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Optimization of the technique for the short-term culture of equine hepatocytes

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Summary

The aim of this study was to establish the optimal conditions for the culture of equine hepatocytes in a monolayer configuration. The obtained results show that the rate of MTT metabolism correlated with the number of cultured cells and a linear increase of MTT reduction rate was observed in cases when the cell density varied between 1.25×10^4 to 5×10^4 viable cell/well of 96-well plate. Hepatocytes reached the optimal cell attachment rate and MTT reduction at a cell density of 5×10^4 cells/well. The number of attached cells to a plastic culture dish was also related to incubation time. The greatest ability of hepatocytes to attach to the culture dish was observed after 10 h of incubation and it was found to be 84.1 ± 2.5% of seeded hepatocytes. It was also found that fetal bovine serum was more efficient than horse serum for the attachment of equine isolated hepatocytes in a monolayer culture. The highest rate of cell attachment (assessed microscopically and with MTT reduction test) was observed when cells were plated with the culture medium supplemented with FBS or HS at a concentration of 5%. However, medium supplementation with higher than 5% serum concentration (10% of FBS or HS) significantly decreased MTT reduction rate. The rate of MTT metabolism and cell attachment in hepatocytes cultured in WE supplemented with FBS or HS was also dependent on the plating time and were the highest after 10 h of seeding.

Keywords: hepatocytes, equine

Recently, a method for the preparation of intact equine hepatocytes has been developed (1). Livers harvested from slaughtered horses yield viable hepatocytes, thus representing an unlimited resource of horse liver cells for studies. The elaborated method of equine hepatocytes preparation is rather uncomplicated and seems applicable in most laboratories. However, the successful use of equine isolated hepatocytes for pharmacological and toxicological studies requires the determination of optimal conditions for cell cultivation. The majority of studies utilizing hepatocyte primary monolayer cultures derived from both homoeothermic and poikilothermic animal species have been already performed. The conditions necessary to achieve optimal attachment and metabolic activity of cells in monolayer culture have been only investigated extensively for teleost (14), chicken (7), rat, mouse, hamster, rabbit (11), sheep (6) and human hepatocytes (8). As significant species differences in optimal monolayer culture conditions have been observed, the optimal conditions for short-term cultures of equine hepatocytes have been investigated.

Material and methods

Chemicals. Williams' Medium E (WE), trypan blue solution 0.4% w/v, fetal bovine serum (FBS), horse serum (HS), 5.6-carboxyfluorescein diacetate, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), penicillin G sodium salt, streptomycin sulfate and amphotericin B solution were purchased from Sigma Chemical Co (St Louis MO, USA). All other reagents and chemicals were purchased from commercial suppliers POCh Gliwice (Poland).

Liver obtainment. Livers were obtained from 12 horses undergoing routine slaughtering processes at the local slaughterhouse („Jasan" Company, Jasło, Poland). All the procedures were conducted in full compliance with the local ethical commission and the livers were procured with the oversight of the General Veterinary Inspectorate. Horses were of both sexes, 4-13-years-old, various thoroughbreds and weighing from 350 to 700 kg. The livers were excised 30 min after the death of the animal at the latest. Part of the liver lobe was prepared according to Human Liver Transplantation Protocols (2, 5, 12, 15). At the slaughterhouse, liver segments were prepared as described previously (1) and transported to the laboratory. The interval from horse death to hepatocyte preparation was never more than 7 h.

