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The comparison of the effectiveness of the criterions used to determine the *in vitro* maturation of bovine oocytes

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Summary: In this study, totally 200 bovine oocytes aspirated from the slaughtered ovaries were used as the material. *In vitro* maturation process was carried out in bovine serum albumine added (%0.6) tissue culture media at 39 C° under 5% CO_2 atmosphere for 24 hours. *In vitro* fertilization was carried out in modified Tyrode's albumine lactate pyruvate media with similar conditions for 20 hours. Oocytes were placed into seperated-tagged petri dishes in ten-a-piece and every oocytes were evaluated for the cumulus expansion degrees (none-0, slight-1 and progressive-2), extursion of polar body (none-0 and existence-1) and fertilization (male and female pronuclei absence-0 and existence-1). After the fertilization process, 91 of 200 oocytes were fertilized (45.5%). Fertilization was observed in 4 of 42 unexpanded oocytes (9.5%) ($p<0.05$), although 42 of 95 progressively expanded oocytes did not showed fertilization (44.2%) ($p<0.01$). Harmoniously, fertilization was observed in 5 of 63 oocytes not extured polar body (7.9%) ($p<0.01$), while 46 of 135 polar body observed oocytes did not fertilized (%34.1) ($p<0.01$). The results of cross-matching evaluation of mentioned criterions showed that none of the 29 unexpanded and polar body unapparented oocytes were fertilized ($p<0.001$) and 24 of 74 progressively expanded and polar body detected oocytes were not fertilized (%32.4) ($p<0.01$). As a result, the predefined criterions (cumulus expansion degree, polar body extursion) were unsufficient in acceptable maturation consideration both individually and cross-matching as positive errors could be done and it was evident that the best maturation criterion was the fertilizability.

Key words: Bovine, cumulus expansion, *in vitro* maturation, oocyte, polar body

Sığır oositlerinin *in vitro* maturasyonunun belirlenmesinde kullanılan kriterlerin etkinliğinin karşılaştırılması

Özet: Çalışmada, mezbahadan toplanan ovaryumlardan aspire edilen toplam 200 sığır oositi kullanıldı. *In vitro* maturasyon, %0.6 sığır serum albumini katılmış doku kültürü vasatı içerisinde, 39°C'de, %5 CO_2 atmosferinde 24 saatte gerçekleştildi. *In vitro* fertilizasyon işlemi ise modifiye Tyrode'nin albumin laktat piruvat vasatında aynı inkubasyon koşullarında 20 saatte gerçekleştirildi. Çalışmaya alınan oositler 10'arlı gruplar halinde işaretli petrilerde inkübe edilerek, her oosit, maturasyon sonrası kumulus ekspansiyon derecesi (yok-0, hafif-1, ileri-2), kutup hücresinin görülmesi (yok-0, var-1) ve fertilizasyonun ortaya konması (dişi-erkek pronukleuslar yok-0, var-1) açısından değerlendirildi. Çalışma sonucunda, *in vitro* maturasyon ve fertilizasyon işlemine alınan toplam 200 oositten 91'inde (%45.5) fertilizasyon saptanmıştır. Kumulus ekspansiyonunu göstermeyen 42 oositten 4'ünde fertilizasyon gözlenirken (%9.5) ($p<0.05$), aksine kumulus ekspansiyonu ileri düzeyde olan 95 oositten 42'sinde (%44.2) ($p<0.01$) fertilizasyon şekillenmemiştir. Benzer şekilde, kutup hücresi görülmeyen 63 oositten 5'inde (%7.9) ($p<0.01$) fertilizasyon şekillenirken, kutup hücresi belirlenen 135 oositten 46'sında (%34.1) fertilizasyon bulgusuna rastlanmamıştır ($p<0.01$). Bu iki kriterin beraber incelenmesi sonucu kumulus hücre ekspansiyonu olmayıp, kutup hücresi görülmeyen 29 oositten hiçbirinde (%0) fertilizasyon tespit edilemezken ($p<0.001$), ileri derecede ekspansiyon gösterip, kutup hücresi belirlenen 74 oositten 24'ünde (%32.4) fertilizasyon saptanamamıştır ($p<0.01$). Sonuç olarak, kullanılan kriterlerin tek başına maturasyonun ortaya konmasında yeterli olamadığı, beraber kullanılmaları durumlarında dahi pozitif hatalar meydana geldiği ve en doğru maturasyon yeteneği belirleme yönteminin fertilizasyon olduğu kanaatine varıldı.

