FETUIN: A TOOL TO STUDY THE BLOCK TO POLYSPERMY[1]

FETUINA: UM INSTRUMENTO PARA ESTUDAR O BLOQUEIO DA POLISPERMIA

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SUMMARY

*The effect of fetuin, an α_1-glycoprotein that has protease inhibitor activity, on the block to polyspermy was determined. Cumulus-oocyte complexes from eCG primed mice were matured in vitro in the presence of 0, 0.01, 0.1, 1 and 10mg/ml of fetuin in modified TCM 199. Both **in vivo** and **in vitro** matured oocytes were fertilized in the presence of fetuin and incubated for 6 and 24h. Fetuin present in a concentration of 1mg/ml in the fertilization but not in the maturation medium was able to induce polyspermy in 52.4% of the eggs. There was a positive relationship between concentration of fetuin in the fertilization medium and the proportion of polyspermic eggs ($p<0.05$). A significant interaction between 0.1 and 1mg/ml of fetuin during maturation and fertilization was observed ($p<0.05$). The results of these experiments demonstrate the inhibition of the block to polyspermy using a protease inhibitor during the fertilization.*

Key words: *murine, block to polyspermy, fetuin, supernumerary sperm*

RESUMO

*No presente experimento, foi determinado o efeito da fetuina, uma α_1-glicoproteina com capacidade de inibir protease, no bloqueio da polispermia. Complexos cumulus-oócitos de murídeos superovulados com eCG foram maturados **in vitro** na presença de 0, 0,01, 0,1, 1 e 10mg/ml de fetuina em TCM 199 modificado. Oócitos maturados **in vivo e in vitro** foram fecundados na presença de fetuina e incubados por 6 e 24 horas. Fetuina presente em uma concentração de 1mg/ml durante a fecundação, mas não no meio de maturação, foi capaz de induzir polispermia em 52,5% dos óvulos. Houve uma relação positiva entre a concentração de fetuina no meio de fecundação e a percentagem de óvulos polispérmicos ($p<0,05$). Uma interação significativa entre 0,1 e 1,0mg/ml de fetuina durante a maturação e fecundação foi observada ($p<0,05$). Os resultados destes experimentos demonstram a inibição do bloqueio da polispermia utilizando um inibidor de protease durante a fecundação.*

Palavras-chave: *murino, bloqueio da polispermia, fetuina, espermatozóide supernumerário.*

INTRODUCTION

A complex sequence of events is required to obtain successful fertilization and embryonic development. To prevent the penetration of more than one sperm, the egg undergoes changes at the level of the zona pellucida (zona reaction) and vitelline membrane (vitelline block). The zona reaction is believed to be mediated mainly by a protease and glycosidase from the cortical granules (WASSARMAN, 1987; YANAGIMACHI, 1994). These substances from the cortical granules are released into the perivitelline space after sperm attachment to the vitellus. The contents of the cortical granules seem to cause hydrolysis of the glycoproteins

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of the zona pellucida. The changes of these glycoproteins result in termination of the sperm receptors, termination of the ability to induce an acrosome reaction and a block to the sperm binding (BLEIL and WASSARMAN, 1980; DUCIBELLA *et al.*, 1990).

A precocious block to sperm penetration is observed when mouse oocytes are cultured ***in vitro*** without serum (DE FELICI and SIRACUSA, 1982). Fetuin was identified as the compound present in serum that is able to prevent hardening of the zona pellucida (SCHROEDER ***et al.***, 1990). If fetuin inhibits the exocitosis of the cortical granules during fertilization, this α_1-glycoprotein can be used to study the mechanisms of the block to polyspermy. For this reason, the present work investigates the effect of fetuin on the inhibition of the block to polyspermy during oocyte maturation and/or fertilization.

