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REVIEW ARTICLE

DEVELOPMENT OF ENZYME IMMUNOASSAY AND ITS ROLE IN REPRODUCTIVE ENDOCRINOLOGY

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Radioimmunoassay is a powerful technique developed in 1960 by Solomon Berson and Rosalyn Yallow, who first devised a R1A for the measurement of insulin concentration in plasma1. The technique was far more sensitive and specific than any method existed at the time. RIA is used to analyse many biological substances that are present in minute amounts, including steroids, cyclic nucleotides, peptide hormones, viruses, bacterial antigen, enzymes, prostaglandins and drugs.

Inspite of certain advantages of RIA in terms of sensitivity, specificity and wide applicability2, it has a number of disadvantages including high capital cost, as the devises used are very expensive and their maintenance in developing countries is extremely difficult task. Beside radiation hazards are a constant threat to personnel working in RIA laboratory. Decomposition of radiolabeled proteins upon storage is also a serious problem in RIA, affecting the sensitivity and reproducibility of assay system.

To overcome these problems, it would not be surprising to develop alternate analytical procedures which in principal was to some extent similar to RIA but the label instead of being a "radioisotope" was in an enzyme. Reagent enzymes and antibodies formed against specific molecules can be combined to determine the concentration of a variety of molecules to which antibodies are formal. Such analytical procedures are known as enzyme immunoassay (EIA). The reagent enzyme can be linked to antibodies or antigens such that complex possess immunological or enzymatic activity. Antibodies can be raised in vertebrate animals when injected with specific proteins (antigens) foreign to them. Macromolecules other than proteins can also be antigenic. Low molecular weight compounds by themselves do not elicit antibodies, but do so if covalently linked to a carrier protein before injection. The term hapten designates the low molecular weight substances with the antibody produced against the carrier protein complex. Enzyme immunoassay are either heterogeneous or homogeneous, reduces the cost of assay system and the personnel working in EIA laboratories are not subjected to radioactive hazards.

When the first report of the use of the enzyme as labels for immunoreagents appeared, the traditional RIA users found it hard to accept that these could really challenge isotopes in terms of sensitivity, and so it was largely left to the infectious disease workers to pioneer and establish these new tcchniques.3 Freed from the dictates of classical RIA the area of non-isotopic labelled reagent tests were explored, using labelled antibody rather than labelled hapten/antigen. This permitted the development of tests based on use of excess of the labelled reagents. This approach is theoretically more sensitive[4] and advantageous in that enzymes and other labels can be coupled to virtually

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antibody, using a common procedure. Rather curiously these developments initiated in the under developed areas of infectious diseases and Veterinary Medicine and are being increasingly accepted by the clinical chemistry and endocrinological laboratories. The EIA has a very high sensitivity and comparison with RIA based on tritiated tracer and shows good correlation (4 = 0.78)[5].

Enzyme labelled immunoassays depend on the conjugation of an enzyme to an antigen without significantly impairing either the enzyme activity or antigen-antibody building and also on the amplification which is inherent in all enzyme substrate reactions.

Enzyme immunoassay techniques for measuring progesterone have been described by Dray et al[6]. More recently Sauer et al had described a method for precisely determining progesterone levels in whole bovine milk using microliter plates without the need for prior extraction of steroid[7]. This allowed large number of samples to be assayed with the minimum of equipment since the whole procedure is conducted in disposable antibody-coated polyvinyl microliter plates and no centrifugation is required for separation of antibody-bound free steroid. The practicality and reliability of heterologous microliter plate EIA systems had been demonstrated by their use in the analysis of progesterone[8].

Sauer et al described a homologous microliter plate enzyme immunoassay (EIA) for the direct determination of progesterone in whole milk[8]. Aspects of the assay are discussed in relation to progesterone profiles obtained from several cows over an ocstrous cycle. The B-galactosidase conjugate used was prepared from the 11 a-hemisuccinate derivative of 11 a-hydroxy-progesterone and was subsequently compared with heterologous conjugates prepared in a similar way using 11 a-hemimaleate, 11 a-Glucuronide-B- galactosidase conjugate provided standard curves with the steepest slope. Approximately 1/10 the binding by 50% when compared with the homologous hemisuccinate conjugate. The results suggest that the use of a glucuronide bridge for linking progesterone to enzyme may reduce bridge-recognition phenomena sufficiently for a visual distinction to be made between luteal and follicular phase concentrations of progesterone.

