EFFECT OF THE LOCAL ADMINISTRATION OF PROTEOLYTIC ENZYMES ON THE RUPTURE OF THE OVARIAN FOLLICLE IN THE DOMESTIC FOWL, *GALLUS DOMESTICUS*

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Summary. An attempt was made to demonstrate whether proteases administered locally to the ovarian follicle of the hen would result in its rupture. Using 'ovulation-suppressed' pullets prepared with a daily injection of PMSG, a large ovarian follicle was brought to the surface and treated with the enzyme solutions. When 'collagenase' and non-specific proteases were injected into the follicle wall or applied on a small piece of filter-paper placed on the follicular surface, rupture was induced in almost all the follicles within 2 to 3 hr, whereas no rupture occurred in the follicles treated with hyaluronidase, lysozyme or saline solution. It is suggested that proteolytic enzymes might be involved in the mechanism of normal rupture of the ovarian follicle in the hen.

INTRODUCTION

Although it is well established that ovulation in vertebrates is under the control of the anterior pituitary, the mechanism of rupture of the ovarian follicle has not been clearly elucidated. On the basis of the results obtained mainly in mammals, it has been proposed that the tearing of the follicle wall results from increased follicular pressure caused by contraction of perifollicular muscle fibres, or by movement of fluid into the follicle, from necrotic changes, or from enzymatic degradation of follicular tissue (see Rondell, 1970). In fowls, *Gallus domesticus*, Phillips & Warren (1937) suggested that the pressure resulting from the prolonged tension of the muscle fibres of the follicular membrane might be one of the factors responsible for ovulation. It has also been found in the hen that rupture can be induced in the follicle excised before the expected time of ovulation (Phillips & Warren, 1937; Neher, Olsen & Fraps, 1950; Ogawa & Nishiyama, 1969). This finding indicates that an assumption involving the movement of fluid into the follicle to cause follicular rupture might be excluded. Nalbandov (1964) stated that LH might cause ovulation by initiating ischaemia in the wall of the follicle, based on the results of LH-induced ovulation in hypophysectomized hens (Opel & Nalbandov, 1961).

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In the last decade, several studies have suggested the participation of proteolytic enzyme in the rupture of the follicular membrane in mammals (Espey & Lipner, 1965; Espey & Rondell, 1968; Rondell, 1970), but no such evidence is as yet available for birds.

The present investigation in the hen was undertaken to find out whether some proteolytic enzymes administered locally to the ovarian follicle would result in its rupture.

MATERIALS AND METHODS

White Leghorn pullets, aged 7 to 12 months, were kept in individual cages and fed freely with a commercial diet and water. The laying birds were injected daily with PMSG for preparing hens with sustained follicular growth but suppressed ovulation. According to the procedure used by Imai (1973), the pullets with regular clutch sequences were pretreated with a daily subcutaneous injection of 75 i.u. purified PMSG (Peamex, Tomoda Pharmac. Co., Tokyo) until the 2nd day after the cessation of egg laying. On the 3rd day after the cessation of egg laying (24 hr after the final injection of PMSG), an incision was made in the lateral abdomen of the bird under anaesthesia with sodium pentobarbitone (Nembutal, Abbott Lab., Illinois; 20 mg/kg body wt, intravenously). The ovaries, with several large follicles and no sign of rupture, were exposed and a follicle of more than 3 cm diameter was brought to the surface and subjected to the enzyme treatment.

The proteolytic enzyme preparations used in the present work were as follows: Pronase, bacterial alkaline protease (Kaken Chemical Co., Tokyo); Nagarse, bacterial alkaline protease (Nagase & Co., Osaka); and crude Collagenase (Worthington Biochem. Corp., New Jersey). Besides protease, hyaluronidase and lysozyme (both preparations from Worthington Biochem. Corp.) were also used. The three kinds of proteases and lysozyme were dissolved in 0.9% saline solution, pH 7.2, adjusted with 1 N-NaOH to a given concentration (Tables 1 and 2). Hyaluronidase was dissolved in 0.9% saline solution, pH 5.3, acidified with 1 N-HCl to the concentration of 1 or 4 mg/ml.