MATERIALS AND METHODS

Cumulus-oocyte complexes were isolated from 22-to 34-day-old ICR mice. The mice received intra-abdominally 5IU of equine chorionic gonadotropin (eCG) a 44 to 52 hours prior to collecting the oocytes to be matured ***in vitro*** or prior to injecting 5IU of human chorionic gonadotropin (hCG) when collecting oocytes matured ***in vivo***. For ***in vitro*** maturation, the oocytes were isolated from the follicles. The ovaries were removed from 5 mice for each replication and placed into 8ml of modified TCM 199 medium containing 1mg/ml of fetuin covered by 4ml of silicon oil in a tissue culture dish. The TCM 199 with glutamine was modified by adding sodium pyruvate (0.2mM) and 100 units of penicillin and 50µg of streptomycin/ml of medium. After isolation, the cumulus-oocyte complexes were divided randomly into different groups, washed 2 times in 200µl and transferred to 200µl of maturation medium. The oocytes were matured in modified TCM 199 in which fetuin was added at levels from 0.0 to 10.0mg/ml in according to the experimental design. The incubation period was 15.5 hours and the oocytes were maintained under a temperature of 37°C a humidity of 100% and an atmosphere of 5%CO_2 in air. To obtain ***in vivo*** matured oocytes, the females were euthanized by cervical dislocation 14 to 16 hours after injection of hCG and the oocytes matured ***in vivo*** were released by puncturing the ampulla of the oviduct with two sterile 30 G½ needles. Cumulus cell-oocyte complexes matured ***in vivo*** or ***in vitro*** were fertilized ***in vitro*** using a method previously described by HOPPE and PITTS (1973).

To determine the frequency of polyspermy, the eggs were stained with aceto orcein (0.75% of orcein in 45% of glacial acetic acid in PBS; DONAHUE, 1968) after the incubation period. The number of sperm heads decondensed in the ooplasm or the number of pronuclei was observed under phase microscopy (40-2000x).

The study of the inhibition of the block to polyspermy using fetuin during oocyte maturation was designed as a randomized complete block. Five treatment groups were investigated simultaneously in 3 to 5 replications. Each time that the oocytes in each treatment were matured and fertilized simultaneously was treated as one replication or block. Oocytes were matured in the presence of 0.01, 0.1, 1 and 10mg/ml of fetuin. For the control group, the oocytes were matured ***in vivo***. After 6 hours of incubation with sperm, the eggs were washed and either fixed in 10% formalin for staining or cultured in M-16 for an additional 18 hours to determine the embryo development to the two cell stage. The percentage of embryos that cleaved to two cells were calculated based on the total number of penetrated eggs.

The inhibition of the physiological barrier to polyspermy was also verified using fetuin during the fertilization period. Oocytes matured ***in vivo*** were randomly divided into five different treatments. The only difference among treatments was the level of fetuin in the fertilization medium. The fetuin concentrations examined were 0, 0.01, 0.1, 1 and 10mg/ml. The eggs were incubated with sperm for 6 hours. Following incubation with sperm, the eggs were washed and fixed in 10% formalin for staining or cultured in M-16 for a total of 24 hours. These two experiments (6 or 24 hours incubation period) with five treatments were replicated four different times.

Two experiments designed by randomized complete block with three crossed factors were conducted to investigate the degree of inhibition of the block to polyspermy using fetuin during oocyte maturation and fertilization. The factors studied were block, concentration of fetuin during maturation and concentration of fetuin during fertilization. In one experiment, the eggs were washed and fixed for staining after 6 hours of incubation with sperm. In the other experiment, the eggs were washed and cultured in M-16 for a total 24 hours after the same period of incubation with sperm. In both experiments, oocytes were matured and fertilized in 0.1 and 1mg/ml of fetuin.

The percentage of penetration, polyspermy and embryo development to the two cell stage was transformed by PROC RANK (CONOVER and IMAN, 1981) in the SAS statistical program to use parametric tests. The relationship between independent (concentrations of fetuin or time of egg incubation) and dependent variables (percentage of eggs penetrated, polyspermic or developing to the two cell stage) was tested using a polynomial regression.

RESULTS

The optimal level of fetuin during oocyte maturation was 1mg/ml (Table 1). However, the percentage of polyspermic eggs increased while the number of embryos that reached two cells decreased when the concentration of fetuin was 10mg/ml in the course of oocyte maturation ($p<0.05$ in relation to the control group). Most of the penetrated eggs matured in the presence of high levels of fetuin had more than one sperm head decondensed in the ooplasm or more than one male pronuclei.

Using a protease inhibitor (fetuin) during fertilization, the percentage of polyspermic eggs increased in a dose dependent manner (Table 2).

Table 2 - Effect of protease activity inhibition during the fertilization period on sperm penetration, polyspermy and first cleavage with oocytes matured **in vivo** and fertilized in different concentrations of fetuin..

