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Effect of Bovine Seminal Ribonuclease and Bovine Pancreatic Ribonuclease A on Bovine Oocyte Maturation

TOMÁŠ SLAVÍK,[1]* JOSEF MATOUŠEK,[1] JOSEF FULKA,[1] AND RONALD T. RAINES[2]
[1]*Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Czech Republic*
[2]*Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706-1569*

ABSTRACT Bovine seminal ribonuclease (BS-RNase) contains the M×M (noncovalent dimer) and M=M (free monomer) in constant ratio. The aim of this work was to evaluate the effect of BS-RNase, its monomer and dimer forms, and also various mutants of this enzyme on meiotic completion in cattle oocytes. It was found that BS-RNase has irreversible effects on the meiotic maturation of bovine oocytes in vitro, particularly on the completion of meiosis. The effect of BS-RNase is dose-dependent. In medium supplemented with 1 μg/ml, the results were comparable with those of the control (70% MII oocytes after 24 hr of culture). Whereas 5 μg/ml reduced the number of MII oocytes to 50%, 10 and 25 μg/ml arrested this process completely. The M×M form and RNase A at 5 μg/ml inhibited the maturation rate by 71 and 48%, respectively, but a less significant effect was observed for the M=M form, or the carboxymethylated monomers MCM31 and MCM32 (21%, 16%, and 42% MII oocytes, respectively, in comparison with control). These data demonstrate that bovine ribonucleases can have variable detrimental effects on the maturation of bovine oocyte. *J. Exp. Zool. 287:394–399, 2000.*

Bovids have high levels of two homologous secretory ribonucleases (EC 3.1.27.5): bovine seminal ribonuclease (BS-RNase) and bovine pancreatic ribonuclease A (RNase A). These enzymes can have significant effects on animal physiology. For example, the aspermatogenic activity of BS-RNase has been demonstrated in males from several species (Matoušek et al., '73; Dostál and Matoušek, '73; Leone et al., '73; Matoušek et al., '78; Hlinak et al., '81; Matoušek, '94). Preliminary investigations of the embryotoxic effect of BS-RNase have been carried out in female mice, guinea pigs, rabbits, and pigs (Matoušek and Grozdanovič '73; Matoušek et al., '73a,b). Here we continue this analysis and report on the effects of wild-type and mutant forms of BS-RNase and RNase A on bovine oocyte maturation.

BS-RNase has been prepared from a synthetic gene in *Escherichia coli* (Kim and Raines, '93). This preparation has the same antiembryonic activity as that of BS-RNase isolated from seminal plasma (Kim et al., '95a). In addition, mutant forms of the recombinant enzyme are embryotoxic (Kim et al., '95a). The cytotoxicity of BS-RNase is related to its quaternary structure. BS-RNase is isolated as a dimer that is cross-linked by two disulfide bonds. Monomeric BS-RNase and bovine pancreatic ribonuclease A (RNase A), which is a monomer, are not cytotoxic (Vescia et al., '80; Tamburini et al., '90; Kim et al., '95a). In contrast, artificially dimerized RNase A is cytotoxic but to a lesser extent than is BS-RNase (Bartholeyns and Baudhuin, '76; Bartholeyns and Zenebergh, '79; Vescia et al., '80; Di Donato et al., '94).

At equilibrium, dimeric BS-RNase is a mixture of two distinct quaternary forms, M=M and M×M (Piccoli et al., '92). The conversion of M=M to M×M entails the exchange of N-terminal helices between subunits, as occurs during the artificial dimerization of RNase A. We and others have demonstrated that it is the M×M form that is respon-

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*Correspondence to: T. Slavík, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, 277 21 Liběchov, Czech Republic. E-mail: slavik@iapg.cas.cz

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sible for the cytotoxicity of BS-RNase (Cafaro et al., '95; Kim et al., '95a). We have also demonstrated that the ribonucleolytic activity of BS-RNase is necessary for its cytotoxic activity (Kim et al., '95a).