Anahtar sözcükler: *İn vitro* maturasyon, kumulus ekspansiyonu, kutup hücresi, oosit, sığır

Introduction

Techniques for producing embriyos by *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) processes are now being used in many laboratories worldwide. Viable embriyos can be produced from oocytes by aspiration of follicules or by slicing the ovaries (1, 6).

IVM is the most important and critical section of in vitro embriyo production, as it is known to be a faster mimicking of the processes forming in vivo. Oocyte maturation is a part of the oogenesis and folliculogenesis and defined as the phase where the ability to be fertilized and to form a viable individual is gained. However, only a limited rate of the oocytes maturated in vitro could

reach the blastocyst stage (transfer stage). Since, this dilemma oriented the researchers towards some early criterions (before fertilization) to determine the fertilizability of the maturated oocytes. The mean problem considered by most investigators is which oocyte could be used in the embryo production process. Some investigators accept the cumulus expansion degree and polar body extursion as the defined criterions of the IVM. But as the percentage of the reaching of the blastocyte stage of the expanded and polar body exturted oocytes is different and lower than the maturation accepted oocytes, there should be another criterion for the maturation assention (11,12).

The objective of this study was to compare the criterions of the acceptable maturation. These criterions are cumulus expansion degree, polar body extursion and *in vitro* fertilization.

Materials and Methods

Ovary collection and oocyte aspiration

In this study, totally 200 Grade I and II (> 2-3 layers of cumulus cell and homologous ooplasm) bovine oocytes aspirated from the ovaries collected from Çubuk municipality slaughterhouse (Ankara), were used as the material. Ovaries were taken to the laboratory in the antibiotic added (Sigma- A5955) transport media-filled-thermoses within 5 hours. The aspiration was done with 18 G needle attached 5 ml syringe. Approximatelly 0.5 ml of the maturation media was taken into the syringe before the aspiration.

In vitro maturation

In vitro maturation was carried out in bovine serum albumine (Sigma- A1933) added (%0.6) tissue culture media-199 (Sigma- M2520) was equilibrated in 39° C for 3 hours before the experiment starts. The process was done at 39° C under 5% CO_2 atmosphere for 24 hours and the oocyte-media complex was covered with mineral oil (Sigma- M8410). After the maturation the oocytes were washed with HEPES added fertilization media and the oocytes were placed into seperated-tagged petri dishes in ten apiece and every oocytes were evaluated for the maturation criterions.

In vitro fertilization

The oocytes were placed into the modified Tyrode's Albumine Lactate Pyruvate media (TALP) (T2397-S, Sigma Co., EU) for *in vitro* fertilization. Meanwhile, the swim-up selection (Gordon, 1994) was carried out with the frozen/thawed sperma for the selection of the motile ones. IVF was carried out in oocyte and 10 µg/ml heparin (for capacitation) added TALP with similar conditions of the IVM but for 20 hours. For every 20 oocyte, 20 µl motile and capacitated sperma was added.

Experimental design

As shown in details in Figure 1, the oocyte groupings were conducted in the course of the study by; expansion degrees (none-CE0, slight-CE1 and progressive-CE2), extursion of polar body (none-PB0 and existence-PB1) and fertilization (absence-F0 and existence-F1).

Statistical evaluation

The statictical evaluation and comparison of the results were done by the Chi-square test.

Results

As mentioned before different maturation rates were observed by the prediction of 3 different criterions (cumulus expansion only, polar body extursion only and both) (Table 1) (Figure 2).