Preparation of equine hepatocytes and short-term culture of the cells. Hepatocytes were prepared by the method described previously (1). Cell viability was assessed by trypan blue dye exclusion and 5.6-carboxyfluorescein diacetate tests. Only hepatocyte suspensions that were > 75% viable in both tests were cultured in monolayer configuration. Isolated hepatocytes were resuspended in various plating media consisting of WE supplemented with FBS or HS in concentrations of 0, 2.5, 5 and 10% (v/v), 1000 U/ml penicillin G, 68.6 μM strep-

tomycin sulfate and 2.7 μM amphotericin B. For optimization of cell attachment, the hepatocyte suspensions were prepared at densities of 1.25×10^5, 2.5×10^5, 5×10^5 and 7.5×10^5 viable cells/ml. Thereafter, plating medium and cell suspensions were dispensed into 96-well plates (Corning Incorporated, New York, USA) at a volume of 0.1 ml (0, 1.25×10^4, 2.5×10^4, 5×10^4 and 7.5×10^4 viable hepatocytes/well) and incubated at 37°C in 5% CO_2/95% air. Cell attachment rate and plating efficiency of hepatocytes were assessed after 1, 2, 4, 6, 10 and 12 h of plating by a microscopic evaluation and MTT reduction test. Cell attachment rate was determined by calculating the percentage of attached cells after 1, 2, 4, 6, 10 and 12 h plating at 37°C in 5% CO_2/95% air. After plating, the medium was removed and microscopic images were captured using inverted light microscopy (Olympus MO21, Tokyo, Japan) at magnification × 280. The number of hepatocytes that adhered at 2 randomly selected sites of known constant area calibrated using a graticule were counted for each well using direct light microscopy. The total number of hepatocytes which attached to each well was calculated by multiplying the mean number of cells counted by the ratio of the total surface area of the wells divided by known constant area. This value was then expressed as a percentage of the number of viable hepatocytes originally introduced to each well (1.25×10^4, 2.5×10^4, 5×10^4 and 7.5×10^4 viable hepatocytes/well).

MTT was assayed according to the method described by Supino (17) with some modifications (1).

Statistics. All data were analyzed using Statistica for Windows (version 5.1 Pl). Results were expressed as mean values ± standard deviations (SD) of the mean. Values of $p < 0.01$ or $p < 0.05$ were considered significant. All data were first proven to fit the assumption of normality. Differences between groups were tested by an analysis of variance (ANOVA). Means found to be significant by ANOVA were compared using a Duncan pairwise multiple comparison procedure.

Results and discussion

The effect of different plating densities (1.25×10^4, 2.5×10^4, 5×10^4 and 7.5×10^4 viable cells/well) on MTT reduction in freshly isolated hepatocytes seeded onto 96-well plates is shown in fig. 1. MTT reduction rate was strictly related to the cell density and showed a direct relationship with the plating density between 1.25×10^4 to 5×10^4 cells/well. MTT reduction observed at 7.5×10^4 cells/well was not co-linear to other values and was slightly higher than at 5×10^4 cells/well but there was not a statistical significance in MTT reduction between these two plating densities (fig. 1). Moreover, at a plating density of 5×10^4 cells/well cells formed a confluent monolayer, were rounded, established both close and had associations with 3-5 neighbors (as assessed microscopically). A further increase of the plating density to 7.5×10^4 cells/well yielded more densely-packed monolayers and some cells piled up in a double layer. These observations are in agreement of Berry et al. (3) who reported that 5×10^4 cells/well was the optimal cell density for rat monolayer culture. Koebe et al. (10) reported high values of MTT reduction in monolayer culture of porcine hepatocytes at a density of 5×10^4 viable cell/well and suggested usage this density for monolayer culture of porcine hepatocytes. In contrast, Gomez-Lechon and Castell (8) presented lower cell densities (from 2.5×10^4 to 4×10^4) to be the best for cultivating human hepatocytes in monolayer culture.

