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HISTOCHEMICAL STUDIES ON THE EFFECT OF ZINC DEFICIENCY IN THE RAT OVARY

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SUMMARY

Adult female rats were given a zinc deficient diet for six weeks. During this period their ovaries were compared with those of normally fed rats. The histochemical activities of two zinc metalloenzymes, namely alkaline phosphatase (APase) and carbonic anhydrase (CA) were markedly lowered three and two weeks after starting feeding, respectively. In vitro, reactivation to the control level of activity of both enzymes by exogenous zinc added to the incubation medium suggests absence of zinc from the apoenzymes is the cause of decreased activity rather than decreased synthesis of the apoenzymes.

INTRODUCTION

In an endeavor to obtain more data concerning the pathophysiology of the ovary during Zn-deficiency and the sametime gain insight into the role of Zn metalloenzymes in gonadal function, histochemical studies were performed on the ovaries of Zn-deficient and normal rats. The histochemical activities of two enzymes, APase and CA, were chosen in the present work, since both are Zn-metalloenzymes (1,2), present in various parts of the ovary (3,4) and are involved in an indirect way in the process of ovarian steroidogenesis (3,5).

MATERIALS AND METHODS

A total of 114, apparently healthy, adult virgin female Albino rats, 6-8weeks old were used. They were

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isolated from the Animal Breeding Centre, College of Medicine, University of Baghdad, fed on a normal diet, obtained from the General Estate Interprise for Forage, Baghdad, Iraq, and tap water, ad libitum.

A detailed precedure of animal treatment, diet, weight measurements and serum zinc can be found in reference (6) which are detailed as follows:

After recording their weights, two ml of blood was taken from them to estimate serum zinc. After that the animals were kept on normal diet for ten days, then divided into controls (42 rats) and a group of rats (72 rats) fed on zinc deficient diet. The control group was continued on normal diet and tap water ad libitum, while the zinc-deficient group was fed on zinc-deficient diet and deionized water ad libitum for six weeks. The zinc deficient diet is composed of egg white of one boiled egg daily for each rat to which 28.485gm sucrose, 0.145gm: multivitamins (SDI-Iraq), 0.145gm sodium chloride and 0.145gm potassium chloride were added.

The animals were weighed weekly using electronic balance and carefully examined for signs of deficiency.

Before animal sacrifice two ml of blood were taken from tail vessels for estimation of serum zinc. Seven control and 12 Zn deficient rats were sacrificed weekly during the six weeks period.

Under ether anaesthesia the ovaries were removed and mounted on a piece of cork with a drop or two of 10% gum acacia (BDH Chemicals Ltd Pool England). Each cork with tissue on top was quenched in liquid nitrogen.

Seven to eight micron thick sections were cut by cryostat, mounted on clean coverslips, and left to dry at room temperature for 15-30 minutes before incubation. Then, the histochemical activity of APase (calcium -

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* Merck.

** Diamox, Pearl River, NY 10965.

cobalt method)(7) and for CA(8) were demonstrated on these sections. Tests of specificity of the enzymatic reactions were done by either omitting the substrate or by addition of specific inhibitor to the incubation media (i.e. 40mM DL-phenyl alanine(*) for APase(9), and 10^{-5}M sodium acetazolamide** for CA(10,11) and, boiling sections for 10 minutes prior to incubation for both.

The effect of exogenous Zn on the activities of APase and CA was tested on sections from both normal and Zn-deficient tissues by preincubating the sections for 15, 30 and 60 minutes in aqueous solutions of 10mM $ZnSo_4$ (BDH, Analar).

RESULTS

The results of serum zinc and animal weights are found in another communication (6).

The appropriate incubation time required for the development of reaction product for APase with optimal intensity and distribution in various components of the ovary of normally fed animals was found to be five minutes (Table 1). Results of normal ovarian tissue reaction for APase compared with those given Zn-deficient diet for six weeks are arranged in Table-2-. Observations obtained from studying this table shows that APase histochemical activity of normally fed animals is principally located in theca interna (Fig.1), vascular endothelium of the corpus luteum (Fig.2) and of the endocrinocytus interstitialis. While the oocyte, germinal epithelium and granulosa cells were virtually negative.

