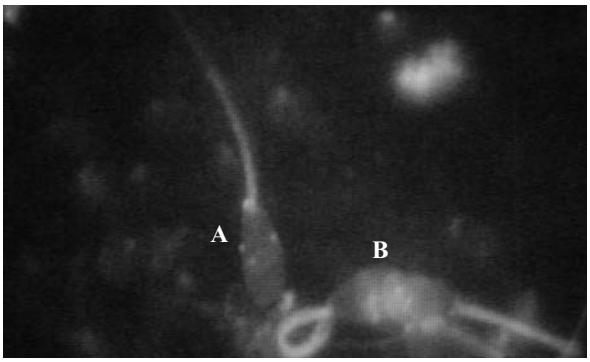

FIGURE 1. COMPLEMENT RECEPTOR gC1qR DISTRIBUTION AND EXPRESSION ON BOVINE SPERM PLASMA MEMBRANE. INDIRECT IMMUNOFLUORESCENCE. EXPERIMENTAL GROUP INCUBATED WITH HEPARIN (50µg/mL OF mDM) A: HOMOGENEOUS FLUORESCENCE DISTRIBUTION ALL OVER THE HEAD. B: FLUORESCENCE DISTRIBUTION OVER THE ACROSOME REGION. FLUORESCENCE INTENSITY ASSESSED AS HIGH (X1000) / DISTRIBUCIÓN Y EXPRESIÓN DEL RECEPTOR DE COMPLEMENTO gC1qR EN LA MEMBRANA PLASMÁTICA DE ESPERMATOZOIDES BOVINOS. INMUNOFLUORESCENCIA INDIRECTA. GRUPO EXPERIMENTAL INCUBADO CON HEPARINA (50µg/mL DE mDM) A: DISTRIBUCIÓN HOMOGÉNEA DE LA FLUORESCENCIA SOBRE TODA LA CABEZA. B: DISTRIBUCIÓN DE LA FLUORESCENCIA EN LA REGIÓN ACROSOMAL. INTENSIDAD DE LA FLUORESCENCIA VALORADA COMO ALTA (X 1000).

Complement receptor gC1qR expression on bovine sperm plasma membrane

In order to obtain the fluorescence intensity data, each replicate specimen (3 per aliquot) was assigned a fluorescence intensity category (low, medium, high) predominant during observation (see FIG. 1-3 for intensity standards). The frequency distribution for fluorescence intensity is shown in TABLE III.

Statistically significant differences (P<0.05) between control and treatment groups in low and high fluorescence intensity categories were observed. Also, in treatment group, frequencies spanned medium-high fluorescence intensity categories, whereas in control group, frequencies spanned low-medium fluorescence intensity categories. There were only

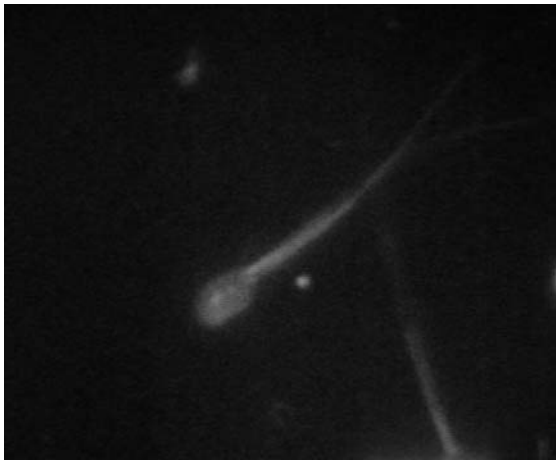

FIGURE 2. COMPLEMENT RECEPTOR gC1qR DISTRIBUTION AND EXPRESSION ON BOVINE SPERM PLASMA MEMBRANE. INDIRECT IMMUNOFLUORESCENCE. EXPERIMENTAL GROUP THAT WAS NOT EXPOSED TO HEPARIN. HOMOGENEOUS FLUORESCENCE DISTRIBUTION ALL OVER THE HEAD. FLUORESCENCE INTENSITY ASSESSED AS MEDIUM (X1000) / DISTRIBUCIÓN Y EXPRESIÓN DEL RECEPTOR DE COMPLEMENTO gC1qR EN LA MEMBRANA PLASMÁTICA DE ESPERMATOZOIDES BOVINOS. INMUNOFLUORESCENCIA INDIRECTA. GRUPO EXPERIMENTAL NO INCUBADO CON HEPARINA. DISTRIBUCIÓN HOMOGÉNEA DE LA FLUORESCENCIA SOBRE TODA LA CABEZA. INTENSIDAD DE LA FLUORESCENCIA VALORADA COMO MEDIA (X 1000).

