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Monitoring of Variable Stages of Ovarian Cells in Buffalo Using Double Stained SDS-PAGE

Mohammed Baqur S. A. Al-Shuhaib
Department of animal resources/college of agriculture/Al-Qasim Green University, Iraq
baquralhilly_79@yahoo.com

Abstract

this study focused on applying gel electrophoresis parameter as primary indicator to estimate and to pursue the approximate growth stage of the variable ovarian samples of buffalos. Several buffalo ovaries were taken from the local slaughter houses. Different positions of ovarian cells were isolated from each ovary. Samples were subjected to electrophoresis on denaturing polyacrylamide gel. Optimizations were taken place in matter of proteins concentration and staining techniques. Several ovarian bands were resolved and the monitoring of the resulting protein profile was taken place. The developmental stages of ovarian cells were highlighted electrophoretically. Additionally, comparative electrophoresis was performed between the SDS-PAGE profile of amniotic fluid of buffalo and its corresponding bovine fluids. Concerning buffalo oocytes electrophoretic profile, significant differences were obtained among variable stages of development. On the other hand, it's possible to use electrophoresis to compare between buffalo follicular fluid and its corresponding bovine counterpart.
Keywords - *buffalo, electrophoresis, oocytes, PAGE, profile*

الخلاصة

تهدف هذه الدراسة الى التركيز على معيار الترحيل الكهربائي في الهلام لاستخدامه كدليل أولي لتقدير ولمتابعة المرحلة العمرية التقريبية للعينات المبيضية المتغايرة للجاموس. أخذت عدة مبايض من الجاموس من المذبح. عزلت مواضع مختلفة من الخلايا البيضية من كل مبيض. تم ترحيل العينات في هلام متعدد الأكريلامايد الممسوخ. تم تحسين تركيز البروتين وتقنيات التصبيغ في الهلام. وقد تم تحديد عدة حزم مبيضية وتم تعقب النمط البروتيني الناتج. وعلى هذا الأساس, تم تسليط الضوء على كل مرحلة من مراحل الخلايا المبيضية وفقا للترحيل الكهربائي بالهلام. بالاضافة الى ذلك, تم اجراء ترحيل كهربائي مقارن بين نمط SDS-PAGE للسائل الجريبي للجاموس مع نظيره الموجود في الأبقار. ووفقاً لنمط الترحيل الكهربائي لخلايا الجاموس المبيضية, وجد أن هنالك عدة فروقات معنوية بين مراحل متنوعة من التطور. ومن الجانب الآخر, يمكن أن يستخدم الترحيل الكهربائي للمقارنة بين سائل الجريبي للجاموس ونظيره في الأبقار.

الكلمات المفتاحية: جاموس، ترحيل كهربائي، بيضة، نمط SDS-PAGE، صورة جانبية.

I. Introduction

Mammalian oocytes, especially of domestic animals are in great demand due to increasing importance for many experimental purposes, since they are utilized as recipient cells for transgenesis studies [Gupta *et al.*, 2005]. Although several papers described some biological fractions of follicular fluids [Roberts *et al.*, 1975; Vince *et al.*, 2004], buffalo ovarian proteins have received little attention with respect to electrophoretic techniques. However, it was noticeably known that there were significant changes in the activity of many oviductal components which interact with their final phenotype [Kille *et al.*, 1973]. Thus, these changes should be focused on with respect to the final perspective in the gel, since this technique is one of the most non-cost effective available techniques that are routinely used in detecting the least changes taking place in the final phenotype of the resolved protein [Laemmli, 1970].

Several stages of ovarian developments of some mammals, such as pigs [Kihara *et al.*, 2000; Huang *et al.*, 2002], and mare [Fahiminiya *et al.*, 20011] were studied extensively. These papers focused on many biochemical and physical changes of

ovarian follicles in several types of mammals [Cabrera *et al.*, 1985; Riley *et al.*, 2004; Spitzer *et al.*, 1996]. Though complete electrophoretic profile was made in mammals including humans [Anahory *et al*., 2002; Lee *et al*., 2005], the electrophoretic pattern of variable buffalo oocytes development was not focused on despite of the fact of the spreading of many accumulated papers that correlated with the monitoring of ovarian changes in variable growth stages in many related animals. Thus, this study aims to target the presence or absence of some proteins in electrophoretic protein profile among variable follicular development stages. Add to that, this paper focuses on the possibility of using the SDS-PAGE as a comparative tool between the follicular fluids of buffalo and their related bovine fluids.