Table 1. Maturation rates observed in study groups with different criterions
Tablo 1. Çalışma gruplarında değişik kriterlere göre elde edilen maturasyon oranları

Maturation criterion	Maturation
Cumulus expansion only (Group 1)	158/200 (79%)[a]
Polar body extursion only (Group 2)	135/200 (67.5%)[b]
Both (Group 3)	122/200 (61%)[c]

[a,b,c] Different superscripts in same column shows statistically difference ($p<0.05$)

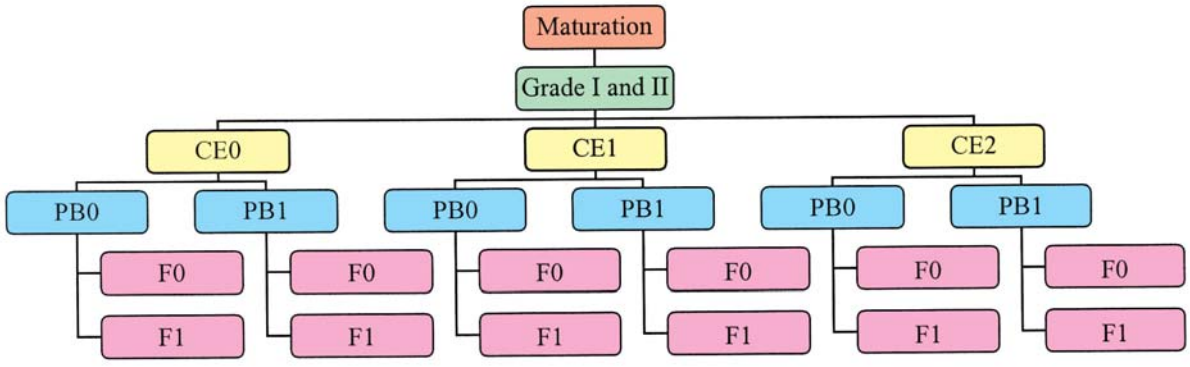

Figure 1. Experimental and statistical design of the study
Şekil 1. Çalışmanın deneysel ve istatistiksel düzeni

As seen in Table 1, the maturation rates in oocytes were found 79% (158/200) when evaluated by cumulus expansion (CE) criterion, where the same rate was 67.5% (135/200) by polar body extursion (PB) criterion and a statistical difference was observed ($p<0.05$). On the other hand, maturation detection results were statistically lower (122/200, 61%) ($p<0.05$) in cross evaluation (with both CE and PB) when compared with individual criterions (Figure 2).

The polar body extursion rates were significiantly higher in moderate (CE1) and fully expanded (CE2) oocytes when compared with unexpanded (CE0) oocytes ($p< 0.001$) (Table 2).

The fertilization rates were found significiantly higher in moderate (CE1) and fully expanded (CE2) oocytes when compared with unexpanded (CE0) oocytes ($p< 0.001$) (Table 3). Contrary any statistical difference were determined between moderate and fully expanded oocytes.

Table 2. Relation between cumulus expansion and polar body extursion
Tablo 2. Kumulus ekspansiyonu ile kutup cisimciğinin atılması arasındaki ilişki

Cumulus expansion	Polar body extursion		
	(0)	(1)	Total
(0)	29 (69%)[a]	13(31%)[a]	42
(1)	15(23.8%)[b]	48(76.2%)[b]	63
(2)	21(22.1%)[b]	74(77.9%)[b]	95
Total	65	135	200

(Chi-square=32,421. a,b: p<0,001)

Table 3. Relation between cumulus expansion and fertilization sucsess
Tablo 3. Kumulus ekspansiyonu ile fertilizasyon başarısı arasındaki ilişki

Cumulus expansion	Fertilization		
	(0)	(1)	Total
(0)	38(90.5%)[a]	4(9.5%)[a]	42
(1)	30(47.6%)[b]	33(52.4%)[b]	63
(2)	41(43.2%)[b]	54(56.8%)[b]	95
Total	109	91	200

(Chi-square=28,053. a,b: p<0,001)