Thirty years ago, Roth ('67) discovered that mammalian cells contain ribonuclease inhibitor (RI), a protein that binds tightly to monomeric but not dimeric BS-RNase (Murthy and Sirdeshmukh, '92; Kim et al., '95b; Murthy et al., '96). RI appears to act as a sentry that protects cellular RNA from invading secretory ribonucleases (Blackburn and Moore '82; Hofsteenge, '94). A key to the cytotoxicity of BS-RNase is likely to be its ability to evade cellular RI (Cafaro et al., '95; Di Donato et al., '95; Kim et al., '95b,c).

Monomers of RNase A, like those of BS-RNase, are bound tightly by RI and are not cytotoxic. Onconase, which is a monomeric ribonuclease isolated from the eggs of the Northern leopard frog (*Rana pipiens*) resists RI and is cytotoxic (Ardelt et al., '91; Wu et al., '93; Boix et al., '96). X-ray diffraction analysis revealed that extensive contacts between RNase A and RI occur in the region near Asp31 of RI and Lys31 of RNase A (Kobe and Deisenhofer, '95). This contact appears to contribute to the stability of the RI·RNase A complex. Here we tested the effect of MCM31 and MCM32, which are monomers with a carboxymethyl group (–CH_2CO_2–) on Cys31 or Cys32 (Matoušek et al., '96), and other ribonucleases on bovine oocyte maturation in vitro.

MATERIALS AND METHODS

Preparation of ribonucleases

Wild-type BS-RNase was isolated from seminal vesicle fluid by precipitation with acetic acid and ammonium sulfate and subjected to chromatography on columns of CM Sephadex C-50 and Sephadex G-100 (Dostál and Matoušek, '73). The purity of BS-RNase was assessed by peak homogeneity during chromatography; by disc electrophoresis in an acrylamide gel; by starch-gel electrophoresis; by immunoelectrophoresis in an agar gel; and by ultracentrifugation (Dostál and Matoušek, '73). Recombinant BS-RNase was produced in *E. coli* as described (Kim and Raines, '93).

Two distinct quaternary forms of BS-RNase dimer, designated as M×M and M=M (Piccoli et al., '92), were purified from bull seminal plasma as described (Tamburrini et al., '86). Briefly, the inter-subunit disulfide bonds of the purified enzyme were reduced with a 10-fold molar excess of reduced dithiothreitol, and the resulting protein was subjected to gel filtration chromatography to separate monomer from the noncovalent dimer. The M=M form was prepared by air oxidation of monomer and purified by gel filtration chromatography. Similarly, the M×M form was prepared by air oxidation of noncovalent dimer and purified by gel filtration chromatography. This method of purification yielded M=M and M×M that were >90% free of the other form, as judged by selective reduction followed by gel filtration chromatography (Kim et al., '95a).

Oligonucleotide-mediated site-directed mutagenesis was used to change the TGT codon of Cys31 or Cys32 to the TCT codon of serine as described (Kim et al., '95a). The resulting plasmids, pLSR31 and pLSR32, were used to produce C31S BS-RNase and C32S BS-RNase, respectively. MCM31 and MCM32 BS-RNase monomers were prepared from C32S and C31S BS-RNase as described (Matoušek et al., '96).

Assay of oocyte maturation in vitro

Cattle oocytes were obtained from ovaries of normally cycling animals after slaughter. The ovaries were transported at about 20°C to the laboratory within 2 hr, and once there they were rinsed briefly with 95% (v/v) ethanol and repeatedly with saline solution containing antibiotics (streptomycin and penicillin). Ovaries were cut and scarified under phosphate-buffered saline containing heparin (100 IU/ml) and isolated under a stereomicroscope. Oocytes were evaluated, and only those with a compact cumulus were used for culture under an atmosphere of N_2 (85% v/v), O_2 (10% v/v), and CO_2 (5% v/v) at 38.5°C for 24–26 hr (Kubelka et al., '88). The composition of culture medium was the same as described by Pavlok et al. ('88). This medium contained 80 ml of TC 199 (Sevac, Prague), 39 ml of 7.5% (w/v) $NaHCO_3$ (Sevac, Prague), 1,500 mg of HEPES (acid form; Serva, Heidelberg), 600 mg of lactic acid (calcium salt; Serva, Heidelberg), 200 mg of pyruvic acid (sodium salt; Serva, Heidelberg), 25 IU/ml penicillin (potassium salt), and 25 IU of streptomycin sulfate, and was made up to 1.00 l with Nanopure-filtered water. Before it was used for culture, the medium was supplemented with bovine serum (BOS; 15% v/v), FSH (0.2 IU/ml; Calbiochem, La Jolla, CA) and with one of selected ribonucleases in the concentration 0, 1, 5, 10, or 25 μg/ml.

