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Periimplantation-like growth and development of mouse blastocysts in medium containing horse serum[1]

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Summary. Horse serum (HS) supported growth and differentiation of blastocysts as well as or better than fetal bovine serum (FBS) ($p < 0.01$) as measured by a) the onset of trophoblastic outgrowth, b) the size of the resultant outgrowths, c) the size of nuclei in trophoblasts after their outgrowth and d) the size of the inner cell mass in outgrowths. Moreover, polyamines were found to be approximately 10–100 times less toxic to embryos when added to medium containing HS than when the medium contained FBS.
Key words. Mouse blastocysts; culture medium; horse serum; polyamine synthesis; trophoblastic outgrowths.

We describe here the use of horse serum (HS) as an alternative to fetal bovine serum (FBS) as a component of medium used to grow periimplantation mouse blastocysts through the stage of trophoblastic outgrowth in vitro. The developmental events which occur during culture of these blastocysts are analogous to periimplantation events which occur in utero[2,3]. Thus, this culture system is useful for examining the biochemical mechanism of nidation, at least as it relates to the blastocyst. Moreover, when examining biochemical events associated with periimplantation development in vitro it is sometimes necessary to add enzyme inhibitors, substrates and or products to the culture medium[4–7]. Because serum contains enzymes and other biochemicals it is possible that substances added to the medium may interact with components of the serum in the medium. For example, cleavage-stage embryos[4] and blastocysts[5] appear to be growth-inhibited[5] and/or killed[4,5] when spermine an/or spermidine (polyamines) are added to medium containing bovine serum albumin[4] or FBS[5]. Presumably, the added polyamines are oxidized by polyamine oxidase present in FBS[8,9] and bovine serum albumin preparations[10]. As is the case for other cell types[8,9,11], the oxidation products of polyamines appear to be particularly inhibitory and/or toxic to the cells of blastocysts[5]. While these toxic substances are produced from polyamines in the presence of bovine sera and products of bovine sera[8–10], the same toxic effect has not been observed for other cell types when HS is substituted for FBS[8,9]. Thus we examined the effect of polyamines on the growth and development of mouse blastocysts in vitro in medium containing HS instead of FBS.

Materials and methods. Swiss, ICR mice (Harlan, Sprague Dawley, Inc.) which had been induced to ovulate and mate, were ovariectomized before 12.00 h on the 4th day post coitus

Effect of serum type and concentration on outgrowth formation by 'delayed implanting' mouse blastocysts cultured in Dulbecco's modified Eagle medium