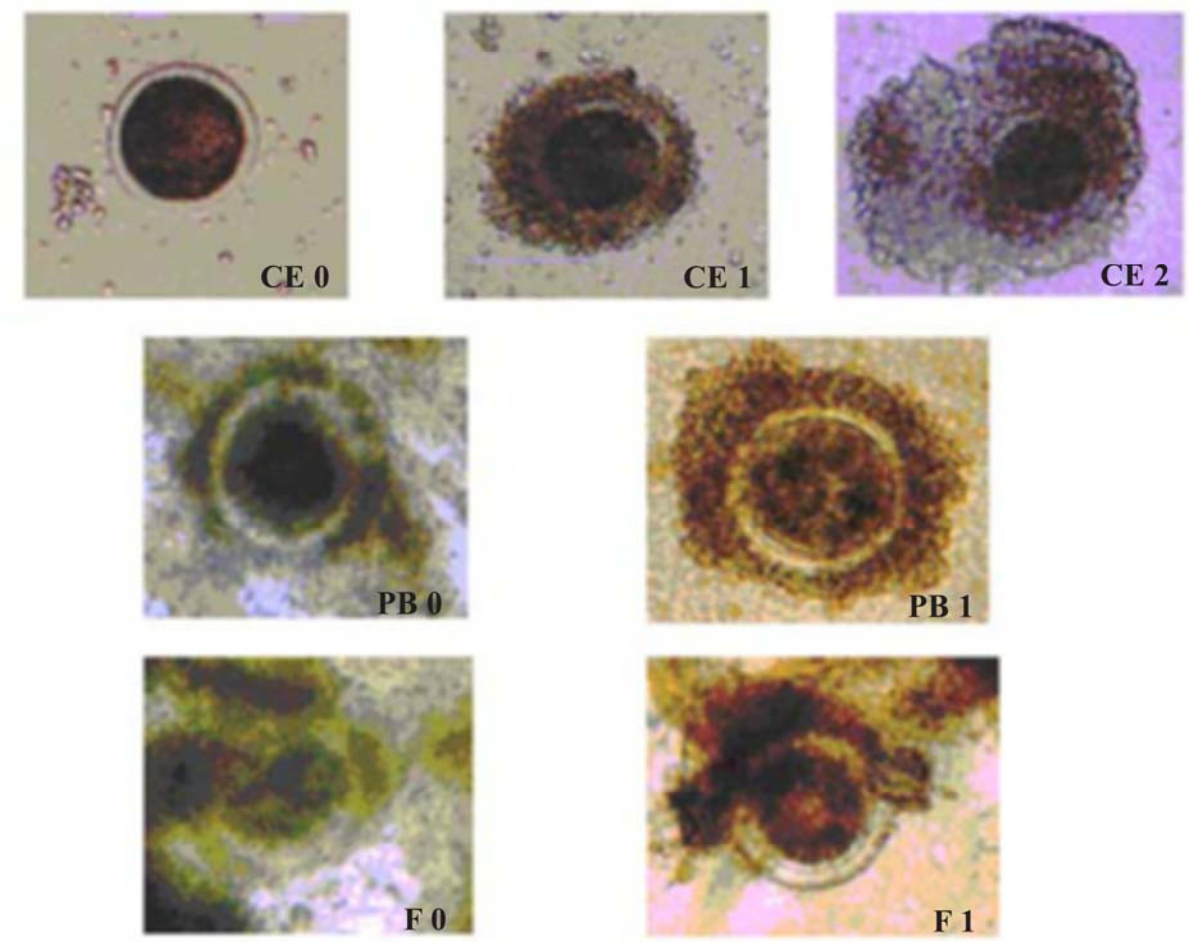

Figure 2. Different expansion degrees (none-CE0, slight-CE1 and progressive-CE2), extursion of polar body (none-PB0 and existence-PB1) (Narrow showing polar body) and fertilization (absence-F0 and existence-F1) (Narrows showing pronucleuses).
Şekil 2. Değişik ekspansiyon dereceleri (yok-CE0, hafif-CE1 ve ileri-CE2), kutup cisimciğinin atılması (yok-PB0 ve görülmesi-PB1) (oklar kutup cisimciğini gösteriyor) ve fertilizasyon (yok-F0 ve görülmesi- F1) (Oklar pronukleusları gösteriyor).

A statistical difference were found between polar body unexturded (PB0) and exturded (PB1) oocytes when compared by means of fertilization success ($p<0.001$) (Table 4).

Table 4. Relation between polar body extursion and fertilization sucsess
Tablo 4. Kutup hücresi ile fertilizasyon başarısı arasındaki ilişki

Polar body extursion	Fertilization		
	(0)	(1)	Total
(0)	60(92.3%)[a]	5(7.7%)[a]	65
(1)	49(36.3%)[b]	86(63.7%)[b]	135
Total	109	91	200

(Chi-square=55,509. a,b: p<0,001)

After the fertilization process, 91 of 200 oocytes were fertilized with a fertilization rate of 45.5%. In Group 1, the overall fertilization rate was found as 55.1% (87/158) (Table 3), whereas fertilization success was 63.7% (86/135) in Group 2 ($p<0.01$) (Table 4). However same parameter was significantly higher in Group 3 (82/122, 67.2%) (Table 5) ($p<0.05$) (Figure 2).