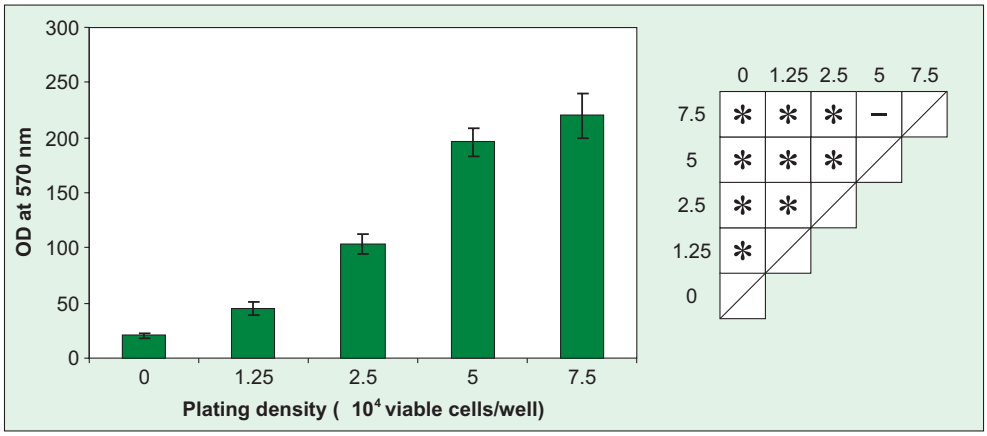


Fig. 1. Effect of different plating cell densities on MTT reduction in hepatocytes seeded onto 96-well plates. (*) $p < 0.01$ vs. another plating density, (–) no statistical difference

The number of attached cells to a plastic culture dish was also related to the plating time (fig. 2). Four hours after seeding, only approximately 50% of seeded viable hepatocytes attached to the plastic culture dish, as judged microscopically. The attachment rate significantly increased between 4 and 6 h of incubation. The highest number of cells attached to the plastic culture dish was observed after 10 h of incubation and was found to be 84.1 ± 2.5% of seeded hepatocytes. Similar results were presented by Ostrowska et al. (13) who showed very high values of human hepatocyte attachment to 96-well plates precoated with type I collagen (92 ± 1.7% after 6 h of plating). However, hepatocytes from various species usually express a different ability to attach. Guilluozo et al. (9) reported that dog liver cells attached to a culture dish coated with fibronectin only in 40%, human in 57.5

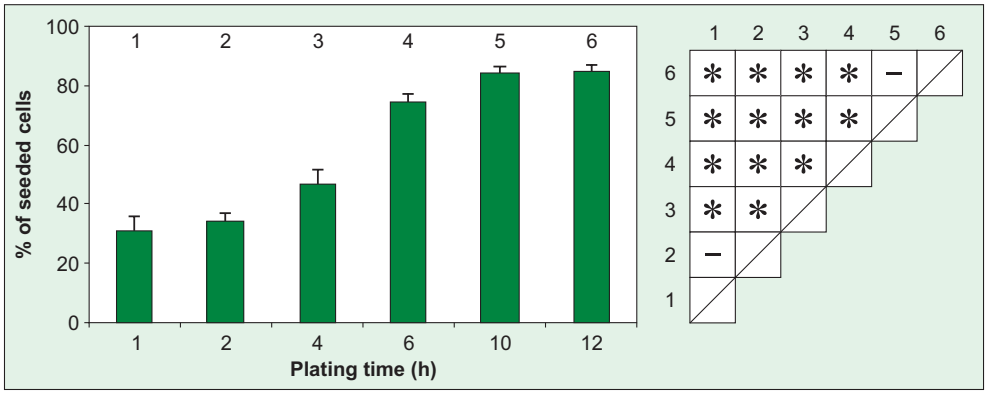


Fig. 2. Effect of plating time on cell attachment rate (assessed microscopically) in monolayer cultures of isolated hepatocytes.). (*) $p < 0.05$ vs. another value, (–) no statistical difference

± 12.9%, and rat in 68 ± 12.1% of the total number of seeded cells (after 6 h of plating). Although, it has to be pointed out that the values of cell attachment rate and MTT reduction are often quite difficult to compare due to significant differences between plating time, a substrate used for cell adhesion, and an expression of results.