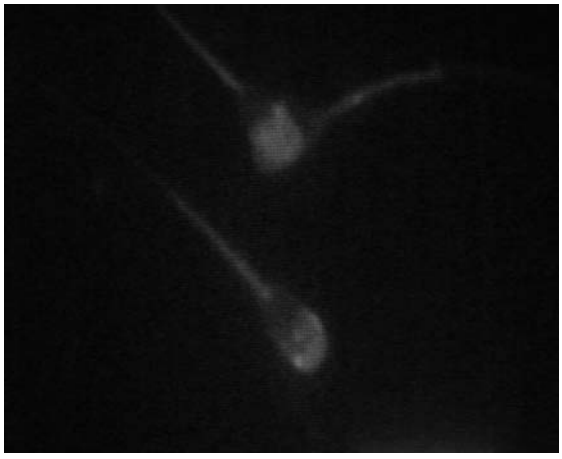

FIGURE 3. COMPLEMENT RECEPTOR gC1qR DISTRIBUTION AND EXPRESSION ON BOVINE SPERM PLASMA MEMBRANE. INDIRECT INMMUNOFLUORESCENCE. EXPERIMENTAL GROUP THAT WAS NOT INCUBATED WITH HEPARIN. FLUORESCENCE DISTRIBUTION MAINLY OVER THE ACROSOME REGION. FLUORESCENCE INTENSITY ASSESSED AS LOW (X1000) / DISTRIBUCIÓN Y EXPRESIÓN DEL RECEPTOR DE COMPLEMENTO gC1qR EN LA MEMBRANA PLASMÁTICA DE ESPERMATOZOIDES BOVINOS. INMUNOFLUORESCENCIA INDIRECTA. GRUPO EXPERIMENTAL NO INCUBADO CON HEPARINA. DISTRIBUCIÓN DE LA FLUORESCENCIA EN LA REGIÓN ACROSOMAL. INTENSIDAD DE LA FLUORESCENCIA VALORADA COMO BAJA (X 1000).

TABLE III

gC1qR EXPRESSION ON BOVINE SPERM PLASMA MEMBRANE/EXPRESIÓN DEL gC1qR EN LA MEMBRANA PLASMÁTICA DE LOS ESPERMATOZOIDES BOVINOS

	Low	Medium	High
without heparin	6[a]	3	0[a]
with heparin*	0[b]	5	4[b]

Results expressed over 9 assessed specimens (3 per aliquot). Values with different superscripts differ with a statistical significance of $P<0.05$ within the same category. * Heparin 50 µg/mL mDM.

treatment group specimens whose fluorescence intensity was assessed as high (4/9), likewise, there were only control group specimens with a low fluorescence intensity (6/9) (TABLE III).

The increased fluorescence intensity after heparin-induced capacitation (TABLE III) indicates that there is a capacitation-related augmentation of gC1qR on the sperm plasma membrane, due to either an increase in its expression [13, 16] or a greater antibody accessibility to the molecule [4], this behaviour is similar to that reported in humans [13].

Finally, it is important to mention that in humans it has been reported that anti-gC1qR antibodies partially inhibit sperm penetration of ZP-free hamster oocytes [8]. The possibility therefore exists for the participation of gC1qR in sperm-oocyte interaction to be similar to that reported in humans. Other functional possibilities include: (i) protective function (as do other complement molecules present in sperm) [6, 13, 19, 21] or (ii) that its function includes both, as some defensins from the epididimis in rats and primates [26].

CONCLUSION

Complement receptor gC1qR is a molecule present on bovine sperm that shows a tendency to redistribute after capacitation, migrating to the acrosome region of the sperm plasma membrane. Moreover, either gC1qR expression or its accessibility to antibodies increases after capacitation. This suggests that gC1qR could have a role in primary sperm-oocyte interaction in bovines.

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