The exposed follicles were treated with 0.1 ml of the enzyme solution by the following two methods: (1) injection into the deep or the shallow part of the intrafollicular wall (injection method—Exp. 1); or (2) dropping onto a small piece of filter-paper (about 2 × 10 mm) which was placed on the stigma or the non-stigma region of the follicle (filter-paper method—Exp. 2). In Exp. 1, the treated follicle was usually returned to the peritoneal cavity and observed for about 2 hr after the enzyme injection. Follicular rupture was indicated by tearing of the follicular membrane followed by an outflow of yolk material. If the follicle which received the enzyme solution did not rupture within the period of observation, it was checked again on the next day (about 20 hr after the injection). If no rupture had occurred by this time, the treatment given to the follicle was assumed to have no effect in causing rupture. In Exp. 2, the follicle treated with the enzyme was covered by a wet cloth to avoid drying and observed for about 3 hr after the application. Follicles which received 0.9% saline solution served as controls in both experiments.

RESULTS

Experiment 1 (injection method)

In most of the follicles injected with 100 μ g (0.1 ml of the 1 mg enzyme/ml solution) of the proteolytic enzymes, rupture was usually induced at the site of injection about 1 hr after the treatment. This contrasts with the control results using saline, in which no rupture was observed within 20 hr after treatment (Table 1). There was an indication of possible differences between the different enzymes in their effectiveness in inducing rupture. The mode of injection also appeared to influence the effect, a deep injection being possibly more effective than a shallow one, judging from the rate of occurrence of rupture or the time required for induction of rupture. Hyaluronidase and lysozyme were found to be ineffective in inducing follicular rupture even if 400 μ g were administered to the follicle by deep injection.

Table 1. Effects of enzymes injected locally into the ovarian follicle wall of the domestic fowl

Enzyme	Injection	Concentration (mg/ml)	Vol. of injection (ml)	No. of follicles			Time from injection to rupture (min)	
				Injected	Ruptured	%	Mean	Range
Pronase	S*	1	0.1	7	7	100	25.4	11 to 60
	D*	1	0.1	8	8	100	13.3	8 to 20
Nagarse	S	1	0.1	6	4	67	15.0	14 to 16
	D	1	0.1	11	11	100	13.9	10 to 16
Collagenase	S	1	0.1	6	4	67	51.8	26 to 69
	D	1	0.1	16	12	75	45.9	7 to 126
Hyaluronidase	D	1	0.1	4	0	0		
	D	4	0.1	5	0	0		
Lysozyme	D	1	0.1	4	0	0		
	D	4	0.1	4	0	0		
Saline	S or D		0.1	6	0	0		

* S = shallow; D = deep. (See text for explanation of injection method).

Experiment 2 (filter-paper method)

In the first trial of this experiment, Pronase and Collagenase were each applied simultaneously to two different sites of the follicle, one on the stigma and the other on the non-stigma region, at the same concentration. Rupture was induced fairly soon only at the site of application on the stigma in all the treated follicles, except for one in which no rupture occurred in any site during the observation period until 3 hr after the Collagenase treatment (Table 2). By contrast, no sign of rupture was detected in the saline-treated controls.

In the second trial, the follicle was subjected to the simultaneous application of Pronase at three different concentrations, 1, 2 and 3 mg/ml, on three different sites of the non-stigma region. Rupture was always observed at the site receiving 2 mg/ml solution (Table 2).

In Trial 3, Pronase was administered simultaneously to two different sites on the follicle, one on the stigma with 1 mg/ml solution and another on the non-stigma region with 2 mg/ml solution. In this case, rupture was induced at the

Table 2. Effects of enzymes applied on filter paper placed on the stigma or the non-stigma regions of follicles of the domestic fowl

Enzyme	Site of application	Concentration (mg/ml)	Vol. of application (ml)	No. of follicles			Ruptured at	Time from application to rupture (min)	
				Treated	Ruptured	%		Mean	Range
Trial 1 Pronase Collagenase Saline	Stigma	1	0.1	5	5	100	Stigma	64.5	10 to 92
	Non-stigma	1	0.1						
	Stigma	1	0.1	5	4	80	Stigma	104.0	78 to 137
	Non-stigma	1	0.1						
Trial 2 Pronase	Stigma		0.1	4	0	0	—		
	Non-stigma		0.1						
	Non-stigma	1	0.1						
	Non-stigma	2	0.1						
Trial 3 Pronase	Non-stigma	3	0.1	3	3	100	Site receiving 2 mg	121.3	82 to 187
	Non-stigma								
	Non-stigma	1	0.1						
	Non-stigma	2	0.1	3	3	100	Stigma	76.6	39 to 130

site of application on the stigma (Table 2). In general, when the filter-paper method was used, the period from the enzyme treatment to the occurrence of follicular rupture tended to be longer than with the injection method.

