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Research Article

The Effects of Various BSA Levels in Different Media on Development in In Vitro Culture of Mouse Embryos*

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Abstract: Various BSA (bovine serum albumin) levels and media effects on development of 2-cell stage mouse embryos in in vitro culture were investigated in this 2 stage study. In the first stage, 1400 2-celled mouse embryos were cultured in 2 various BSA-bearing culture media for 96 h and directly observed microscopically. In the second stage, 1024 2-celled mouse embryos cultured for the same period and cell numbers (nuclei of cells) were determined by staining techniques.

Embryos were cultured in both stages in M 16 and Whitten's media containing 0 (control), 0.3, 1, 3, 9, 18 and 36 mg/ml of BSA. The highest blastocyst rate was 94.57% ± 7.43, observed in 3 mg/ml BSA-containing Whitten's medium group ($P < 0.05$). The highest hatching + hatched blastocyst rates were obtained in the same group with 67.70% ± 25.22.

The highest nucleus number in the M 16 medium was in the 1 mg/ml BSA group (95.45 ± 9.61). In the M16 medium group, the 3 mg/ml BSA group showed the highest development rate in the first stage of the study; however, the nucleus numbers in this group (81.37 ± 18.31), were significantly lower than the nucleus numbers of the 1 mg/ml BSA group ($P < 0.05$).

It was concluded that the optimal benefit to in vitro culture of 2-cell mouse embryos could be maintained when BSA was used as a protein source at 1 mg/ml and 3 mg/ml doses, and that M16 or Whitten's media could be used successfully as a culture medium. Developmental evaluations of in vitro cultured mouse embryos could be confusing when only the native route was employed, and a staining technique could give more reliable results.

Key Words: Mouse, embryo, medium, BSA, protein

Fare Embriyolarının İn Vitro Kültüründe Değişik Medyumlardaki Farklı BSA Oranlarının Gelişime Etkisi

Özet: İki aşamadan oluşan bu çalışmada, iki hücreli fare embriyolarının in vitro kültüründe faklı BSA (bovine serum albümin) oranlarının ve medyumların etkisi araştırıldı. İki hücreli fare embriyoları birinci aşamada (1400 adet), çeşitli oranlarda BSA içeren iki farklı medyumda 96 saat süreyle kültüre edildi ve ardından gelişim düzeyleri direkt mikroskobik bakı ile kontrol edildi. İkinci aşamada ise 1024 adet iki hücreli fare embriyosu aynı süreyle kültüre edildi ve boyama teknikleri kullanılarak embriyoların gelişim devreleri ve hücre sayıları (hücre çekirdek sayıları) kontrol edildi.

Embriyolar her iki aşamada, M 16 ve Whitren's medyumlarının 0 (kontrol), 0.3, 1, 3, 9, 18 ve 36 mg/ml miktarlarında BSA içeren gruplarında kültüre edildi. En yüksek blastosist oranı Whitten's medyumunun 3 mg/ml BSA içeren grubunda % 94,57 ± 7,43 olarak saptandı ($P < 0,05$). En yüksek hatching + hatched blastosist oranı % 67,70 ± 25,22 ile yine aynı grupta gözlendi.

Çalışmanın ilk aşamasında M 16 medyumu için saptanan en yüksek gelişim oranı, 3 mg/ml BSA içeren grupta saptanmıştı. Oysa çalışmanın ikinci aşamasında, M 16 medyumu için belirlenen en yüksek hücre çekirdek sayısının (hücre sayısı) 1 mg/ml BSA grubunda olduğu (95,45 ± 9,61) ve bunun 3 mg/ml grubundaki değerden (81,37 ± 18,31) daha üstün olduğu belirlendi ($P < 0,05$).

Sonuç olarak denilebilir ki, iki hücreli fare embriyolarının in vitro kültüründe protein kaynağı olarak BSA'nın 1 ve 3 mg/ml dozlarda optimum derecede yararlı etkiler sağladığı ve kültür medyumu olarak gerek M 16 ve gerekse de Whitten's kültür medyumlarının başarılı bir şekilde kullanılabileceği söylenebilir. Fare embriyolarının gelişimsel muayenelerinde natif muayene yönteminin yanıltıcı sonuçlar verebildiği ve hücre boyama tekniğinin daha sağlıklı sonuçlar verdiği söylenebilir.