In ovarian sections obtained from animals kept three weeks on Zn-deficient diet, lower staining for APase was obtained in theca interna up to 15 minutes after incubation. This reduction was gradually increased with the progress of deficiency period (Fig.3). The reduction of APase activity was also noted in the granulosa cells of atretic follicles, luteal cells, secondary interstitial tissue and blood vessels of the corpora lutea (Fig.3).

Table (1): Alkaline phosphatase optimal incubation time of various ovarian components.

Types of cells	Granular reaction minutes	Diffuse reaction minutes
1- Granulosa cells of growing follicles.	-	15
2- Granulosa cells of atretic follicles.	5	10
3- Teca interna.	-	5
4- Corpus luteum cells.	5	10
5- Blood vessels of corpora lutea and endocrinocytus interstitialis.	-	5
6- Primary interstitial tissue	-	15
7- Secondary interstitial tissue.	5	15
8- Ova.	-	-
9- Germinal epithelium	-	-

Table (2): Subjective double blind assessment of APase activity in various components of ovaries of normal and Zn-deficient rats.

Ovarian components	weeks on Zn deficient diet						
	control	one	two	three	four	five	six
1- Granulosa cells of growing follicles.	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^
2- Granulosa cells of atretic follicles.	+2	+2	+2	+1	+1	–	–
3- Theca interna.	+5	+5	+5	+4	+3	+3	+2
4- Blood vessels of corpora lutea and endocrincoytus interstitialis.	+3	+3	+3	+2	+2	+1	+1
5- Luteal cells.	+1	+1	+1	$\overset{-}{+}$ ^	$\overset{-}{+}$ ^	–	–
6- Ova.	–	–	–	–	–	–	–
7- Germinal epithelium.	–	–	–	–	–	–	–

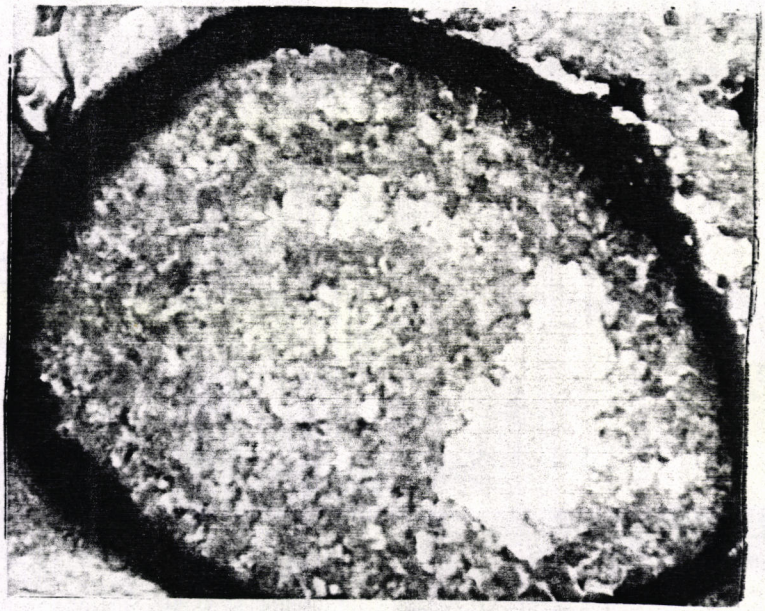

Fig (1): Normal growing follicle showing intense reaction product in theca interna, but weak staining in granulosa cells. (X256).

