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Research Article

The Influence of Epidermal Growth Factor on Maturation and Fertilisation of Bovine Oocytes In Vitro*

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Abstract: The aim of the present study was to evaluate the efficiency of different doses of epidermal growth factor (EGF) on maturation and fertilisation of bovine oocytes in vitro. A total of 1209 high quality oocytes (follicles 2-8 mm in diameter, with unexpanded cumulus cells) were obtained by aspiration from ovaries collected at a slaughterhouse. The oocytes were then assigned into 6 groups: 1, 10, 100, and 1000 ng/ml EGF groups, as well as positive (EGF replaced with hCG) and negative (TCM 199 only, no supplement) control groups. Maturation and fertilisation rates were calculated at 39 °C in an atmosphere of 5% CO_2 in air with maximum humidity for 24 and 20 h, respectively. Based on the cumulus expansion, no significant difference was found between the EGF groups. However, the highest dose of EGF (1000 ng/ml) had a lower percentage of 1st polar body extrusion than all other doses ($P < 0.05$). Considering monospermic fertilisation and non-fertilisation rates, a further significant difference ($P < 0.05$) was found between all the EGF groups. Moreover, there were also significant differences ($P < 0.05$) between the positive and negative control groups for all the parameters given. In conclusion, the data suggest that the EGF used as a media additive (more favourable at 100 ng/ml) on IVM can be useful for IVF of cattle oocytes.

Key Words: Bovine, oocyte, epidermal growth factor, IVF, IVM

Epidermal Büyüme Faktörünün Sığır Oositlerinin In Vitro Maturasyonu ve Fertilizasyonuna Etkisi

Özet: Bu çalışmanın amacı, farklı dozlardaki epidermal büyüme faktörünün epidermal growth factor - EGF, sığır oositlerinin in vitro maturasyonu ve in vitro fertilizasyonu üzerindeki etkinliğinin araştırılmasıdır. Çalışma materyalini mezbahada kesilen sığır ovaryumlarından aspirasyon ile toplanan 1209 adet A kalite oosit (2-8 mm'lik folliküllerden cumulus ekspansiyonu gerçekleşmemiş oositler) oluşturdu. Oositler 1, 10, 100, ve 1000 ng/ml EGF grupları ile pozitif (EGF yerine hCG) ve negatif (TCM 199 tek başına) kontrol grupları şeklinde 6 grupta işlem gördü. Maturasyon ve fertilizasyon oranları, 39 °C'de % 5 CO_2 içeren maksimum neme sahip ortamda sırasıyla 24 ve 20 saatte hesaplandı. Kumulus ekspansiyonu dikkate alındığında EGF grupları arasında önemli fark olmadığı görüldü. Ancak oositler 1. kutup hücresi yönünden incelendiğinde, 1000 ng/ml EGF grubunun diğer EGF ve control gruplarıyla farklı olduğu tespit edildi ($P < 0,05$). Monospermik ve fertilize olmayan oositlerin oranlarındaki fark tüm EGF gruplarında önemli bulundu ($P < 0,05$). Aynı zamanda pozitif ve negatif kontrol gruplarında elde edilen tüm paramatreler arasındaki fark önemli bulundu ($P < 0,05$). Sonuç olarak sığır oositlerinin IVM'sinde vasat katkısı olarak kullanılan EGF'nin (en başarılı 100 ng/ml) IVF için uygun olabileceği kanısına varıldı.

Anahtar Sözcükler: Sığır, oosit, epidermal büyüme faktörü, IVF, IVM

Introduction

Mammalian oocytes collected from the antral follicles can complete meiotic maturation in vitro. However, subsequent development of cattle oocytes matured in vitro is lower than that of those matured in vivo. This is probably due to suboptimal media for the oocytes. In vivo conditions cannot be mimicked totally in vitro, but developmental capabilities of oocytes can be improved by supplementation of maturation media with various hormones, growth factors, sera, cells, follicular liquid, and other additive substances (1,2).