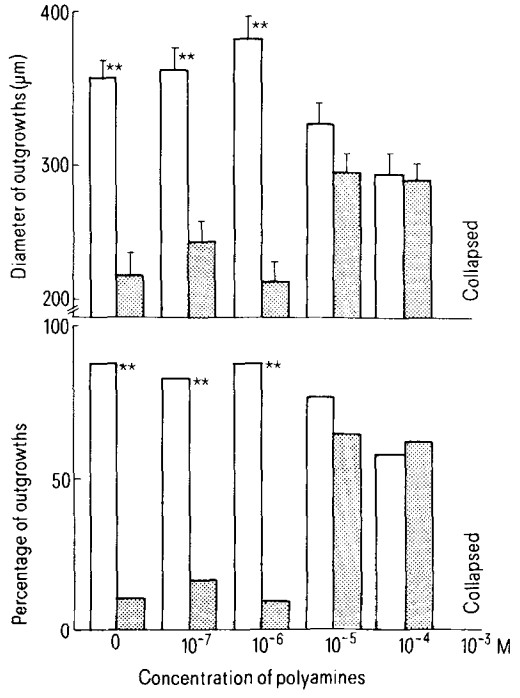
Parameter measured and duration of culture[b]	Source, type and concentration of serum[a]									
	Grand Island Biological Co.				HyClone (Logan, Utah)					
	Horse serum		Fetal bovine		Horse serum		Fetal bovine$_1$		Fetal bovine$_2$	
	3%	10%	3%	10%	3%	10%	3%	10%	3%	10%
Percentage of outgrowths after 42 h	91.1 (45)	97.7 (44)	28.9 (45)	58.7 (47)	21.7 (69)	50.0 (66)	34.4 (64)	38.1 (63)	37.3 (55)	47.6 (63)
Diameter of outgrowths (μm) after 70 h (mean ± SE)	316 ± 19 (15)	391 ± 17 (20)	306 ± 17 (20)	365 ± 17 (20)	276 ± 15 (25)	357 ± 15 (25)	304 ± 15 (25)	342 ± 15 (25)	287 ± 15 (25)	325 ± 17 (20)
Diameter of trophoblastic nuclei (μm) after 70 h (mean ± SE)	22.6 ± 1.6 (20)	25.6 ± 1.6 (20)	22.3 ± 1.6 (20)	27.0 ± 1.6 (20)	21.2 ± 1.4 (25)	23.5 ± 1.4 (25)	23.5 ± 1.4 (25)	27.5 ± 1.4 (25)	23.0 ± 1.4 (25)	23.0 ± 1.4 (25)
Diameter of inner cell mass (μm) after 70 h (mean ± SE)	97.1 ± 7.0 (17)	103.6 ± 7.0 (17)	113.7 ± 7.0 (17)	119.1 ± 7.0 (17)	114.9 ± 6.3 (22)	121.7 ± 6.3 (22)	100.1 ± 6.3 (22)	124.6 ± 6.3 (22)	106.9 ± 6.3 (22)	110.2 ± 6.3 (22)

[a] The total number of blastocysts cultured (1st row) or measurements made (last 3 rows) is shown in parentheses. Pooled data from 4 or 5 experiments are presented. [b] All parameters had larger values at higher serum concentrations ($p < 0.01$ for rows 1, 2 and 3; $p < 0.05$ for row 4). Horse serum from Grand Island Biological Co. produced more rapid onset of outgrowth than other sera ($p < 0.01$). No other statistically significant differences were observed.

(p.c.) as described previously[12,13]. The day of copulatory plug detection was designated day 1 p.c.

On days 8–13 p.c. the ovariectomized mice were sacrificed by cervical dislocation and blastocysts were flushed from the animals' excised uteri with Dulbecco's modified Eagle medium (GIBCO) supplemented with 100 U/ml penicillin G and 50 µg/ml streptomycin sulfate (MEM)[14,15]. The medium also contained either 3 or 10% horse serum, fetal bovine serum or dialyzed bovine serum (HyClone, Logan, Utah or GIBCO). Groups of 5–25 embryos were washed then cultured in the depression of a Maximov slide containing 1–2 ml of fresh medium by methods which have been described elsewhere[5,14–16]. Some media also contained methylornithine (MORN; Calbiochem), methylglyoxal bis-(guanylhydrazone) (MGBG; Sigma Chemical Company), putrescine (Sigma), spermidine (Sigma) and/or spermine (Sigma) at concentrations indicated in the text, figure and table. Cultured embryos were examined periodically for the onset of trophoblastic outgrowth. In some cases, a microscope equipped with an ocular micrometer was used to measure the diameter of 5 or more representative outgrowths, inner cell masses or trophoblastic nuclei in each culture. In other experiments, blastocysts were incubated with a mixture of ^{3}H-amino acids (NET-250, New England Nuclear) for 2 h then processed to determine the radioacitivity they accumulated as described previously[13,14]. All experiments were repeated 3 or more times. Data from replicate experiments were combined since the results were similar each time a treatment was used.

The fraction of embryos which had formed trophoblastic outgrowths under the various culture conditions were compared statistically with two-by-two contingency tables[5,17]. The mean diameters of outgrowths, inner cell masses or trophoblastic nuclei or the cpm of radioactive amino acids accumulated per blastocyst were compred with analysis of variance coupled with a multiple range test[17,18].