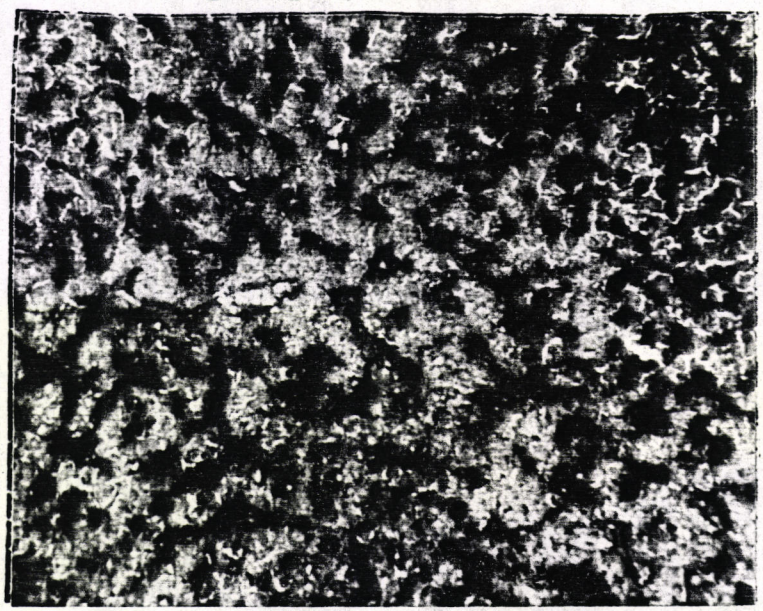

Fig (2): Corpus luteum showing intense reaction in blood vessels, but faint reaction in luteal cells of normal rat ovary. (X403.2).

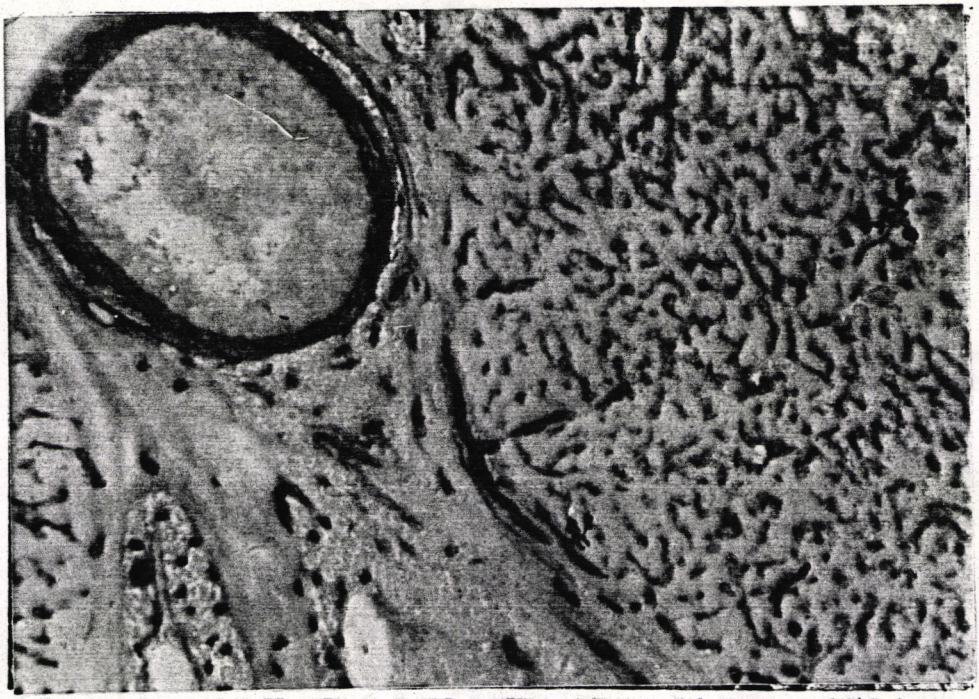

Fig (3): APase activity in rat ovary six weeks after starting the deficiency regime, showing part of corpus luteum and one follicle. Note the reduced reaction product as compared with Fig. 1 and 2. (X163.8).

Fig (1-3): APase activity in eight micron thick, fresh frozen section of rat ovary.

Regarding CA activity, Table-3- shows the main loci of CA activity which include the granulosa cells of atretic and healthy follicles (Fig. 4,5), theca externa of mature follicles, corpora lutea, luteal cells, interstitial tissue and to a smaller fegree theca interna cells. Data presented in Table -3- shows that CA activity is lower in Zn-deficient ovary than in controls. This reduction in the activity was apparent at the end of the second week and was maximum at the sixth week (Fig.6) of diet treatment.