* This study is summarised from the first author's PhD thesis

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The IVM conditions of cattle oocytes markedly affect their abilities to be fertilised and undergo subsequent embryonic development (3,4). Lonergan et al. (5) concluded from published studies that the amount of epidermal growth factor (EGF), as an important growth factor, ranged from 2 to 15 ng/ml in the sera of various species. However, there is little information on the concentration of EGF in cattle serum (5). Nevertheless, various doses (1, 10, 30, 50, 100 ng/ml) of EGF have been used in maturation media, even though the optimal dose is not known (5-8). Therefore, the objective of the present study was to determine the optimal dose (from 1 to 1000 ng/ml with 10-fold increments) of EGF in TCM 199 medium for in vitro maturation of cattle oocytes.

Materials and Methods

All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless indicated otherwise.

Oocyte recovery and IVM

Cumulus oocytes complexes (COCs) were obtained by aspiration of 2 to 8 mm follicles from ovaries collected at a slaughterhouse. All the oocytes used were completely surrounded by unexpanded cumulus cells. They were washed 3 times with Dulbecco's phosphate buffered saline (Gibco Lab., Grand Island, NY, USA). Selection of oocytes to be used was performed under a stereomicroscope according to the method described by Brackett and Zuelke (3). The characteristics of 'high' quality oocytes used were as follows: the presence of intact zona pellucida, uniform cytoplasm and cumulus appearance, and cumulus cellular zone in 4 or more layers tightly surrounding the oocytes. The groups comprising the animal material and the contents of media used in these groups are given in Table 1. Considering the contents of media, negative (TCM 199 only) and positive (EGF replaced with hCG) control groups (C + and C-, respectively) were also included herein to test the medium itself or the supplement (EGF) whether they could work properly for IVM or IVF.

The oocytes were randomly assigned into individual treatment drops (200 µl, 10-15 COCs/drop) and incubated for 24 h for IVM in the culture plates. All the incubations were performed at 39 ºC in media under paraffin oil in an atmosphere of 5% CO_2 in air with maximum humidity. After maturation, cumulus expansion (CE) and the 1st polar body (Pb) of all the samples were visually assessed under the stereomicroscope. No assessment was performed for the COCs in those groups to be used for fertilisation.

IVF:

Fertility-proven (Lalahan Livestock Central Research Institute, Ankara, Turkey), frozen-thawed bull semen in 0.25 cc straws (20×10^6 total sperm with a minimum of 50% post-thawing motility) was prepared for IVF according to the method reported by Hansen (9) using a swim-up procedure that was slightly modified in our laboratory. Spermatozoal concentrations (per ml) were then determined using a haemocytometer. At the 24th h of incubation, the oocytes that were considered mature were washed twice in Hepes TALP medium for detaching the cumulus cells at the most exterior side. Next, the oocytes were transferred into IVF-TALP media in plates. Spermatozoal capacitation was obtained during the fertilisation by using 10 mg/ml heparin (Sigma, H 3149) that was added to the fertilisation media.

After the oocytes within the plates were taken out of the incubator, they were immediately placed onto a hot plate adjusted to 37 ºC. For each millilitre of the culture media, 1×10^6 spermatozoon and 25 µl of penicillamine-hypotaurine-epinephrine (PHE) mixture (comprising 20 mM D-penicillamine, 10 mM hypotaurine, and 1 mM epinephrine) were added to these plates. For fertilisation, the media covered by mineral oil were incubated at 39 ºC under an atmosphere of 5% CO_2 in air for 20 h.

Following the IVF, the oocytes were denuded by pipetting. To assess the pronuclear status, the oocytes were firstly denuded by repeated pipetting, then fixed in ethanol:acetic acid (3:1), and stained with orcein (1%) afterwards. Finally, the presumed zygotes treated with destaining solution were identified by the presence of 2 pronuclei (monospermic fertilisation, MF), more than 2 pronuclei (polyspermic fertilisation, PF), or the absence of any pronucleus (non-fertilised, NF), according to Leibfried-Rutledge et al. (10).