Anahtar Sözcükler: Fare, embriyo, medyum, BSA, protein

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Introduction

Medium is a critical factor in the in vitro culture of mammalian embryos (1-3). It was reported that it is necessary to add proteins to the in vitro culture media to meet the needs of embryos, and bovine serum albumin (BSA) and fetal calf serum (FCS) like animal origin sera could be added for this purpose, although not more than 10%, otherwise it could be detrimental to the embryos (4,5).

Researchers have used various protein sources in various quantities for the in vitro culture of mouse embryos, and have achieved various results (6 -12).

In the in vitro culture of mouse embryos, sera proteins, especially BSA, have been reported to support development thanks to their trace elements such as fatty acids. BSA also captures toxic and inorganic ions and mediates CO_2 transport (2,12-14).

Although morphological examination is important in the evaluation of embryonic development, subjective criteria and confusing results might be obtained. The ideal examination is therefore achieved by counting the cell nuclei (15).

This study was planned to culture in vivo developed 2-cell stage mouse embryos in vitro for 96 h, to observe the in vitro developmental stages and to determine the best medium for embryonic development, the effects of various BSA levels and the optimal BSA amount.

Materials and Methods

A total of 122 female CB6 hybrid (F_1) mice, 6-8 weeks of age, were used in the study. Two-cell stage embryos with a normal appearance, collected by means of M 2 flushing medium, were passaged 3 times with gas equilibration (CO_2 5%, O_2 5%, N_2 90%) and without BSA culture medium to eliminate the BSA traces of M 2 medium. Two blastomere embryos were incubated in M 16 and Whitten's media groups containing respectively 0 (control), 0.3, 1, 3, 9, 18 and 36 mg/ml BSA under identical circumstances (a CO_2 5%, O_2 5%, N_2 90% gas environment and nearly 100% humid incubator at 37 ºC) for 96 h.

The study was carried out in 2 stages. The first stage consisted of the recovery and culture of embryos in various BSA - bearing different culture media for 96 h and of developmental checks by direct microscopy (native examination) every 24 h. At this 2-cell stage, embryos were collected and 14 treatment groups were established, 7 for each media group as mentioned above. The effects of BSA as a protein source on the in vitro development of embryos, and its optimal concentration, were tested in this way.

In the second half of the study, since the native examination technique can only provide information about the morphology of the embryos, a calculation of cell nuclei counts (cell numbers) was employed to make a detailed determination of the developmental stages of in vitro cultured embryos. For this calculation embryos were stained with 2% aceto-orcein dye in their different developmental stages, and the cell nuclei were counted by a phase-contrast microscope at x400 magnification.

Statistical analyses were done by using the t-test and ANOVA variance analysis technique.

Results

In the first stage of the study, 1400 2-cell stage embryos (Figure 1), 96 unfertilized oocytes and 33 degenerated cells were recovered from the oviducts. During the first 24 h of in vitro culture there was no statistically significant difference between the mean development rates of the M 16 and Whitten's media samples (Tables 1 and 2).

During the first 24 h there was no difference in development among the M16 and Whitten's media groups. The highest development rates during the 48, 72 and 96 h culture periods were observed in the 3 and 1 mg/ml BSA groups (Tables 1 and 2).

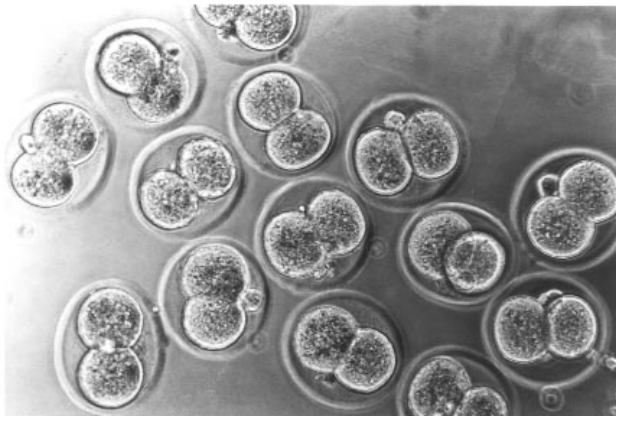

Figure 1. Two-cell stage mouse embryos recovered from the oviduct, x20 magnification.

Table 1. Average development rates of embryos cultured in M 16 medium.