Preincubation of ovarian sections of Zn-deficient rats for 30-60 minutes in aqueous solution containing 10mM $ZnSO^{4}$ resulted in reactivation of both enzymes (APase and CA) histochemical staining to a level comparable to normal, while histochemical staining in normal tissue was not much affected by this preincubation procedure.

Total inhibition of APase activity of normal and Zn-deficient tissues was obtained by 40mM DL-phenylalanine. Sodium acetazolamide in 10^{-5}M concentration caused total inhibition of CA activity.

DISCUSSION

Observations obtained from studying Table -2- shows that APase histochemical activity is principally located in theca interna, vascular endothelium of the corpus luteum and of the endocrinocytus interstitialis. This distribution is largely in accord with that described by others, in rats (12-14), and in fowl (15). While in the oocyte, germinal epithelium and granulosa cells of healthy follicles were virtually negative. This result is more or less in agreement with others (13,16).

Restoration of APase activity, after preinculation of sections from Zn-deficient rat ovaries in $ZnSO_4$ solution fr half an hour to one hour, suggested that Zn is associated with the enzyme as a readily dissociable cofactor. Reduction in the activity of the enzyme

Table (3): Subjective double blind assessment of CA activity in various components of ovaries of normal and Zn-deficient. rats.

Ovarian components	weeks on Z-deficient diet						
	control	one	two	three	four	five	six
1- Granulosa cells of growing follicles.	+3	+3	+2	+1	+1	+1	+1
2- Granulosa cells of atretic follicles.	+5	+5	+4	+3	+2	+2	+1
3- Theca interna.	+1	+1	+1	±1	-	-	-
4- Theca externa of Graafian follicles.	+4	+4	+3	+3	+2	+2	+1
5- Luteal cells.	+2	+2	+1	+1	±1	±1	-
6- Smooth muscle cells around corpus luteum.	+4	+4	+3	+3	+2	+2	+1
7- Primary interstitial tissue	+1	+1	±1	-	-	-	-
8- Secondary interstitial tissue.	+2	+2	+1	+1	+1	±1	-

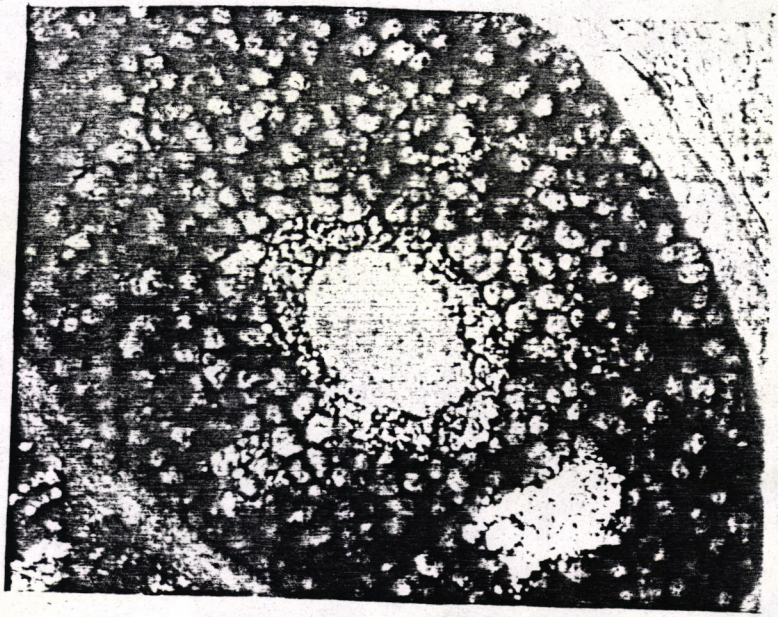

Fig (4): CA activity in normal, healthy growing follicle showing the intense reaction product in granulosa cells, weak reaction in theca interna, and absence of reaction product in the ovum. (X320).

