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EFFECT OF SODIUM GLUTAMATE ON THE MORPHOLOGY AND HISTOCHEMISTRY OF EXPERIMENTAL GLIOPATHY INDUCED *IN VITRO* BY AMMONIA AND SODIUM MALONATE

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In a previous *in vitro* study Opalski cells and Alzheimer cells, type II, similar to those occurring in spontaneous and experimental hepato-cerebral pathology, were found to appear under the influence of exogenous copper and ammonia salts and sodium malonate, added to the standard culture medium (Mossakowski et al., 1970; Renkawek et al., 1973). Inhibition of the gliotoxic effect of the above mentioned substances by alfa-oxoglutarate and a remarkable reduction of glutamic dehydrogenase activity in the pathological glial cells led us to the conclusion that insufficient detoxication of ammonia is the main pathogenic factor of Wilsonian and hepatogenic gliopathy (Mossakowski et al., 1972; Renkawek et al., 1973).

As glutamate is one of the steps of natural detoxication of ammonia into glutamine in the central nervous system, it seemed interesting to study its influence on the formation and morphological and histoenzymatic picture of experimental gliopathy due to ammonia and malonate and to compare it with that of alfa-oxoglutarate.

MATERIAL AND METHODS

Experiments were carried out on *in vitro* cultures of glial tissue from the cerebellum of newborn rats (Kraśnicka, Mossakowski, 1965). The studies were performed in four experimental and two control groups of 1- and 3-week-old cultures.

I. E x p e r i m e n t a l g r o u p. The cultures of appropriate age were transferred for 24—120 hrs to standard miedium, containing ammonium chloride in a final concentration of 100 mg percent.

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II. Experimental group. Cultures of the same age were placed for 3—9 days in medium containing sodium malonate in a concentration of 1 mM/13.5 ml medium *).

III. Experimental group. The cultures were treated identically as those in group I, with the only difference consisting in the presence of sodium 1-glutamate in a 1 mM concentration in the medium.

IV. Experimental group. Cultures were grown in conditions analogous to those in group II, but the medium contained additionally 1 mM sodium 1-glutamate.

Two control groups consisted of cultures of appropriate age grown in standard medium and in that with addition of sodium 1-glutamate in the concentration used in experimental group. The latter control has been applied in order to ascertain if sodium glutamate itself has any effect on the morphological and histoenzymatic picture of glial cells *in vitro*.

Cultures of experimental groups I and III were kept in appropriate medium for 24, 48, 72 and 120 hrs and then used for histological and histochemical studies.

Cultures from groups II and IV were grown in experimental medium for 3, 6 and 9 days. The difference in incubation time resulted from the slower and weaker cytotoxic effect of sodium malonate, observed in our previous investigations. The duration of the culture in medium containing sodium glutamate alone was analogous with that in experimental cultures of groups III and IV. The cultures of all experimental and control groups were stained by the methods mentioned in Table 1.

Table 1. Histological and histochemical methods used

Method	Reference
Toluidine blue	
Cresyl violet	
Hematoxylin-eosin	
Sudan black B	
Periodic acid Schiff (PAS)	
Alcian blue method (Lison)	
Succinic dehydrogenase activity	— Scarpelli et al. (1958)
Glutamate dehydrogenase activity	— Pearse et al. (1961)
Glucose-6-phosphate dehydrogenase activity	— Hess et al. (1958)

RESULTS

The morphological and histochemical picture of glial cultures, grown in medium containing exogenous ammonia (group I) and sodium malo-

*) Malonate concentration was estimated by the complete inhibition of succinic dehydrogenase activity in glial cells *in vitro*.

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nate (group II) did not differ from that known from our previous investigations (Mossakowski et al., 1970; Renkawek et al., 1973). Numerous pathological glial cells in the form of both Opalski cells and Alzheimer cells type II were present. Opalski cells were large, oval or round with abundant granular cytoplasm and excentrically placed dark, small nuclei. Multinuclear forms were also present. Their cytoplasm contained abundant, coarsely granular PAS-positive material, as well as fine granules positively stained with alcian blue. High activity of glucose-6-phosphate dehydrogenase, and weak of both succinic and glutamic dehydrogenases was the most common histoenzymatic feature. Weaker activity of succinic dehydrogenase was typical of Opalski cells from cultures grown in medium containing sodium malonate, while in those kept with exogenous ammonia reduction of glutamate dehydrogenase activity was more pronounced.

In both experimental groups prolonged exposure to the action of cytotoxic agents resulted in an increased number of Opalski cells and Alzheimer cells type II. At the same time non-specific degeneration of all cellular elements, present in the cultures was visible, this being more significant in cultures grown with exogenous ammonium chloride. Intermediate forms between Opalski cells and normal astrocytes were present in both experimental groups. They were more numerous in cultures grown with sodium malonate.