Statistical Analysis:

Data obtained were analysed statistically by chi-square test and regression analysis using MINITAB statistical software for Windows (Release 11.2, PA, USA). Differences between the groups were considered significant when $P < 0.05$.

Results

Following maturation, the proportion of oocytes with cumulus expansion (CE) amongst the EGF groups was highest (95.52%) in the 1 ng/ml EGF group, while it was lowest (89.47%) in the 1000 ng/ml EGF group. There were numerical, but not statistically significant, differences amongst all the EGF groups. By contrast, a significant difference ($P < 0.05$) was found between the C (+) and C (-) groups (Table 1, $P < 0.001$).

The highest proportions of 1st Pb expulsion were in the 100 ng/ml (77.27%), 10 ng/ml (76.47%), and 1 ng/ml (71.64%) EGF groups, with no significant differences between them. Amongst the EGF groups, the proportion of oocytes with the 1st Pb was lowest (55.26%) in the 1000 ng/ml group. Significant differences ($P < 0.05$) were found between the 1000 ng/ml EGF group and all the other EGF groups as well as between the C (+) and C (-) groups (Table 1, $P < 0.001$).

The proportion of oocytes with monospermic fertilisation (MF) was highest (66.02%) in the 100 ng/ml EGF group, while it was lowest (49.15%) in the 1000 ng/ml EGF group. There were significant differences ($P < 0.05$) between all EGF groups as well as between the C (+) and C (-) groups (Table 2, $P < 0.001$; Figure).

The proportion of oocytes with polyspermic fertilisation (PF) was highest (7.77%) in the 100 ng/ml EGF group, while it was lowest (3.81%) in the 1 ng/ml group. Since the proportion of PF was so low, in terms of the analysis of statistical differences, this criterion could not be taken into account (Table 2).

Discussion

In the present study, the effects of different concentrations of EGF on maturation and fertilisation of cattle oocytes in TCM 199 medium were investigated. The results show that (i) the IVM medium alone (no additives) works properly, (ii) supplementation (of IVM medium) with different doses (1 to 1000 ng/ml) of EGF, as compared to controls (negative/positive), was more favourable during IVF in particular, and finally (iii) the optimum EGF concentration in the IVM medium was 100 ng/ml for IVF.

Considering data on maturation (IVM) in the literature, Harper and Brackett (6), using EGF at doses of 1, 10, and 100 ng/ml in the maturation media, determined that the proportions of CE and M II oocytes in these groups were 63.6% and 71.8%, 66.7% and 76.45%, and 70.5% and 76.5%, respectively. Lonergan

Table 1. Maturation rates of cattle oocytes using different EGF concentration groups (along with positive and negative controls) in TCM 199.

Maturation Groups	No. oocytes Cultured	CE (n)	%	Pb (+) (n)	%
1 ng/ml EGF *	67	64	95.52[c]	48	71.64[d]
10 ng/ml EGF *	51	47	92.16[c]	39	76.47[d]
100 ng/ml EGF *	66	61	92.42[c]	51	77.27[d]
1000 ng/ml EGF *	76	68	89.47[c]	42	55.26[b]
Positive control **	117	93	79.49[b]	75	64.10[c]
Negative control ***	140	95	67.86[a]	59	42.14[a]

* in TCM 199 with BSA (6 mg/ml), ** in TCM 199 with BSA (6 mg/ml) and, hCG (1 IU/ml), *** only TCM 199

CE: Number of COCs with partial-full cumulus expansion ($P < 0.05$), Pb (+): Number of COCs with the 1st polar body expulsed ($P < 0.05$).

[a-c]: The values having different superscripts within the same column are significantly different ($P < 0.05$).

Table 2. Fertilisation rates of cattle oocytes using different EGF concentration groups (along with positive and negative controls) in TCM 199.