Fetuin (mg/ml)	Egg Cultured for 6h			Egg Cultured for 24h			
	Total No.	Penet.[1] (%)	Polysp.[2] (%)	Total No.	Penet. (%)	Polysp (%)	Cleavage. (%)
0.00	130	90.7	15.2[a]	108	76.8	9.6[a]	84.3[a]
0.01	71	88.7	17.5[a]	74	81.1	1.7[a]	96.7[a]
0.10	80	82.5	12.1[a]	82	86.6	8.4[a]	88.7[a]
1.00	116	90.5	52.4[b]	66	81.8	24.1[a]	74.1[a]
10.00	117	82.0	56.2[b]	49	83.7	82.9[b]	0.0[b]

[1]Percentage of eggs penetrated by one or more sperm.
[2]Percentage of eggs with more than one sperm in the ooplasm or more than one male pronuclei.
[ab]Statistic differences between treatment and control (0mg/ml fetuin concentration) groups are indicated in each column with different lower case letters ($p<0.05$).

A positive linear relationship was observed between concentration of fetuin and percentage of polyspermic eggs at 6 hours ($F=19.69$; $p=0.0004$) and 24 hours ($F=18.72$; $p=0.0005$) of culture. At the end of the 6-hour egg-sperm incubation period, many sperm were still bound to the zona pellucida. At concentration of 1mg/ml of fetuin during fertilization, the percentage of polyspermic eggs decreased to levels statistically similar to the control group (***in vivo*** matured) when they were cultured for 24 hours. However, the number of polyspermic eggs was constantly high following 6 and 24 hours of culture and cleavage to two cells when fetuin was present in a concentration of 10mg/ml.

The effect of enzymatic inhibition during both oocyte maturation and fertilization is demonstrated in Table 3. In a dose dependent manner, fetuin inhibited the block to polyspermy only when present during fertilization. Significant numbers of polyspermic eggs were observed only when the concentration of 1mg/ml of fetuin was used during fertilization. The level of 0.1mg/ml of fetuin was not enough to inhibit the physiological barrier to polyspermy even when higher concentrations of protease inhibitor were used during oocyte maturation.

Table 1 - Effect of the inhibition of proteases using fetuin during oocyte maturation on the subsequent physiological block to polyspermy.

Fetuin (mg/ml)	Egg Cultured for 6h			Egg Cultured for 24h			
	Total No.	Penet.[2] (%)	Polysp.[3] (%)	Total No.	Penet. (%)	Polysp (%)	Cleavage. (%)
In vivo[1]	122	92.6[a]	7.1[a]	69	97.1[a]	7.5[a]	89.5[a]
0.01	81	38.3[b]	3.2[a]	65	36.9[b]	4.2[a]	66.7[a]
0.10	86	59.3[b]	15.7[a]	72	58.3[b]	19.1[a]	61.9[a]
1.00	80	77.5[a]	9.7[a]	69	65.2[b]	8.9[a]	84.4[a]
10.00	73	42.5[b]	45.2[b]	51	74.5[a]	44.7[b]	7.9[b]

[1]Oocytes were matured **in vivo** and fertilized **in vitro** in a fetuin-free medium.
[2]Percentage of eggs penetrated by one or more sperm.
[3]Percentage of eggs with more than one sperm in the ooplasm or more than one male pronuclei.
[ab]Statistic differences between treatment and control (**in vivo**) groups are indicated in each column with different lower case letters ($p<0.05$).

Table 3 - Effect of protease inhibition on the percentaghe of eggs penetrated, polyspermic and/or developed to two cells using fetuin in the course of maturation and fertilization **in vitro**.

Fetuin (mg/ml)	Egg Cultured for 6h			Egg Cultured for 24h			
	Total No.	Penet.[1] (%)	Polysp.[2] (%)	Total No.	Penet. (%)	Polysp. (%)	Devel. (%)
1M[3]/1F[4]	37	83.8[a]	32.3[a]	63	57.1	30.5[a]	58.3[ab]
0.1M/0.1F	38	57.9[b]	4.5[b]	64	43.7	7.1[b]	64.3[ab]
0.1M/1F	35	82.9[a]	51.7[a]	66	48.5	18.7[a]	46.9[a]
1M/0.1F	37	59.5[b]	13.6[b]	63	49.2	3.2[b]	74.2[b]

[1]Percentage of eggs penetrated by one or more sperm.
[2]Percentage of eggs with more than one sperm in the ooplasm or more than one male pronuclei.
[3]M means that the oocytes were matured in that specific concentration of fetuin.
[4]F means that the eggs were fertilized in that specific concentration of fetuin.
[ab]Statistic differences among treatments are indicated in each column with different lower case letters ($p<0.05$).