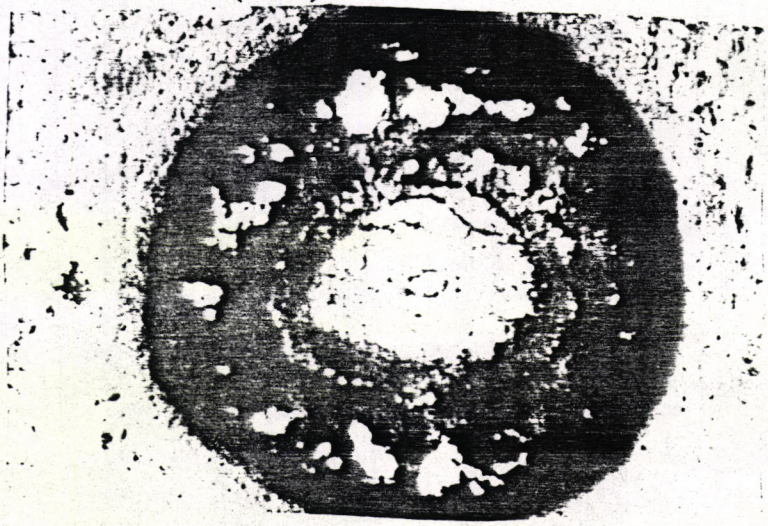

Fig (5): CA in normal, early atretic follicle showing intense reaction product in granulosa cells (X320).

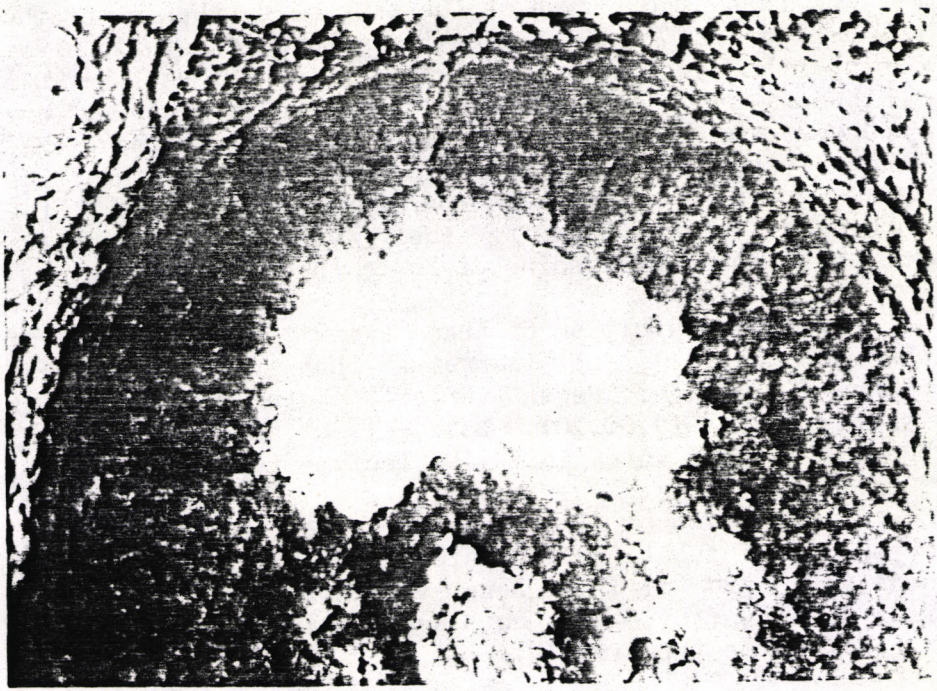

Fig (6): CA activity in rat ovary six weeks after starting the deficiency regime, showing marked reduction in the staining intensity of granulosa cell of antral follicle (Cf. with Fig 4), (X320).

Fig (4-6): CA activity in eight micron thick fresh frozen section of rat ovary.

therefore appears to involve either lack of incorporation of Zn into the enzyme at the time of synthesis, or the removal of Zn from a normally saturated metalloenzyme during the deficiency period. It is behieved that DNA synthesis and hence ENA and protein synthesis, are regarded to be primary defects in Zn-dificiency (17), then it seems reasonable to expect that the synthesis of this enzyme would also be impaired. However, the unchanged activity during the first two weeks might be due to firmness of binding of Zn to the enzyme protein.