Groups BSA (mg/ml)	Initial Embryo Numbers (n)	Developing Rates (%) *			
		24 h	48 h	72 h	96 h
0	100	100.00 ± 0.00 [a]	74.36 ± 33.07 [c]	40.26 ± 32.08 [d]	33.43 ± 30.69 [c]
0.3	100	100.00 ± 0.00 [a]	89.31± 20.25 [ab]	70.85 ± 28.87 [bc]	65.57 ± 27.44 [b]
1	100	100.00 ± 0.00 [a]	95.83 ± 14.43 [a]	85.92 ± 16.82 [a]	79.22 ± 16.64 [ab]
3	100	100.00 ± 0.00 [a]	100.00 ± 0.00 [a]	88.01 ± 16.06 [a]	84.74 ± 17.61 [a]
9	100	97.92 ± 7.22 [a]	87.95 ± 20.14 [ab]	78.41 ± 21.36 [ab]	77.02 ± 19.79 [ab]
18	100	100.00 ± 0.00 [a]	81.06 ± 23.44 [bc]	68.48 ± 30.56 [bc]	45.94 ± 32.20 [c]
36	100	98.81 ± 4.13 [a]	73.39 ± 29.22 [c]	63.59 ± 32.47 [c]	37.80 ± 26.07 [c]

[a, b, c, d] Vertical columns with different letters have significant differences between them ($P < 0.05$) (ANOVA variance analysis)

* Rates calculated considering initial embryo numbers.

Table 2 Average development rates of embryos cultured in Whitten's medium.

Groups BSA (mg/ml)	Initial Embryo Numbers (n)	Developing Rates (%) *			
		24 h	48 h	72 h	96 h
0	100	98.33 ± 5.77 [a]	88.23 ± 15.79 [abcd]	75.13 ± 30.53 [c]	63.88 ± 35.06 [c]
0.3	100	100.00 ± 0.00 [a]	94.04 ± 10.18 [ab]	89.35 ± 16.62 [ab]	88.00 ± 13.87 [ab]
1	100	98.81 ± 4.13 [a]	90.69 ± 13.20 [abc]	89.50 ± 12.92 [ab]	86.97 ± 13.29 [ab]
3	100	100.00 ± 0.00 [a]	96.52 ± 6.70 [a]	94.57 ± 7.43 [a]	94.57 ± 7.43 [a]
9	100	100.00 ± 0.00 [a]	84.58 ± 25.21 [cd]	78.33 ± 30.53 [bc]	78.33 ± 30.53 [b]
18	100	98.33 ± 5.77 [a]	85.97 ± 18.00 [bcd]	72.57 ± 18.65 [cd]	51.96 ± 17.10 [c]
36	100	98.61 ± 4.81 [a]	81.07± 21.31 [d]	60.29 ± 32.96 [d]	32.64 ± 22.70 [d]

[a, b, c, d, e] Vertical columns with different letters have significant differences between them ($P < 0.05$) (ANOVA variance analysis)

*. Rates calculated considering initial embryo numbers.

At the end of the culture period, when the highest ''hatching + hatched'' blastocysts (Figure 2) rates were compared among the groups containing the same BSA levels, the highest rate was observed in the 3 mg/ml BSA group (Table 3).

Fifty CB6 F_1 hybrid female mice were used in the second stage. The yields of this stage were 1024 2-cell stage embryos, 127 unfertilized oocytes and 79 degenerated cells.

In the medium M 16, at the end of the 96 h culture period, the highest cell count average was observed in the 1 mg/ml BSA group ($P < 0.05$) (Table 4).

In Whitten's medium, at the end of the culture period, the highest cell count average (Figure 3) was observed in the 1 and 3 mg/ml BSA groups ($P < 0.05$) (Table 5).

In the comparison of the M 16 and Whitten's media groups with same BSA content, the cell numbers of developing embryos at 24, 48, 72 and 96 h were similar (Tables 4 and 5).