Fertilization was observed in 4 of 42 unexpanded oocytes (9.5%) ($p<0.001$), although 41 of 95 progressively expanded oocytes did not showed fertilization (43.1%) ($p<0.001$). Harmoniously, fertilization was observed in 5 of 65 oocytes not extured polar body (7.7%) ($p<0.001$), while 49 of 135 polar body observed oocytes did not fertilized (%36.2) ($p<0.001$). The results of cross-matching evaluation of mentioned criterions showed that none of the 29 unexpanded and polar body unapparented oocytes were fertilized ($p<0.001$) and 24 of 74 progressively expanded and polar body detected oocytes were not fertilized (%32.4) ($p<0.001$) (Table 5).

Table 5. Fertilization rates when examined with both criterions (CE and PB)
Tablo 5. Tüm kriterlerle (CE ve PB) değerlendirildiğinde fertilizasyon başarısı arasındaki ilişki

Maturation criterion	Fertilization		
	(0)	(1)	Total
CE0-PB0*	29 (100%)	0 (%)	29
CE0-PB1*	9 (69.3%)	4 (30.7%)	13
CE1-PB0*	14 (93.3%)	1 (6.7%)	15
CE1-PB1	16 (33.3%)[a]	32 (66.7%)[a]	48
CE2-PB0	17 (81%)[b]	4 (19%)[b]	21
CE2-PB1	24 (32.5%)[a]	50 (67.5%)[a]	74
Total	109(64.5%)	91(45.5%)	200

(Chi-square=17,348. a,b: p<0,001; * Chi-square test could not be performed as the data in the former rows-1,2 and 3- were low)

Discussion and Conclusion

Hoshi (4) has reviewed the post-maturational changes in the basis of cumulus expansion degree (gained volume to all dimensions) and considered the cumulus expansion as the outer-sign of the maturation, while giving some doubts on the qualifiedness. In addition, Nandi et al. (7) have stated that the most-used criterion for maturation evaluation in buffalo and bovine oocytes.

Hunter and Moor (5) divided the maturated cumulus-oocyte complexes according to the expansion rates (fully expanded- expanded 3 times of oocyte diameter, moderate expanded- expanded 2 times of oocyte diameter and slightly expanded- attached cumulus cells to zona pellucidae) and found a strong positive relation between fertilization rates and cumulus expansion rates.

Gordon and Lu (3) have notified that cumulus expansion should not be used individually for estimating the maturation as unexpanded oocytes could be fertilized, where some of the unfertilized oocytes could be fully expanded. Similarly, Gordon (2), informed the same insufficiency of cumulus expansion degree, but this paradox would be reached over by taking the care on first polar body extursion as well.

Shamsuddin et al. (10) set forth the result that the GVBD occured oocytes could be fertilized, since the time interval between GVBD and first polar body extursion is very fast and hard to decide which oocyte is maturated or going to be.

Ün (13), has reported that 31% of unexpanded and 18,5 % of unextruded first polar body oocytes were fertilized and stated the fact that the real evaluation of maturation is fertilization. Moreover, the recent studies have shown that it is more proper to estimate the maturational competence with the embriyonic development competence or better, with the number of alive calves 3 months after birth (4, 8, 9).

In the present study, similar with all workers above, fertilization was observed in 4 of 42 unexpanded oocytes (9.5%) ($p<0.001$), although 41 of 95 progressively expanded oocytes did not showed fertilization (43.1%) ($p<0.001$). Harmoniously, fertilization was observed in 5 of 63 oocytes not extured polar body (7.9%) ($p<0.001$), while 49 of 135 polar body observed oocytes did not fertilized (%36.2) ($p<0.001$). The results of cross-matching evaluation of mentioned criterions showed that none of the 29 unexpanded and polar body unapparented oocytes were fertilized ($p<0.001$) and 24 of 74 progressively expanded and polar body detected oocytes were not fertilized (%32.4) ($p<0.01$).

As a result, the predefined criterions (cumulus expansion degree, polar body extursion) were

unsufficient in acceptable maturation consideration both individually and cross-matching as positive or negative errors could be done and it was evident that the best maturation criterion was the fertilizability.

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