Effect of polyamines on inhibition of growth of 'delayed implanting' blastocysts by methylornithine and MGBG. Embryos were cultured in MEM containing 10% horse serum (GIBCO) (open bars) or this medium plus 10 mM methylornithine and 200 µM MGBG (filled bars). The polyamines, putrescine, spermine and spermidine, were added at the indicated concentrations. The mean diameter (µm) ± SE of outgrowths after 66 h (upper pannel) or the percentage of blastocysts that had formed outgrowths after 40 h (lower pannel) are shown for pooled data from 3 or more independent experiments (total of approximately 68 embryos per group). Methylornithine and MGBG significantly inhibited growth of embryos at polyamine concentrations marked with double asterisks ($p < 0.01$).

Results. While polyamines (or their oxidation products) are approximately 10 times less toxic to blastocysts in medium containing 10% dialyzed than in non-dialyzed fetal bovine serum (10^{-5} M polyamines are toxic in non-dialyzed FBS), 10^{-4} M spermine and spermidine are toxic to these embryos in both of these sera[5]. Thus, we were unable in our previous study[5] to attempt to completely reverse the effects of inhibitors of polyamine synthesis with spermine and spermidine. HS supported periimplantation-like development of delayed implantation mouse blastocysts in vitro as well as or better than FBS (table) but may not interact with spermine and spermidine to produce toxic substances[8,9]. Thus, we attempted to completely reverse the inhibitory effects of methylornithine (an inhibitor of ornithine decarboxylase) and MGBG (an inhibitor of S-adenosyl methionine decarboxylase) on activation and growth of diapausing blastocysts with polyamines in medium containing horse serum.

In contrast to our previous report utilizing fetal bovine serum, 10 mM methylornithine plus 200 µM MGBG did not appear to completely prevent activation and growth of blastocysts in medium containing horse serum. Nevertheless, these inhibitors slowed the onset of trophoblastic outgrowth by delayed implantation blastocysts in vitro ($p < 0.01$) and the resultant outgrowths were smaller than embryos in control cultures ($p < 0.01$; fig). The inhibitors did not, however, have any detected effect on these parameters in the presence of 10^{-4} or 10^{-5} M polyamines. In fact, polyamines favored the onset of outgrowth and increased the size of the resultant outgrowths in the presence of methylornithine and MGBG ($p < 0.01$; fig.). However, polyamines themselves (10^{-4} and 10^{-5} M) also inhibited the onset of outgrowth ($p < 0.01$) and these compounds appeared to be toxic at a concentration of 10^{-3} M as evidenced by collapse and failure of embryos to grow (fig.).

Outgrowths which formed in the presence of 10 mM methylornithine plus 200 µM MGBG appeard to lack trophoblastic nuclei and this effect was reversed by 10^{-4} and 10^{-5} M polyamines ($p < 0.01$; data not shown). Similarly, the inner cell mass was undetected in ⅔ of the embryos cultured with inhibitors of polyamine synthesis and the inner cell mases which were measurable were significantly smaller than in control culture ($p < 0.01$; data not shown). The presence of 10^{-4} M polyamines reversed this apparent failure of the inner cell mass to develop in medium containing methylornithine and MGBG ($p < 0.01$). Finally, inhibitors of polyamine synthesis partially prevented the increase in the capacity of diapausing blastocysts to accumulate radioacitve amino acids when these embryos were incubated in vitro. This effect was also reversed by 10^{-4} M polyamines ($p < 0.01$; data not shown).

Discussion. These data show that HS can be used instead of FBS as a component of the culture medium utilized to grow periimplantation mouse embryos. This could be important for all investigators studying implantation-like events in vitro if the cost of FBS were to become prohibitive. Moreover, in our own investigations, and potentially in the studies of others, HS is a valuable alternative to FBS because compounds which we must add to our medium (i.e., spermine and spermidine) are 10–100 times more toxic to blastocysts in medium containing FBS than when the medium contains HS.