II. Materials and Methods

2.1. Collection of samples: The genital tracts of mature buffaloes slaughtered in Babylon city at unknown times of the oestrous cycle were excised within a few minutes of slaughter and the ovaries were dissected out. Care was taken to prevent contamination with blood and transported to the laboratory while stored in ice. According to diameters and appearance, different developmental stages oocytes membrane proteins were collected. In addition to Corpus Luteum, Fallobian tubules, and ovarian follicles were collected from buffalo ovarian sections. In the later case, the mechanical method of Alpizer and Spicer was used [Alpizer and Spicer, 1993], in which follicles were pierced by 3 ml medical syringes and follicular fluid was aspirated. However, in each case, small pieces of each sample were taken carefully and placed in a sterile centrifuge tube, and immediately kept under -20˚C until processing.

2.2. Lysis of Ovarian samples for electrophoresis: Ovarian samples were lysed by Chang and Cseke method [2004]. All samples were treated with equal volume of denaturing loading buffer in reduced conditions (0.09 M of Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 1% of bromophenol blue, 0.1 M of beta-mercaptoethanol) and immediately minced into small pieces by a sterile blade. Samples were boiled for 4 min before being loaded into each lane. Only blood free samples were considered to prevent contamination. Samples were centrifuged for 30 min at 13500 xg in microcentrifuge to prevent cellular debrises. The supernatant was aspirated and placed in a sterile microcentrifuge tubes.

2.3. Electrophoresis Samples by SDS-PAGE: Proteins were separated using the mini vertical electrophoresis system, version *EcoMini E System* (*Biometra* – Germany). The discontinuous Laemmli (SDS-PAGE) method was applied [1970]. Electrophoresis of buffalos ovary proteins was performed using 10% separating gel {10% of 30:0.8% acrylamide\bis acrylamide, 1.5M tris-Cl pH8.8, 0.4% (w/v) SDS}, and 6% stacking gel {6% of 30:0.8% acrylamide\ bisacrylamide, 1M tris-HCl pH6.8, 0.4% (w/v) SDS}. From 1 µg into 10 µg of samples loaded were by mixing 1:1 V\V with sample loading buffer. Molecular weight pre-stained standards were also routinely loaded (Bioneer Cat # D-2010). Loaded samples were electrophoresed in 1X of running buffer {25 mM Tris pH 8.3, 250 mM glycine, 0.1% (w/v) SDS} in vertical electrophoresis tank at 120V and 30 mA. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. At the end of migration, gels were stained by two techniques, Coomassie blue and silver nitrate.

2.4. Staining by Coomassie Brilliant Blue: The polyacrylamide gel was stained using Coomassie brilliant blue method [Candiano *et al.*, 2004]. The gel was stained by submerging in the staining solution {0.025% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol, and 10% (v/v) glacial acetic acid in deionized water} for 2 hours.

Then, the de-staining buffer {40% (v/v) methanol, and 10% (v/v) glacial acetic acid in deionized water} was used to remove excessive stain. The destainer was repeatedly used until obtaining the clearest background. The polyacrylamide gel was placed on a white plate. And the Photo of the gel was taken by 16 M.P. digital camera (Sony – China).

2.5. Staining by Silver Nitrate: Coomassie stained gels were stained by silver nitrate [Hames, 1998]. After electrophoresis, the stacking gel layer was removed. The polyacrylamide gel was submerged in gel fixing solution {ethanol 40% (v/v), glacial acetic acid 10% (v/v)} for 30 min. After removing the fixer, the sensitizing solution {30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, and 0.125% (v/v) glutaraldehyde} was added, the gel was incubated in the sensitizer for 30 min. Thereafter, the gel was washed three times with bi-distilled water. The gel was stained by silver staining solution {0.25% (w/v) silver nitrate, and 0.015% (v/v) formaldehyde} for 20 min. After discarding the staining solution, the gel was rinsed twice with bi-distilled water. The developer solution {2.5% (w/v) sodium carbonate, and 0.0074% (v/v) formaldehyde}was added with gentle agitation for approximately 4 min, or until the desired bands were developed and until the entire intensity was reached. Once the desired bands were obtained, developing solution was discarded rapidly, and stop solution {1.5% (w/v) disodium EDTA} was added. The polyacrylamide gel was placed on a white plate. And the Photo of the gel was taken by 16 M.P. digital camera (Sony – China).