To test the reversibility of a ribonuclease effect, the ribonuclease-containing medium was replaced

after 24 hr of culture with ribonuclease-free medium. The oocytes were then cultured for an additional 24 hr (Kubelka et al., '88).

At the beginning and at the end of a culture period, some of the oocytes were removed for nuclear evaluation. After fixation in acetalcohol overnight, the oocytes were stained with acetoorcein (2% w/v) and examined by phase-contrast microscopy. Between 14 and 52 oocytes were examined for each type of maturation medium.

The proportion of MII oocytes with extruded polar bodies was used as a unique criterion of maturation. The suitability of this procedure had been demonstrated previously in experiments in which oocytes matured under the identical culture protocol described above resulted in healthy progeny after in vitro fertilization and transfer to foster mothers (Pavlok et al., '88; Slavík and Fulka, '91; Slavík et al., '92).

Fisher's exact test was used for statistical evaluation.

RESULTS

Oocyte maturation in vitro

Maturation rates of oocytes in the each experimental group were compared with meiotic progression to MII in controls. The effect of wild-type BS-RNase was dose dependent (Fig. 1). Oocyte maturation was not affected significantly by 1 μg of BS-RNase per ml. Rather, maturation was comparable with that of control oocytes (70% MII oocytes after 24 hr of culture), and no degeneration was observed under a phase-contrast microscope. In contrast, 5 μg/ml reduced the maturation rate by approximately 1/3 in comparison with controls (70% vs. 43%); 10 and 25 μg/ml arrested this process completely. Moreover, at higher concentrations of BS-RNase, remarkable degenerative changes in the cytoplasm and an atypical configuration of chromatin were observed. In these two groups about half of oocytes had been ruptured during routine fixation, most likely due to disintegration of cytoskeletal structures, and thus they were included in the category of degenerated oocytes. (Fig. 1).

The effect of BS-RNase on oocyte maturation was not reversible. No resumption of the maturation process was observed after replacing the medium containing 5 μg of BS-RNase per ml with ribonuclease-free medium (Fig. 2).

Marked differences were found among the mutant ribonuclease tested. In medium supplemented with BS-RNase, its M×M form, and RNase A at 5 μg/ml, the maturation rate was significantly (**$P > 0.01$, *$P > 0.05$) inhibited by 45% (*), 48% (*), and 71% (**), respectively, in comparison with a control group (100%). On the other hand, a statistically nonsignificant effect was observed for the M=M form, MCM31, and MCM32 (21%, 16%, and 42%, respectively) in comparison with a control group (Fig. 3).

From these results it may be concluded that ribonucleases and their mutants affected resumption of meiosis of bovine oocytes and particularly the completion of this process.

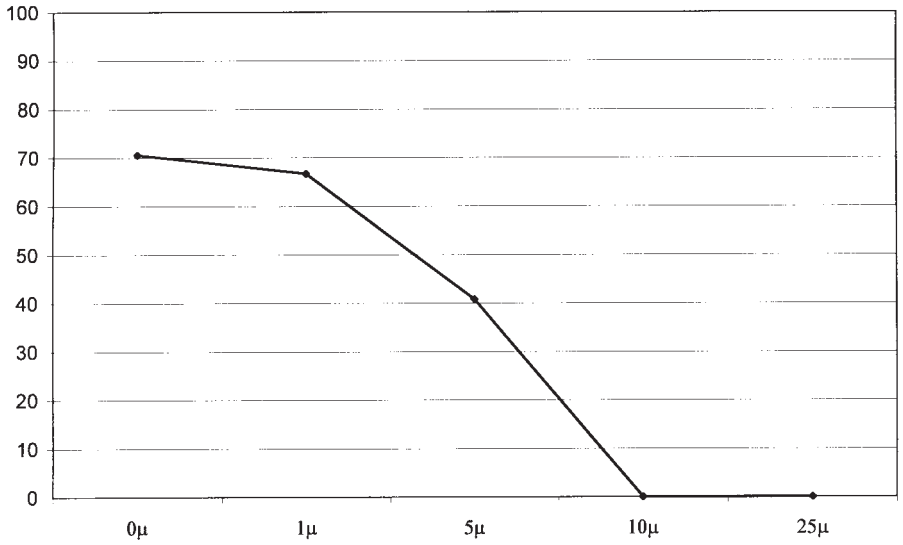

Fig. 1. Effect of added BS-RNase concentration on maturation of bovine oocytes in vitro. Data are the mean values for 24 hr incubation of 14–34 oocytes per group (2 replications).