Despite the potential advantages of using HS instead of FBS for growing mouse blastocysts, it is important to note that the response of blastocysts to added compounds may differ quantitatively if not qualitatively in medium containing HS instead of FBS. For example, the inhibitors of polyamine synthesis, methylornithine and MGBG, appeared to stop activation and growth of diapausing blastocysts in medium containing FBS[5].

However, these inhibitors did not completely prevent growth when the medium contained HS (fig.). The different responses of blastocysts in these 2 sera probably cannot be ascribed to the presence of polyamines in horse serum for we were unable to detect putrescine, spermidine or spermine in any of the sera used in this study (unpublished). Moreover, other cell types have also been shown to respond differently to the effects of inhibitors of polyamine synthesis when grown in medium containing HS instead of FBS[19]. Nevertheless, the results reported here confirm and expand our previous conclusions that polyamine synthesis is required for activation and growth of diapausing blastocysts in vitro. Further studies are required to establish how polyamine synthesis is linked to control of growth in these embryo.

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The generation of new 'white' muscle fibers by budding in the lateral musculature of elvers *Anguilla anguilla* (L.) during normal development

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Summary. The elver is a developmental stage of the European eel Anguilla anguilla (L.). During its growth, relatively large numbers of new muscle fibers are formed in the lateral musculature. We investigated the origin of these fibers. They proved to originate from already existing fibers by budding.
Key words. Muscle fibers; budding; *Anguilla anguilla;* muscle development; eel, European.

The elvers used in the present investigations represent a stage of development of the European eel *Anguilla anguilla* (L.). The larvae migrate from the Sargasso Sea to the West-European coasts. During this journey of 2¾–3 years they develop through larval (Leptocephalus) stages into glass eels which migrate from the sea to fresh water[1]. At that time their length varies from 60–75 mm. During the initial period of freshwater life (elver stage), pigment is gradually developed in the cutaneous layers. The growth occurring in this stage includes growth of the lateral musculature which is characterized by an increase in diameter of muscle fibers as well as a considerable increase in number of fibers[2]. In the lateral muscles of the eel 2 fiber complexes may be distinguished, the superficially situated complex of slow ('red') fibers separated by a fascia from the deep, fast ('white') fiber complex[3–6]. In the white fiber complex new small fibers appear almost immediately after the start of growth. In 8 cm animals 25% of the white fibers consists of small new fibers, in 9 cm animals 50%[2].

Light microscopical investigations show that the first new fibers appear in the deeper region on the white fiber complex and gradually small new fibers appear also in still more superficially-situated regions.

Electron microscopical studies of transverse and longitudinal sections of the white muscle fiber complex in elvers, ranging from 6–15 cm, revealed the total absence of myotubuli or similar stages of muscle fibre development which usually precede the appearance of new small muscle fibers. Myosatellite cells were present, accompanying less than 3% of the muscle fibers and never found in serial arrangement. However, in the transverse sections we searched carefully for satellite cells, an abundance of quite remarkable structural features was found, especially in 80–85 mm specimens. We may classify them as follows:

1. Groups of 6–10 myofibrils are situtated exremely superficially in large muscle fibers. They are separated from the other fibrils in the fibers by a zone of sarcoplasma. In this sarcoplasmatic zone nuclei and mitochondria may be found (fig. A).

2. A similar group of myofibrils is found, while additionally in the sarcoplasmatic zone membrane fragments (M) are present. In some cases invaginations (F) of the sarcolemma partially divide the sarcoplasmatic zone (fig. B).

3. Sarcolemmal membranes are present to such an extent that their presence results in the separation of a small fiber (S) from a large muscle fiber (L). In both fibers nuclei are situated in a peripheral zone of sarcoplasma, and both fibers have contact areas that are restricted in number and extent (fig. C and D, showing the same pair of fibers).