Subhan Qureshi and Mir Hassan Khan studied relationship of corpus luteum size with milk progesterone level in Nili Ravi buffaloes[9]. Milk progesterone level was determined with the help of EIA technique and post-slaughter examination of corpus luteum was used as a criterion for confirmation of EIA diagnosis o corpus luteum size. Single corpus luteum had fairly good relationship with the concurrent milk progesterone level but double corpora luteal did not have any significant relationship with the milk progesterone level.

In another study M. Subhan Qureshey al investigated comparative efficiency of rectal palpation and milk progesterone profiles in diagnosing ovarian contents in mammals.[10] Rectal palpation of ovarian contents was made and milk samples for progesterone were subjected to EIA technique.

Post-slaughter examination of ovarian contents was used as a criterion for confirmation of rectal and EIA diagnosis of ovarian contents. The difference between accuracy of rectal and EIA diagnosis of ovarian contents (corpus luteum) was not significant.

The main problem of enzyme immunoassay (EIA) techniques is the possible interference in the assay of sample constituents other than the hormone to be measured (the so called "matrix effect"). This matrix effect can be solved cither by extracting and purifying the hormone from the sample, or by increasing the sensitivity in order to reduce the sample volume and thus minimizing the matrix effect. Visual reading of the results may be another important requirement, especially in small field laboratories where no spectrophotometer is available. With most EIA techniques the large colour changes required for observation by naked eye can be obtained only by increasing the sample volume; this, however, may also result in in acceptably large matrix effects.

For these two reasons, namely direct addition of the sample and reading of the test results by naked eye, Van Dc Wiel and Koops developed and validated a sensitive, simple and direct (i.e. without extraction) enzyme immunoassay (EIA) system for the measurement of progesterone in bovine milk and blood plasma 1[11]. Progesterone (P) has been analysed by a microlitre plate EIA, employing polyclonal antibodies against P-7 a car- boxyelhylthioether-BSA as the antigen. The enzyme used as a label is horscradishperoxidase (HRP) and the chromogen is tetramethylebenzidine (TMB). Sensitivity of the EIA has been greatly improved by introduction of a heterologous tracer, in which the same antiserum has been sued, the sensitivity is 20 times greater. The detection limit of the assay is 0.4 pg. per well (i.e. 0-40 ng per ml), and 50% reduction of the initial binding is obtained with 2.5-5.0 pg. Results can be obtained cither by spectrophotometric measurement at 450 nm, or by naked eye. Total time needed for hi assay of 40 replicate samples is approximately 3 h. Comparison of the EIA system with a previously validated RIA system gave a regression line EIA = 0.85 RIA + 2.11 (4 = 0.93, n = 400 milk samples), application of the milk - progesterone EIA to pregnancy testing (n=66) gave an accuracy of 79.6% for positive diagnosis and 100% for negative diagnosis.

Comparison of radioimmunoassay and enzyme-linked immunoassay for the measurement of progestogen in equine plasma and milk was made [12]. Milk and plasma samples were obtained every 48 hours from eight pony mares for 40 days after foaling. Progestogen concentrations in milk and plasma were measured using an enzyme-linked immunoassay (ELISA) and compared with radioimmunoassay of the plasma. In general, the two assays showed similar trends considerably. Difficulty could occur in interpreting the results from single samples taken at limes when progestogen concentrations were either rising (i.e. after ovulation) or falling. ELISA could be used on plasma obtained by allowing the erythrocyte to settle for 30 minutes at room temperature or for two days at 4°C.

In another study while the enzyme immunoassay results for the aged serum samples were as expected, the progesterone levels measured by radioimmunoassay were lower than those for fresh serum or plasma, indicating that scrum should be separated as soon

as practicable after sampling if the possibility of obtaining false results is to be avoided[13]. This small trial has shown the Ovucheck Mareside enzyme immunoassay to be accurate for the qualitative assessment of the progesterone content of bovine whole blood, plasma or scrum, provided that, if plasma or serum samples are to be tested, separation is carried out as soon as possible after sampling, the test is simple to carry out and results are available within 45 minutes.

The introduction of AI and embryo transfer technology in the field of buffalo reproduction has brought hormonal measurement and treatment in association with nutrition as a key researeh. Hormonal regulation in buffalo reproduction is increasingly becoming an important tool for monitoring genetic progress and productivity in buffaloes. Currently the methods of hormone measurement that are very sensitive, accurate and convenient to use are those involving the use of immunoassay both radioimmunoassay (RIA) and enzyme immunoassay (EIA).