Total inhibition of APase activity was obtained by 40mM Dl-phenylalanine indicates that this enzyme is of placental, Regan, Regan varient, Nago or intestinal isozyme type (9,18,20), but not of Non-Regan, liver, bone, kidney or intestinal-like isozyme type (18,21).

Inhibition of APase activity after boiling sections prior to incubation indicates that this enzyme is heat sensitive and it is of Regan varient, Non-Regan or Nago isozyme type(18), but not necessarily of placental or Regan isozyme type (18,19).

After thorough literature review, it was found that there was no previous study available concerning APase activity in the ovary of Zn-deficient rat. However, many histochemical studies established a decrease in APase activity in many tissues other than the ovary, after Zn-Depletion (22).

It is evident from table -3-, that the main loci of CA activity included the granulosa cells of atretic and healthy follicles, theca externa of nature follicles, corpora lutea, luteal cells, interstitial tissue and to a smaller degree theca interna cells.

After thorough literature review, it appears that there is only one study (3) demonstrated and localized CA in the ovary which is in agreement with this work.

The particularly intense reaction of CA observed in granulosa cells of atretic follicles and luteal cells of

old corpora lutea, can be attributed to the presence of large number of lysosomes which well known to play an important part in this autolytic process (23). CA had been demonstrated in the lysosomal matrix (24) as it may be involved in the transmembrane H^+ graduent in lysosomes to achieve a low pH essential for maximal activity of lysosomal enzymes.

CA activity, in granulosa cells of healthy follicles, varied in intensity during different stages of follicular growth. The physiological significance of CA in granulosa cells, may be due to participation of the enzyme in metabolism of steroid hormones (3), since this enzyme has an esteric activity (25). The intense staining recorded for CA in the theca externa may be due to the presence of smooth muscle like cells (26) which were found around large antral follcles and many corpora lutea. This layer seems to disappear in old corpora lutea.

Data presented in Table -3- shows that CA activity was lower in the Zn-deficient ovary than in controls. This reduction of the activity which was apparent at the end of the second week would be attributed (as in case of APase) to the general impairment of protein synthesis or lack of sufficient Zn to support the activity of the enzyme. Which was apparent at the end of the second week would be attributed (as in case of APase) to the general impairment of protein synthesis or lack of sufficient Zn to support the activity of the enzyme. However, reactivation of the enzyme following 30 minutes preincubation of the ovaruan sections in $ZnSO_4$, as shown uniquely in this work, may support the second attribute.

There are no available data concerning the effect of Zn deficiency on CA in the ovary nor about its in vitro reactivation. However, reduced CA activity was recorded in many tissues, other than the ovary during Zn depletion (23).

Zn-deficiency causes a definite decrease in histochemically demonstrable enzyme activities of both Apase and CA in most rat ovarian tissues as detailed in this work.

The novel use of in vitro preincubation of tissue sections of Zn-deficient animals, for the period used in this work, favours lack of metal incorporation to form the holoenzymes (particularly CA) rather than decrease in apoenzyme synthesis.

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دراسة كيميانسجية لتاثير عوز الخارصين على مبيض الجرذ

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الخلاصة

احدث عوز مفتعل للخارصين، بحمية غذائية مكونة من بياض بيض أعطي لمدة ستة اسابيع في اناث الجرذ البالغة. وخلال هذه الفترة فحصت مبايض هذه الجرذان نسجيا وقورنت مع مثيلات اعطيت عليقة سوية.

أوضحت هذه الدراسة الكيميانسجية حدوث نقص في فعالية انظيمين من الاناظيم التي يدخل الخارصين في تركيبها هما الفسفاتاز القلوية والانيدراز الكاربونية، بعد ثلاثة أسابيع واسبوعين من بداية الحمية الغذائية على التوالي، كما أوضحت ان اضافة الخارصين الى وسط الحضن يرفع الفعالية الكيميانسجية الى الحد السوي في مقاطع المبايض معوزة الخارصين. تكون بذلك قلة الفعالية مسببة عن غياب الخارصين من صميم كلا الانظيمين لابسبب قلة في تخليقهما.

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