Groups	Oocyte (n)	MF	%	PF	%	NF	%
1 ng/ml EGF	105	44	41.90[c]	4	3.81	57	54.29[d]
10 ng/ml EGF	123	72	58.54[e]	5	4.07	46	37.40[b]
100 ng/ml EGF	103	68	66.02[f]	8	7.77	27	26.21[a]
1000 ng/ml EGF	118	58	49.15[d]	6	5.08	54	45.76[c]
Control (+)	119	43	36.13[b]	7	5.88	69	57.98[d]
Control (–)	124	35	28.23[a]	7	5.65	82	66.13[e]

MF: Monospermic fertilisation ($P < 0.001$), PF: Polyspermic fertilisation, NF: Non-fertilised oocytes ($P < 0.001$).

[a-f]: The values having different superscripts within the same column are significantly different ($P < 0.05$).

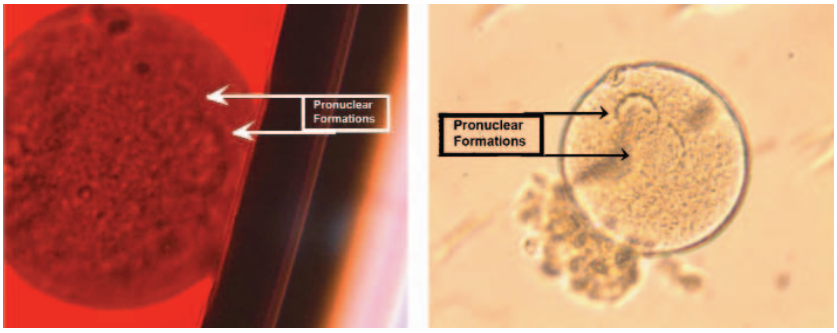

Figure. The appearance of oocytes following monospermic fertilisation during staining (a) and after treatment with destaining solution (b).

et al. (5), using the same concentrations of EGF, obtained 89%, 97%, and 91% M II oocytes, respectively. Additionally, Im and Park (11), using only 10 ng/ml EGF, noted that the proportion of mature oocytes was 85%. Likewise, Mermillod et al. (7), also using 10 ng/ml EGF only, reported that the proportion of M II oocytes was 84 ± 5%. Considering the present results, the proportions of CE observed herein were higher than those reported by Harper and Brackett (6). Those researchers noted that when the EGF was used in defined media it only partially increased the proportion of CE of oocytes. However, many other researchers (5,7,12) reported that EGF markedly increased the proportion of CE, which was in good agreement with the present results. The proportion of mature oocytes found herein was lower than those reported by Mermillod et al. (7) and Im and Park (11). This might have been due to the replacement of FCS with BSA used herein as a source of protein, along with other supplements. Indeed, many researchers have suggested that the FCS contains various hormones and growth factors (12,13). It was also thought that the lower proportions of IVM presented herein, as compared to those reported by Lonergan et al. (5), could have originated from differences in the initial qualities of oocytes used, along with varying experiences of different researchers. There was no statistically significant difference between the proportions of CE in the EGF groups used herein, in parallel with the findings published by Harper and Brackett (6). Additionally, regarding the 1st Pb expulsion no significant difference was found between the 1, 10, and 100 ng/ml EGF groups herein. Likewise, Harper and Brackett (6) and Lonergan et al. (5), using concentrations of EGF identical to those in the present study, also observed that there was no significant difference between the EGF groups based on M II oocytes.

Abeydeera et al. (14) reported that for the IVM of pig oocytes, 10 ng/ml EGF was superior to both 20 and 40 ng/ml. These researchers thought that the use of high concentrations of EGF might lead to a reduction in the number of cell receptors of the oocytes and hence called this condition "down regulation". Indeed, considering the present findings that the proportion of 1st Pb expulsion was significantly lower in the 1000 ng/ml group than in the other (low doses of) EGF groups, we assumed that this may also be considered a down regulation effect.