DISCUSSION

Results of the present study indicate that a serine protease inhibitor (fetuin) in the fertilization medium is able to inhibit the physiological barrier to polyspermy in a considerable number of mouse eggs. There is evidence that the release of protease and glycosidase by cortical granules during exocitosis after sperm penetration catalyze the conversion of ZP2 and ZP3 to $ZP2_f$ and $ZP3_f$ respectively, causing hardening of the zona pellucida and inactivation of sperm receptors (BLEIL ***et al.***, 1981; WASSARMAN, 1987; WASSARMAN, 1990). Therefore, the presence of fetuin in the fertilization medium seems to inhibit temporarily the so called zona reaction or secondary block to polyspermy.

In the course of mouse oocyte maturation, cortical granules appear also to release proteases resulting in a premature block to polyspermy (zona pellucida hardening) when oocytes are matured ***in vitro*** in the absence of serum or fetuin (DUCIBELLA ***et al.***, 1990; SCHROEDER ***et al.***, 1990). In the present experiment, fetuin was able to allow penetration in a dose dependent manner when used in the maturation medium. The ideal concentration of fetuin to obtain penetration of the zona pellucida by the sperm, considering the range and the levels tested in this work, was 1mg/ml of fetuin. This concentration of fetuin is identical with that observed by SCHROEDER ***et al.*** (1990) to impede the hardening of the zona pellucida. In the study performed by SCHROEDER ***et al.*** (1990), the first cleavage was observed in a few eggs when fetuin was in a concentration of 10mg/ml in the maturation medium, which led them to infer that at this level fetuin causes a toxic effect. These results are not inconsistent with the data observed in the present study. However, the percentage of eggs penetrated in the 10mg/ml of fetuin groups was similar to the **in vivo** control group when eggs were cultured for 24 hours in the current work. At the level under consideration, polyspermy was the most important abnormality noticed. Therefore, there are at least two possibilities for the significant level of polyspermy when high concentrations of fetuin is in the oocyte maturation medium. On the one hand, a toxic effect could cause a total disorder in the ooplasm resulting in an inability of the cortical granules to respond to the sperm penetration. On the other hand, a residual effect of fetuin cannot be discarded to affect the block to polyspermy.

The results of many studies involving fetuin usage during oocyte maturation and fertilization could be affected if fetuin has a residual effect in inhibiting the block to polyspermy. In this regard, a factorial experiment was designed to test the interaction between different concentrations of fetuin during maturation and fertilization. If fetuin has a residual effect in the protease activity of the cortical granules during maturation, lower levels of fetuin would be required during fertilization to inhibit the barrier to polyspermy. This was not the case in the situation in which these experiments were performed. A significant interaction was detected when oocytes were matured and fertilized in the presence of 0.1 and 1mg/ml of fetuin. The percentage of polyspermic eggs was significantly high only when 1mg/ml of fetuin was used in the fertilization medium. Also, the concentration of fetuin during maturation did not predispose the oocytes to be more sensitive to low levels of fetuin at the concentrations tested in this study.

In 1974, GALEMBECK and CANN reported that fetuin is a trypsin-like proteases inhibitor, which maintains its activity upon desialicization similar to human α_1-antitrypsin. Therefore, it is expected that fetuin would be able to inhibit the block to polyspermy considering that

proteases play an important role in the physiological barrier to polyspermy (BLEIL *et al.*, 1981; WASSARMAN, 1987; WASSARMAN, 1990). This expected result was observed in the present work when fetuin was present in the fertilization medium. The percentage of polyspermy increased in a positive linear relationship with the fetuin concentration.

The results of the present study demonstrate that fetuin inhibits the exocitosis of the cortical granules during fertilization. Therefore, this glycoprotein is a powerful tool to study the mechanisms of the block to polyspermy, enabling to use zona intact eggs.

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