To improve cell seeding, the culture medium was supplemented with FBS or HS at concentrations ranging between 0 to 10%. Equine hepatocytes were able to attach in a serum-free medium. Serum supplementation, however, significantly improved cell attachment rate and MTT reduction (tab. 1). The highest rates of MTT reduction (independent on plating time) were shown by hepatocytes incubated with FBS or HS in a concentration of 5%. Medium supplementation with higher serum concentration (10% of FBS or HS) significantly decreased MTT reduction. Comparable dependence between plating time and MTT reduction was observed in hepatocytes cultured in the medium supplemented with both FBS and HS. The highest MTT reduction rate showed hepatocytes cultured for 10 h with FBS or HS. Further plating to 12 h slightly decreased MTT reduction. These findings of the effect of serum supplementation on plating efficiency are in agreement with observations performed on monolayer cultures of hepatocyte derived from other species, although there are considerable interspecies differences between cell attachment rate and serum concentration. For instance, rabbit hepatocytes displayed the best attachment, achieving an efficiency of 85.8 ± 1.9% after 2 h plating in WE supplemented with 10% of FBS (11). Optimal attachment rate of hamster hepatocytes was attained in WE with 1% of FBS, and was found to be 74.5 ± 2.3% (11). The rate of attachment in serum supplemented media was relatively slow, with peak attachment occurring 3 h after inoculation. Mouse hepatocytes achieved maximal attachment in WE supplemented with 1% FBS, and was found to be 85.1 ± 5.2% after 2 h of incubation (11). However, higher serum supplementation (10% and 20%) decreased cell attachment rate. Attachment of rat hepatocytes to a plastic culture dish in WE supplemented with 10% of FBS was superior to that achieved in 1% of FBS (71.1 ± 6.9% versus 60.8 ± 5.0%, respectively) (11). In contrast to mammals, fish hepatocytes do not require serum supplementation to achieve high cell attachment rate. Braubeck and Segner (4) reported 93% attachment rate of isolated rainbow trout hepatocytes to culture plates in a serum-free medium.

To summarize, the optimal conditions for cell attachment and MTT metabolism in isolated equine hepatocyte monolayer cultures were found to be at a cell density of 5×10^4 cells/well of 96-well plate in 0.1 WE supplemented with 5% FBS and after cell seeding for 10 h.

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Tab. 1. Effect of plating time and FBS (A) and HS (B) concentration on MTT reduction rate in monolayer cultures of isolated hepatocytes expressed as an optical density of formazan ± SD

A. Concentration of FBS (% v/v)	Incubation time (h)					
	1	2	4	6	10	12
0	44.3 ± 5.1	54.8 ± 4.2	59.5 ± 6.0	62.0 ± 5.3	74.6 ± 7.1	70.1 ± 8.2
2.5	79.2 ± 4.0	87.3 ± 4.4	104.9 ± 4.2	163.5 ± 8.2	180.6 ± 9.1	177.4 ± 5.3
5	79.8 ± 3.2	88.6 ± 3.5	118.3 ± 4.4	172.5 ± 5.2	192.0 ± 6.5	188.3 ± 6.8
10	77.8 ± 2.3	80.3 ± 5.6	104.8 ± 5.2	146.3 ± 5.9	142.8 ± 4.3	134.3 ± 9.4

B. Concentration of HS (% v/v)	Incubation time (h)					
	1	2	4	6	10	12
0	44.7 ± 4,9	54.3 ± 5.2	60.9 ± 6.0	75.1 ± 3.2	80.1 ± 7.1	78.7 ± 8.7
2.5	48.9 ± 2.4	73.9 ± 3.7	97.8 ± 3.9	143.0 ± 7.7	178.8 ± 8.8	171.0 ± 6.1
5	55.3 ± 2.3	77.9 ± 3.1	101.3 ± 4.4	162.6 ± 7.2	163.3 ± 5.5	158.8 ± 6.8
10	50.9 ± 1.5	70.7 ± 5.2	92.6 ± 4.6	141.4 ± 5.4	153.4 ± 6.3	149.7 ± 10.2

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