Discussion

At the end of the in vitro culture, the best embryonic development was observed by native examination in the 3 mg/ml BSA groups of both M 16 and Whitten's media. In the high BSA-bearing groups, our results are in accordance with those of Kraemer and Bowen (5), who reported that a high BSA concentration in long - term in vitro culture impaired embryonic development by affecting the medium pH. Our results also supported the opinions of Brinster (4) and Saito et al. (9) that high BSA

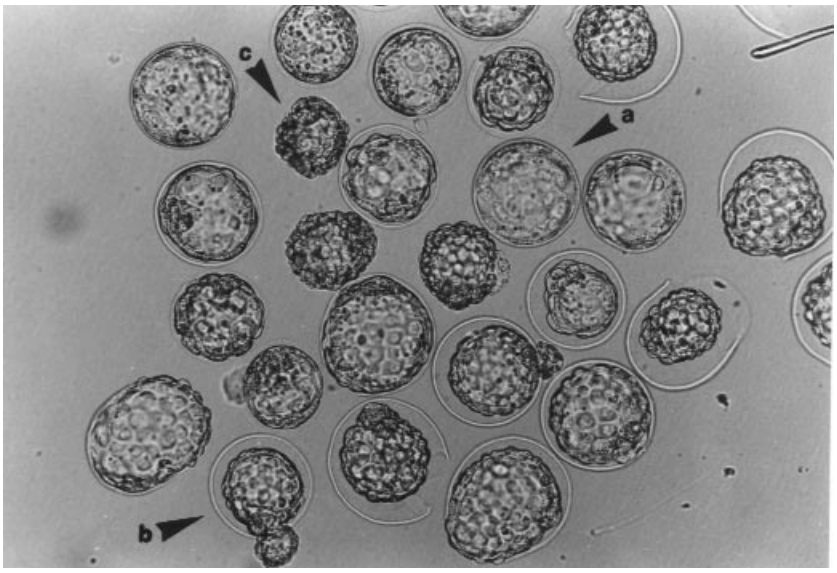

Figure 2. Embryos at various developmental stages x20 magnification.
a: Expanded blastcyst b: Hatching blastocyst c: Hatched blastocyst

Table 3. Hatching + hatched blasocyst rates among the media and groups at 96 h (%)*

Media	BSA (mg/ml)						
	0	0.3	1	3	9	18	36
M 16	9.72 ± 20.47[b]	18.83 ± 21.31[b]	34.05 ± 26.32[b]	44.34 ± 25.94[a]	20.61 ± 18.68[a]	3.27 ± 5.96[a]	5.42 ± 11.39[a]
Whitten's	36.30 ± 36.93[a]	32.55 ± 24.60[a]	55.31 ± 32.35[a]	67.70 ± 25.22[a]	19.05 ± 24.36[a]	0.83 ± 2.89[a]	1.67 ± 5.77[a]

[a,b] Vertical columns with different letters have significant differences between them (P < 0.05) (t test).

* Rates calculated considering initial embryo numbers

Table 4. Cell count averages determined at different times during the in vitro culture period in M 16 medium.

BSA mg/ml	M 16 Medium			
	*24 h	48 h	72 h	96 h
0	4.2 ± 2.14 [c]	11.44 ± 4.36 e	21.26 ± 11.65 [d]	29.6 ± 14.93 f
0.3	7.2 ± 1.6 [b]	23.45 ± 8.14 [c]	43.15 ± 11.88 [c]	69.94 ± 19.09 [c]
1	9.6 ± 3.2 [a]	43.65 ± 9.65[a]	69.60 ± 17.29 [a]	95.45 ± 9.61 [a]
3	7.8 ± 3.9 [b]	34.05 ± 10.61 [b]	57.65 ± 17.54 [b]	81.37 ± 18.31 [b]
9	4.2 ± 2.1 [c]	21.65 ±10.95 [c]	65.00 ± 16.56 [ab]	57.06 ± 23.15 [d]
18	3.8 ± 2.3 [c]	16.20 ±9.38 [d]	53.07 ± 23.84 [b]	58.69 ± 18.75 [d]
36	3.2 ± 1.0 [c]	17.58 ± 10.56 [d]	28.00 ± 11.96 [d]	47.35 ± 15.75 [e]

[a, b, c, d, e] Vertical columns with different letters have significant differences between them. (P < 0.05) (ANOVA variance analysis)

* During the first 24 h of in vitro culture, cell counts of the embryos were calculated by native examination.