KIA IS USED IN THE FOLLOWING AREAS OF ANIMAL REPRODUCTION:

1. Proper Heat Detection:

At the ovarian level, the estrous period is characterized by high estrogen secretion from preovulatory graafian follicles. At the end of estrus, ovulation occurs followed by corpus luteum formation resulting in progesterone secretion. This secretion regresses abruptly some days before the next estrus. the period of corpus luteum activity is called the luteal phase; it lasts 14 to 15 days in ewes and 16 to 17 days in cows. The follicular phase, from the regression of the corpus luteum to ovulation, is relatively short, i.e. two to three days in ewes and goals and three to six days in cows. This short follicular phase does into reflect the true duration of graafian follicle growth. Thus, estrus cycle length is closely related to the duration of the pituitary luteotrophic hormones (LH and prolactic) but by the action of a luteolytic factor, prostaglandin F2a (FGF_{2a}).

The suitability of a progesterone assay in blood plasma for estrus control and pregnancy diagnosis in the swamp buffalo (Bunalus Bubalia) was studied[14]. Progesterone was determined both by radioimmunoassay (RIA) and by enzyme immunoassay (EIA). Blood samples were taken on day 1 (= day of insemination) and on days 24, 27 and 30 alter insemination (p.i.) Normal progesterone values during estrus were lower than 0.5 ng/ml, and generally the same low values were found in case of none pregnancy at days 24, 27 and 30 p.i.

Kamonpatana used two hundred and twenty-two female buffaloes to study the feasibility of using progesterone (P) and oestrone Sulphate (ESO) as a tool for oestrus confirmation and early pregnancy diagnosis [1,5]. The ratio of these steroids at day 0 and 24 were 1. .93 ± 3.20 and 18.80 ± 12.01 of the pregnant group and 1.61+3.20 and 2.42± 4.02 of the non-pregnant group respectively.

Eddy and Clark used the commercially available Lest kit for assaying milk progesterone in the practice laboratory on samples taken daily from cows 17 to 24 days' after service.[16] Improved oestrus detection rates and accuracy were achieved by predicting the onset of oestrus. Calving to conception intervals improved from 115 to 84 days in one herd and from 85 to 74 days in another.

Nasir Hussain Shah et ul undertook a fertility treatment trial of an oestrus buffaloes on a well-managed dairy farm i the low breeding season[17]. Norgestomet implantation and intramuscular injection and oestradiol valerate injection were used for estrus induction. Progesterone determination by means of an enzyme immunoassay (ElA) were used for estrus confirmation. The

overall conception rate was 53.3%.

Mir Hassan Khan cl al observed that progesterone level during oestrus cycle showed an increase after ovulation due to the developing corpus luteum size [18]. An optimum increase was observed till 17thc day, which later dropped rapidly to basal level. The length of oestrus cycle was 21 days. Use of milk progesterone profiles in oestrus detection (milk progesterone < 2ng/ml) helped to achieve a conception rate of 67%.

2. Early pregnancy Test:

Whereas the plasma progesterone content declines as the corpus luteum regresses in the non-pregnant animal, in the pregnant animal it persists or rises. This difference in progesterone levels is the basis for an early pregnancy test in the cows and ewes[19]. A close relationship exists between the progesterone levels in plasma and in whole milk of lactating dairy cattle[20]. Progesterone levels in whole milk are approximately twice those in plasma and reflect the secretory activity of the corpus luteum in the normal cycle and early pregnancy. Thus the possibility of using milk progesterone levels as an aid to pregnancy diagnosis in lactating dairy cattle has been studied extensively[20,21].

In a study the success rate of the pregnancy test from a single milk sample 21,24, 28 or 42 days alter insemination ranged from 77.5 to 100% for non-pregnant cows[22]. Perera, et al collected blood samples from 84 buffalo cows 21 days after fixed time artificial insemination following oestrus synchronization with cloprostcnol[23]. Out of 31 animals diagnosed pregnant by rectal palpation, 28 (90.3 per cent) had been correctly detected by assay at 21 days. Thirty-four (64.2 per cent) of the 53 animals found non-pregnant had been correctly detected by assay.

Singh and Puthiyandy collected milk samples on days 20, 24, 28 and 40 after insemination[2^]. The detection of non-pregnant animals was 100% successful at all times but the diagnosis was correct for 66, 68, 81 and 83% of animals tested on days 20, 24, 28 and 40 respectively and predicted as pregnant.

In another study Nasir Hussain Shah determined a diagnostic level of 10 ng progesterone per ml of milk in order to distinguish between pregnancy and non-pregnancy when the sampling of the milk is done in the period between 20 and 23 days after service[5].