DISCUSSION

The ovarian follicles of the PMSG-pretreated birds used in the present work were maintained in a non-ovulating state for about 20 hr after enzyme administration because no rupture could be induced in any bird by 0.9% saline solution within that period. Proteolytic enzymes were shown to cause a rapid induction of follicular rupture in the hen when administered either by injection into the follicle wall or by infiltration from filter-paper pads.

It has not been clearly demonstrated that collagen fibres exist in the chicken ovarian follicle wall; however, the collagenase-induced rupture in this work might be attributed to the disintegrating action of the enzyme on the connective tissue in the follicle wall. Crude 'collagenase' (Worthington) used here contained not only collagenase but also pepsidase and trypsin-like substance. These contaminants probably also participated in the decomposition of the follicle wall. The other two preparations used in the present work, Pronase and Nagarse, which are non-specific proteases, were also effective in inducing rupture. In rabbits, Espey & Lipner (1965) induced follicular rupture by injections of proteases into the Graafian follicle and suggested that in the normal process, gonadotrophin(s) might initiate follicular production of enzyme(s) capable of degrading the connective tissue of the follicle, and that the progressive decomposition of the wall results in its rupture. On the other hand, hyaluronidase and lysozyme were shown to be ineffective in causing follicular rupture. This finding suggests that the substances normally digested by these enzymes are not involved in maintaining the integrity of the ovarian follicle wall of the fowl.

In Exp. 1, deep injection of the enzyme appeared to be slightly more effective in inducing rupture than shallow injection. The latter was probably made into the theca externa, judging from observation of diffusion of the enzyme to a wide area, while the deep injection was probably into the theca interna and/or stratum granulosum, judging from the more restricted spread of the injected solution. It is not possible, however, to determine whether the difference in effectiveness is due to the structural difference of the injected parts or to different degrees of diffusion of the injected material.

In Exp. 2, the stigma was invariably found to be more responsive than the non-stigma region to the enzyme administration. It may be presumed that the stigma contains structural constituents which are relatively easily attacked and disintegrated by proteases. Thus, it is possible that proteolytic enzymes play a rôle in the mechanisms of follicular rupture in the hen ovary. Further studies, however, are required to ascertain precisely the mechanisms involved in follicular rupture in the hen.

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REFERENCES

- ESPEY, L. L. & LIPNER, H. (1965) Enzyme-induced rupture of rabbit Graafian follicle. *Am. J. Physiol.* **208**, 208.
- ESPEY, L. L. & RONDELL, P. (1968) Collagenolytic activity in the rabbit and sow Graafian follicle during ovulation. *Am. J. Physiol.* **214**, 326.
- IMAI, K. (1973) Effects of avian and mammalian pituitary preparations on induction of ovulation in the domestic fowl, *Gallus domesticus*. *J. Reprod. Fert.* **33**, 91.
- NALBANDOV, A. V. (1964) *Reproductive physiology*, 2nd edn. Freeman, San Francisco and London.
- NEHER, B. H., OLSEN, M. W. & FRAPS, R. M. (1950) Ovulation of the excised ovum in the hen. *Poult. Sci.* **29**, 554.
- OGAWA, K. & NISHIYAMA, H. (1969) Studies on the mechanism of ovulation in the fowl. *Mem. Fac. Agric. Kagoshima Univ.* **7**, 1.
- OPEL, H. & NALBANDOV, A. V. (1961) Ovulability of ovarian follicles in the hypophysectomized hen. *Endocrinology*, **69**, 1029.
- PHILLIPS, R. E. & WARREN, D. C. (1937) Observations concerning the mechanics of ovulation in the fowl. *J. exp. Zool.* **76**, 117.
- RONDELL, P. (1970) Biophysical aspects of ovulation. *Biol. Reprod. Suppl.* **2**, 64.