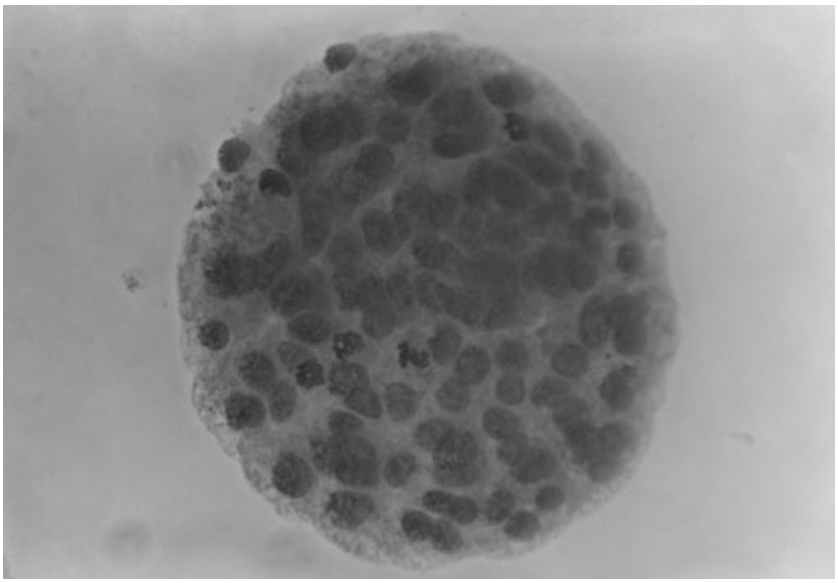

Figure 3. Stained cell nuclei of hatched blastocyst x40 magnification.

Table 5. Cell count averages determined at different times during the in vitro culture period in Whitten's medium.

BSA mg/ml	Whitten's Medium			
	*24 h	48 h	72 h	96 h
0	4.8 ± 1.64 [c]	15.84 ± 12.05 [d]	36.1 ± 6.05 [d]	33.41 ± 9.86 [e]
0.3	7.6 ± 1.23 [b]	31.2 ± 11.93 [c]	69.5 ± 9.61 [b]	80.88 ± 20.42 [b]
1	7.6 ± 3.40 [b]	67.72 ± 22.90 [a]	92.75 ± 7.18 [a]	105.60 ± 11.52 [a]
3	9.6 ± 5.09 [a]	47.18 ± 18.20 [a]	86.3 ± 6.73 [a]	107.65 ± 10.27 [a]
9	4.0 ± 2.51 [c]	23.83 ± 12.38 [c]	57.94 ± 27.18 [c]	60.87 ± 35.59 [c]
18	3.8 ± 2.33 [c]	26.61 ± 3.17 [c]	52.73 ± 34.36 [c]	52.06 ± 21.33 [d]
36	3.4 ± 0.94 [c]	27.07 ± 10.91 [e]	26.18 ± 19.23 [d]	40.83 ± 17.91 [e]

[a, b, c, d, e] Vertical columns with different letters have significant differences between them. ($P < 0.05$) (ANOVA variance analysis)

* During the first 24 h of in vitro culture, cell counts of the embryos were calculated by native examination.

levels damaged embryos during in vitro culture and that very low levels had no effect on embryonic development. In the comparison of hatching + hatched blastocyst rates of embryos cultured in various BSA-containing groups of M 16 and Whitten's media, the highest levels were obtained in groups of both media containing 3 mg/ml BSA. This is in agreement with the findings of Millham et al. (2), who reported that sera or sera fractions enhanced the hatching and hatched blastocyst rates in in vitro mouse embryos.

The cell count results of embryos cultured in Whitten's medium groups were mainly in agreement with native examination results in the same group. On the other hand, in the study in which the cell counts of embryos cultured in M 16 medium were determined, cell count means in the 1 mg/ml BSA group were superior to all other groups ($P < 0.05$). This result agrees with the report of Willey et al. (14) that native examination alone is not a sufficient and satisfactory way to determine the developmental stages of embryos.

The conclusion of this study suggests that both M 16 and Whitten's media can be used for the in vitro culture of 2 - cell stage mouse embryos that native examinations (direct observation) alone can give confusing results in the determination of embryonic development, and that a reliable examination can be carried out by cell count calculation.

During the in vitro culture of 2 - cell stage mouse embryos, the addition of BSA as a protein source to the medium can provide successful results, but optimal results are achieved when BSA is used in the quantities of 1 mg/ml with M 16 medium and 3 mg/ml with Whitten's medium.

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