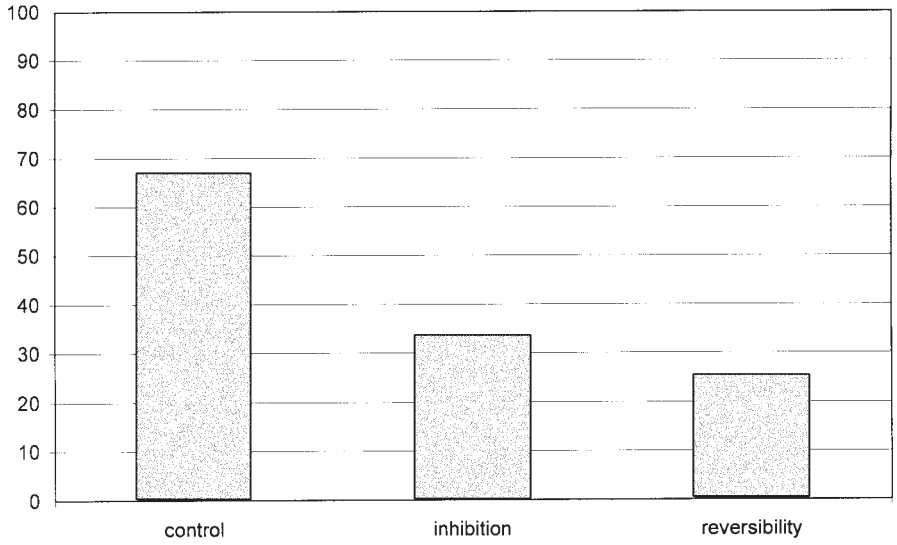

Fig. 2. Reversibility of inhibitory effect of BS-RNase on maturation of bovine oocytes in vitro. The concentration of BS-RNase was 5 μg per ml for 24 hr (inhibition) and 0 μg per ml for additional 24 hr (reversibility). The control cultures contained no added ribonuclease. Data are the mean values for 23–30 oocytes per group (3 replications).

DISCUSSION

In this work, we evaluated the effect of BS-RNase and variants of this enzyme on final period of bovine oocyte formation. The variants were chosen with the particular aim of correlating their quaternary structure with cytotoxic activities during the final part of meiosis.

Bovine seminal ribonuclease at a concentration of 5 μg/ml reduces the rate of bovine oocyte maturation to the MII stage by approximately 58%. Ten and 25 μg/ml stop maturation completely (Fig. 1). Further, the higher concentrations of BS-RNase increases markedly the number of oocytes with degenerative cytoplasmic changes and chromatin disorders.

Mammalian oocyte together with surrounding cumulus cells represents specific experimental model. During growth and meiotic maturation,