Macun (15) reported that the formation of CE and the 1st Pb was 83.77% and 75.1%, respectively, when FSH was added to the TCM 199 medium, while the corresponding proportions were 86.80% and 67.51% when FSH was replaced with hMG. Cetin and Bastan (16) determined that when FSH and oestradiol were added to the same medium, the proportions of the same parameters were 95.14% and 74.76%, respectively. In the present study, however, the proportions of both CE (79.49%) and 1st Pb (64.10%) in the C (+) group were lower than those reported by Macun (15) and Cetin and Bastan (16). This might have been due to the fact that in their studies serum and different hormones, instead of the BSA used in the present study, were added to the maturation media given. Van Tol et al. (17) reported that when only 0.05 IU hCG was added to the medium, there were no marked improvements either in the proportions of CE or meiotic maturation. Unlike in the study by those researchers, we used BSA plus a higher dose (1.0 vs 0.5 IU) of hCG as 2 supplements together in the C (+) group herein. That may explain why we observed significant differences between the C (-) and C (+) groups in terms of both CE and Pb.

In the present study, the proportions of CE and 1st Pb expulsion in the C (-) group were 67.86% and 42.14%, respectively. Some researchers (13,18) reported that when the TCM 199 was used only (no additives) for maturation 40 ± 2.8% to 51.9 ± 3.2% of oocytes reached the M II stage, in good agreement with our results.

Considering fertilisation (IVF), Im and Park (11) reported that when 10 ng/ml EGF was added to the maturation medium the proportions of MF and PF were 68% and 3%, respectively. Izadyar et al. (19) observed that when FSH was added to the media the corresponding proportions were 68% and 10%. In the present study, the greatest proportion (66.02%) of MF was in the 100 ng/ml EGF group. In a previous study (20) in which the effect of various doses (1, 10, and 100 ng/ml) of EGF on blastocyst formation was examined, the highest proportion was also obtained in the 100 ng/ml EGF group. Even though the blastocyst stage is a subsequent step of fertilisation (following the formation of pronuclei), this latter report supports well the present findings. Additionally, Im and Park (11) noted that the most successful group (with 30 ng/ml EGF) in maturation also yielded the same results in MF, in parallel with the present findings. In contrast, it was considered that the lower fertilisation rates obtained herein as compared to those reported by Izadyar et al. (19) might have originated from differences in both the contents of maturation media and sperm selection methods used.

In the present study, the proportions of PF ranged from 3.81% to 7.77%, regardless of the contents of the media used. In a previous study (21), it was reported that when FCS as well as FSH, LH, and oestradiol were added to the maturation medium the proportion of PF was 7.46%, while it was 8.95% when only FCS was used. The present findings are in parallel with those obtained by Sirard et al. (22). Considering other studies reported, the present relatively low proportions of PF might be due to the use of heparin as a capacitating agent (4) and BSA (21) instead of serum as a supplement.

Birler et al. (23) found that when serum and LH were added into TCM 199 medium, the proportions of MF and PF were 38.7% and 0%, respectively, but the corresponding proportions were 39.3% and 27.3% when LH was replaced by FSH in the same medium used therein. In the present study, the proportions of MF and PF in the C (+) group were 36.13% and 5.88%, respectively. Considering their studies, the present findings of the proportion of MF are the same, but the proportion of PF observed herein was lower (than those results with FSH). This might be due mainly to differences in the contents of media and the concentrations of spermatozoa used, as well as varying manipulations during the course of the studies undertaken.

In the present study, the proportions of MF and PF in the C (-) group were 28.23% and 5.65%, respectively. In a previous report (23), it was observed that neither MF nor PF took place when TCM 199 was used alone (no supplement) as the maturation medium. By contrast, Zuelke and Brackett (24) observed that the fertilisation

rate of oocytes was 39.7%. Likewise, Ocana et al. (18) also reported a similar rate (32.7 ± 2.9%). These results are well in line with the present results.

Overall, the present findings suggest that the optimum concentration of EGF to be added into the maturation medium (TCM 199) was 100 ng/ml for IVF of cattle oocytes.

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