III. Results And Discussion

In the present study, the macromolecular components of buffalo ovarian lining cells with particular emphasis on the developmental changes were studied by submitting ovarian components to SDS-PAGE. Thus, several experiments were performed to monitor the age of each ovarian stage using SDS-PAGE. The tiny changes among the variable ovarian stages of buffalo were observed on the polyacrylamide gels after subjecting them to electrophoresis. The noticed bands in protein profile was reflected a proportional increase according to the maturity of each individual stage (Fig. 1). However, since polyacrylamide gel electrophoresis is very sensitive to any tiny changes in protein profile, two variable concentrations of ovarian proteins were applied. In the first one, low concentration ovarian proteins were electrophoresed on SDS-PAGE (Fig. 1). However, application of Coomassie brilliant blue R-250 was not sensitive enough to these changes (Fig. 1, A). As long as the Coomassie stained gel is ready to be stained with silver nitrate and since the staining of gel with silver nitrate is much more sensitive than the staining with Coomassie in many folds [Hames, 1998; Bassam *et al*., 1998], the same gel was exposed to silver staining protocol in order to detect these tiny changes (Fig. 1, B).

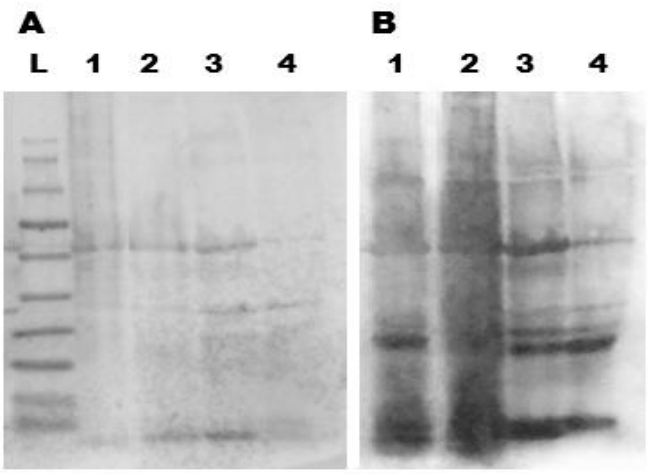

Fig. 1: Comparison of low concentrated ovarian oocyte membranes of buffalo in different developmental stages and other related samples. two dyes (A Comassie blue R-250 and B silver nitrate) stained SDS-PAGE of variable oocyte maturation stages. Lane L: prestained protein ladder, Lane 1: fallobian tubules, Lane 2: Corpus Luteum, Lane 3: tertiary oocytes, Lane 4: secondary oocytes.

Concerning oocytes, the appearance of some additional bands was obvious as long as oocytes develop from secondary to tertiary stage (Fig. 1). This result agrees with the observed changes in protein profile during follicular development in mares [Spitzer *et al.*, 1996].

Indeed, it was obviously recognized that several changes were noticed in protein profile of ovary of buffalo (Fig. 1), in which, an increasing in protein bands numbers by time during development of follicles progressing was observed (Fig. 2A). Consequently, the numbers of detected bands were increased. Although no concomitant reports focused on this electrophoretic progression in buffaloes, the numbers of protein bands were clearly increased. However, the using of high concentration samples was easily detected by Coomassie staining method (Fig. 2A), while the using of silver staining was not highly recommended in this case (Fig. 2B). However, the utilization of silver nitrate was potent enough to detect some faint bands in Corpus Luteum, tertiary and secondary oocytes membrane proteins respectively that weren't detected by Coomassie stain (Fig. 2B). Accordingly, the using of one gel, double-staining techniques are much more potent in detecting electrophoretic profile of ovarian proteins.