Mir Hassan Khan et al found an increase in the milk progesterone level in the first few days of pregnancy. However, instead of declining from about day 17 or so high progesterone concentrations were maintained[1*]. In pregnancy diagnosis milk profiles provided 80% accurate positive and 100% accurate negative results.

3. Introduction of EIA **in Pakistan (NWFP):**

Hormonal regulation in livestock reproduction is increasingly becoming an important tool

for monitoring of genetic progress and productivity in buffaloes. The pioneer work has been done in Pakistan by establishing Enzyme immunoassay laboratory at Veterinary Researeh Institute Peshawar[23]. Procurement and installation of the equipment's was successfully achieved in the year 1986-87. Assay accuracy and precision was checked and was found according to the international standard.

The work was planned to apply EIA techniques in the fields of Animal Production and Health. However, during the year 1987-88, in accordance with the recommendations of the Technical committee of the PARE more emphasis has been placed on animal production aspects.

During the year 1987-88 as many as 80(X) milk samples and 700 blood samples were analysed for determination of progesterone hormones. These samples were of different researeh projects as mentioned below:

1. Progesterone profiles in postpartum buffaloes under different nutritional conditions.
2. Seasonal Variation in the resumption of ovarian activity after calving in Nili-Ravi buffaloes as assessed by progesterone profiles and rectal examination.
3. Effect of GnRH treatment on Reproductive performance in postpartum dairy buffaloes.
4. The accuracy of rectal diagnosis of corpus luteum in Nili-Ravi buffalo.
5. Oestrus synchronization in the Nili-Ravi buffaloes.
6. Progesterone measurement in more milk samples for early pregnancy diagnosis and oestrus detection.

4. Role of Immunoassays in Human Reproduction:

Progesterone is a female sex hormone; it regulates the accessory organs during the menstrual cycle. In non-pregnant women, progesterone is secreted mainly by the corpus luteum and during pregnancy the placenta becomes the major source. Minor sources are the adrenal cortex in both sexes and the testes in sources are the adrenal cortex in both sexes and the testes in males. Progesterone concentrations very considerably among individuals and in the same individual from day to day. Abraham et al found that the functional status of the corpus luteum is normal in females with normal menstrual cycles [26]. The sum of three plasma progesterone values obtained 4-11 days prior to menstruation is > 15 ng/ml. In females with abnormal cycles, the sum was < 15 ng/ml. According to some investigators, measurement of progesterone in saliva may be a better means of evaluating luteal function[27].

During pregnancy plasma progesterone level rise steadily, reaching values as high as 200 ng/ml at term. Although decreased excretion of the urinary progesterone metabolite pregnanediol has been reported in eases of threaten abortion, toxemia of pregnancy and intrauterine fetal death.[28].

Human plasma progesterone levels were measured by two radioimmunoassay procedures employing high specific antiserum as well as one utilizing less specific antiserum and a chromatographic purification procedure [29] (Table-1). El A can be used in studying eases of infertility due to endocrinological disorders in women.

Table-I Human Plasma Progesterone Levels Measured by Radioimmunoassay

Source	Progesterone Concentration (ng/ml ± SD)
Female	
Cycle	
Days 2-10	0.50 ± 0.44
Days 19-23	12.10 ± 4.81
	0.25 ± 0.09
Postmenopausal	
Cycle	0.555 ± 0.265[A]
Days 1-14	0.424 ± 0.187[B]
Days 14-end	7.70 ± 2.49[A]
	7.70 ± 1.90[B]
Pregnancy	
12 week-term	144 ± 66[A]
	104 ± 55[B]
Cycle	
Follicular phase	0.545 ±0.103
Midlureal phase	8.561 ± 4.661
Pregnancy	
16-18 weeks	48.4 ± 18
28-30 weeks	98.0 ± 2
38-40 weeks	178.5 + 48
Male	0.26 +0.08
	0.495 + 0.132
	0.230 + 0.068

A: Without chromatography. B: With chromatography

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ON PASSING AWAY OF A COLLEAGUE

I was going through the papers of JAMA August, 1991 Vol. 3 article "a piece of my mind "fare well" page 360.

Every day we have stories and events which shock us and move our souls – hardened pebbles, we – still roll on with our daily tide of rituals.

I do not think we analyse our routines ever, seriously and even if we, do, do we make any amendments for the better, for us individually and for the mankind in general.

My feeling is we have all fallen prey to any ugly routine and we have become mindless old fashioned machines which need to be changed according to the needs of the time.

DR. FARIDA AZIZ
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