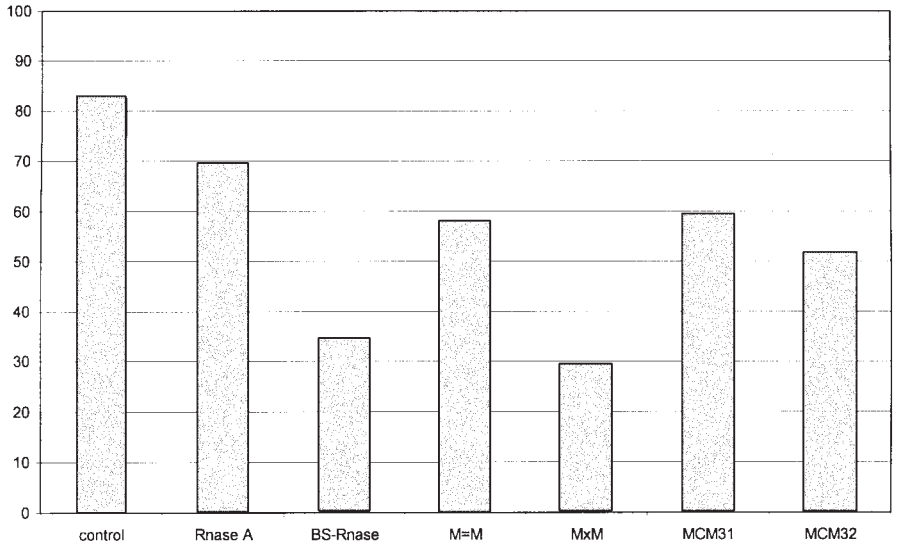

Fig. 3. Effect of ribonucleases on the maturation of bovine oocytes in vitro. The concentration of added ribonuclease was 5 μg per ml. The control cultures contained no added ribonuclease. Data are the mean values for 24 hr incubations of 31–52 oocytes per group (3 replications). Statistically significant differences in comparison with control: $^{**}P > 0.01$, $^{*}P > 0.05$.

oocyte and cumulus cells form functional complexes that are characteristic with mutual interaction between these two cell categories (Eppig, '91). While RNA synthesis in fully grown oocytes, which were used in our experiments, is very low (Crozet et al., '86), cumulus cells behave as normal mitotic cells. Under routinely used culture protocol and also in vivo, they supply the oocyte with nutritional substances and with not yet defined molecular signals, which are responsible for the regulation of meiotic maturation. Therefore, it cannot be excluded that the inhibition of meiosis and degenerative changes in maturing oocytes were caused indirectly as a toxic effect of BS-RNase or its variants upon cumulus cells which were not able to provide oocytes with adequate array of molecules.

In previous work, we showed that the M×M form of BS-RNase has significantly higher cytotoxic activity than did the M=M form (Kim et al., '95b). We therefore proposed that the difference in the cytotoxicity of the two forms derives from their different fates inside the cell. More specifically, the basis of the different activities of M×M and M=M is the ability of M×M but not M=M to remain dimeric in a reducing environment, such as the mammalian cytosol.

In contrast to its relative low cytotoxicity, the M=M form of BS-RNase has significant antimeiotic activity in vitro, similar to that of the M×M form (Fig. 3). The antimeiotic effects as well as antitumor and immunosuppressive activities of the M=M form (Kim et al., '95b) were demonstrated in vitro, where identical cell–cell interactions do not exist. This result contrasts somewhat with a modest aspermatogenic effect reported recently (Kim et al., '95b). The aspermatogenic activity was, however, assessed in vivo, where more complex interactions might influence degradation or inhibition of ribonucleases.

Until this decade, only dimeric ribonucleases were known to have significant cytotoxic activity (Bartholeyns and Baudhuin, '76; Bartholeyns and Zeneberg, '77; Di Donato et al., '94, '95). The demonstrated cytotoxicity of onconase (Ardelt et al., '91; Wu et al., '93, '95) and angiogenin (Matoušek et al., '95), two monomeric ribonucleases, reveals that a dimeric form is not essential for a ribonuclease to be cytotoxic. The aspermatogenic activity of MCM31 and MCM32 (Matoušek et al., '96), which have no effect on oocyte maturation in assays in which other monomeric ribonucleases are active, casts further doubt on the relationship between a dimeric form and cytotoxicity. The key to ribonuclease cytotoxicity appears to be the evasion of cellular ribonuclease inhibitor RI (Cafaro et al., '95; Di Donato et al., '95; Kim et al., '95a,b). Different tissues contain different levels of RI (Lee and Vallee, '93). Thus, a possible explanation for the toxicity of MCM31 and MCM32 for spermatogenic layers is that these cells do not contain enough RI to inactivate invading MCM31 and MCM32. From this perspective, the bovine ova have enough RI to block MCM31 and MCM32. Nevertheless, no explanation is apparent for the sensitivity of these cells to RNase A, which is also a monomer. Perhaps differential adsorption of ribonucleases also plays a role in the cell-specificity of cytotoxicity. Testing this hypothesis will be the basis of the future work.

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