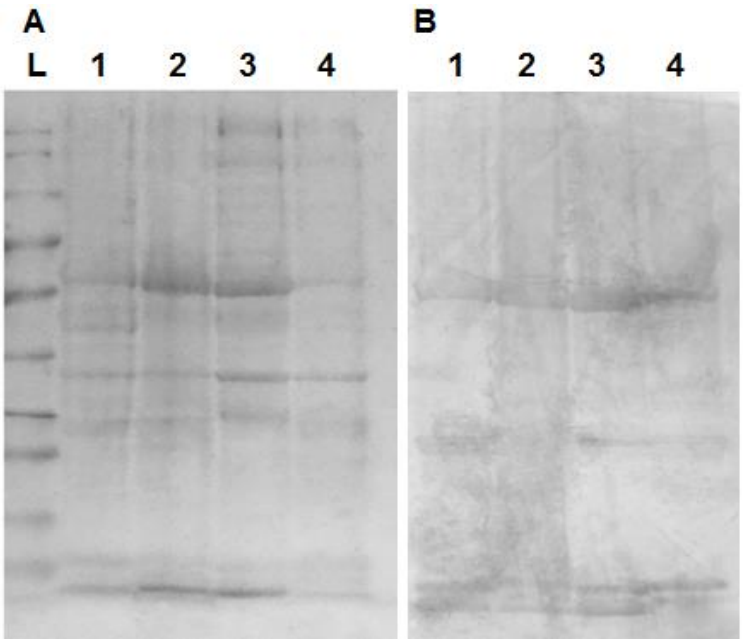

Fig. 2: Comparison of high concentrated ovarian oocyte membranes and other related samples of buffalo in different developmental stages. Two dyes (A; Comassie blue R-250 and B; silver nitrate) stained SDS-PAGE of variable oocyte maturation stages. Lane L: prestained protein ladder. Lane 1: ladder, Lane 2: fallobian tubule, Lane 3: Corpus Luteum, Lane 4: tertiary oocyte membrane proteins.

In addition to the estimation of the progress of ovarian cells of buffalo's samples, a concomitant electrophoretic fluid monitoring of follicular fluids were performed. Thus, variable concentrations of follicular fluids were compared between buffalo and bovine (Fig. 3). In high concentration Coomassie blue stained gels, no obvious differences were obtained (Fig. 3, A). While, the using of lower concentrations with silver nitrate stained gels insures the appearance of several differences between the two cases (Fig. 3, B). It deserves to be noted that high concentration Coomassie stained gels are not amenable to staining with silver, since the sensitivity of silver nitrate for dense bands was not as high as faint bands [Rabilloud, 1990].

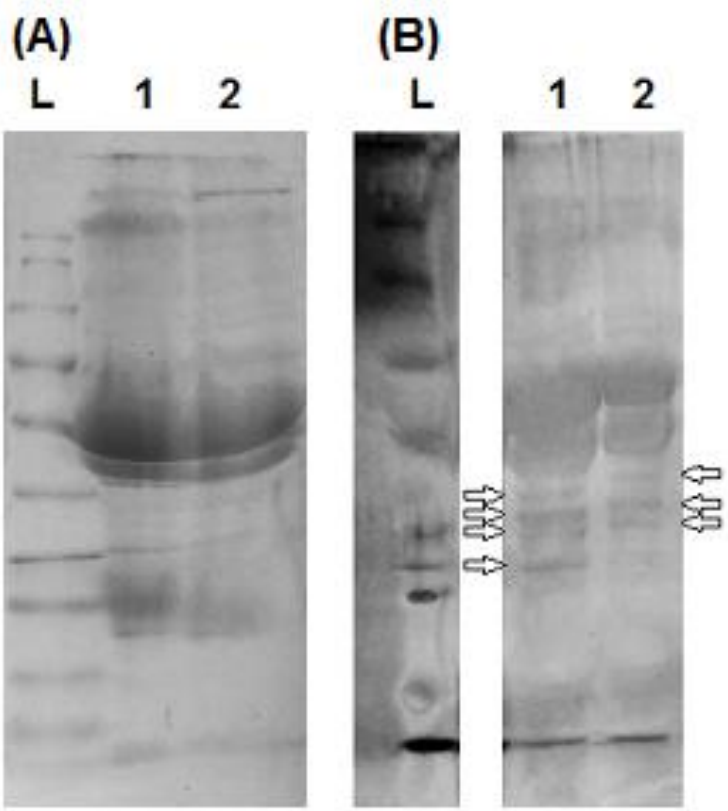

Fig. 3: Follicular fluids comparison between bovine and boffalo as determined by SDS-PAGE. (A); high concentration follicular fluids stained by Coommasie blue dye. (B); low concentrations follicular fluids stained by silver nitrate. Lane L: prestained protein ladder, Lane 1 and 2: low concentrations of follicular fluids for buffalo and bovine respectively.

IV. Conclusion

In conclusion, two significant notes were observed in this report, the first one correlated with the increasing of protein bands as long as oocytes develop in age. Accordingly, it might be possible to determine the age of oocytes electrophoretically using much more standardized criteria such as 2 dimensional gel electrophoresis [Moura *et al.,* 2006]. The second observation was made after the observation of obvious differences between buffalo and its cows corresponding follicular fluids. These electrophoretic differences might pave the way for more evolutionary studies by considering SDS-PAGE as a diagnostic tool to differentiate among variable types of amniotic fluids of mammals.

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