Cultures grown in medium containing both ammonium chloride and sodium glutamate (group III) varied significantly in their morphological and histoenzymatic picture from those kept with ammonium chloride alone, although they also contained both Opalski and Alzheimer cells. The number of cells of the former kind was greately reduced. Alzheimer genous ammonia reduction of glutamate dehydrogenase activity was more pronounced.

In 1-week-old cultures Opalski cells appeared as early as after 24 hrs (Fig. 1), but they were present only in part of the cultures. They were seen only in 7 of 15 cultures. They were numerous (more than 10 per culture) in 4 cultures, while in the other 3 their number per culture was less than five. After 48 hrs numerous Opalski cells were found in 3 of the 19 cultures examined, while single ones were seen in other 5 cultures. This proportion was significantly smaller in cultures kept in medium containing sodium glutamate together with exogenous ammonia for 120 hrs. Opalski cells were found only in 2 of 19 cultures, but they were not in any of them numerous. Morphology and histochemistry of Opalski cells in this experimental group was identical with that described in groups I and II (Figs. 1, 2). In this age group Alzheimer cells type II were not present at any time of observation.

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In 3-week-old cultures the frequency and number of Opalski cells was even less, for instance in the 22 cultures examined at 72 hr of experiment they were present only in 8, occurring always in a number less than 5 per culture. After 120 hrs 4 cultures of the total 22 contained single hypertrophied giant cells. In contrast to the previous age group, 3-week-old cultures contained numerous Alzheimer type II cells, appearing at 48 hrs of experiment (Fig. 3).

Small, round, glial cells lacking processes and showing histochemical properties identical with Opalski cells formed a new pathological feature, not seen in our former observations (Figs. 3, 4). They differed in their shape, size, histochemical properties and lack of fatty degeneration from degenerating astrocytes. The lack of even rudimentary cellular processes and their diameter distinguished them from intermediate forms between normal astrocytes and Opalski cells. They were numerous in 1-week-old cultures, and less common in 3-week-old ones. Prolonged maintenance in medium containing both ammonium chloride and sodium glutamate resulted in significant reduction of their number.

In addition to the above described cellular forms, cultures of both age groups contained numerous glial cells revealing features of non--specific degeneration. Many of them showed significant process fragmentation; their cytoplasm was filled with sudanophilic lipid droplets. Part of the glial population was not changed, showing morphological and histochemical properties typical for glia cultured *in vitro* (Renkawek, 1972).

Fig. 1. One-week culture, 24 hrs in a medium containing ammonia and sodium glutamate. Opalski cell and numerous transitory cells. H—E. × 400.

Ryc. 1. Hodowla 1-tygod., 24 godz. w medium z dodatkiem amoniaku i glutaminianu sodu. Komórka Opalskiego i liczne komórki przejściowe. H—E. Pow. 400 ×.

Fig. 2. Three-week culture, 72 hrs in a medium containing ammonia and sodium glutamate. Glutamate dehydrogenase. Opalski cell with a markedly decreased enzyme activity. × 400.

Ryc. 2. Hodowla 3-tygod., 72 godz. w medium z dodatkiem amoniaku i glutaminianu sodu. Dehydrogenaza glutaminianowa. Komórka Opalskiego ze znacznie obniżoną aktywnością enzymatyczną. Pow. 400 ×.

Fig. 3. Three-week culture, 72 hrs in a medium containing ammonia and sodium glutamate. A group of small, round-shaped, process-free cells. Alcian blue positive deposits in cytoplasm. Visible Alzheimer cells type II (arrow). Alcian blue. × 400.

Ryc. 3. Hodowla 3-tygod., 72 godz. w medium zawierającym amoniak i glutaminian sodu. Grupa drobnych, okrągłych bezwypustkowych komórek. Złogi Alcjan-dodatnie obecne w cytoplazmie. Obok widoczne komórki Alzheimera typu II (strzałka). Błękit alcjanu. Pow. 400 ×.

Fig. 4. One-week culture, 24 hrs in a medium containing ammonia and sodium glutamate. A number of small process-free cells with cytoplasm containing numerous PAS-positive granules PAS. × 400.

Ryc. 4. Hodowla 1-tygod., 24 godz. w medium z dodatkiem amoniaku i glutaminianu sodu. Kilka drobnych komórek bezwypustkowych, których cytoplazma zawiera liczne ziarnistości PAS-dodatnie. PAS. Pow. 400 ×.

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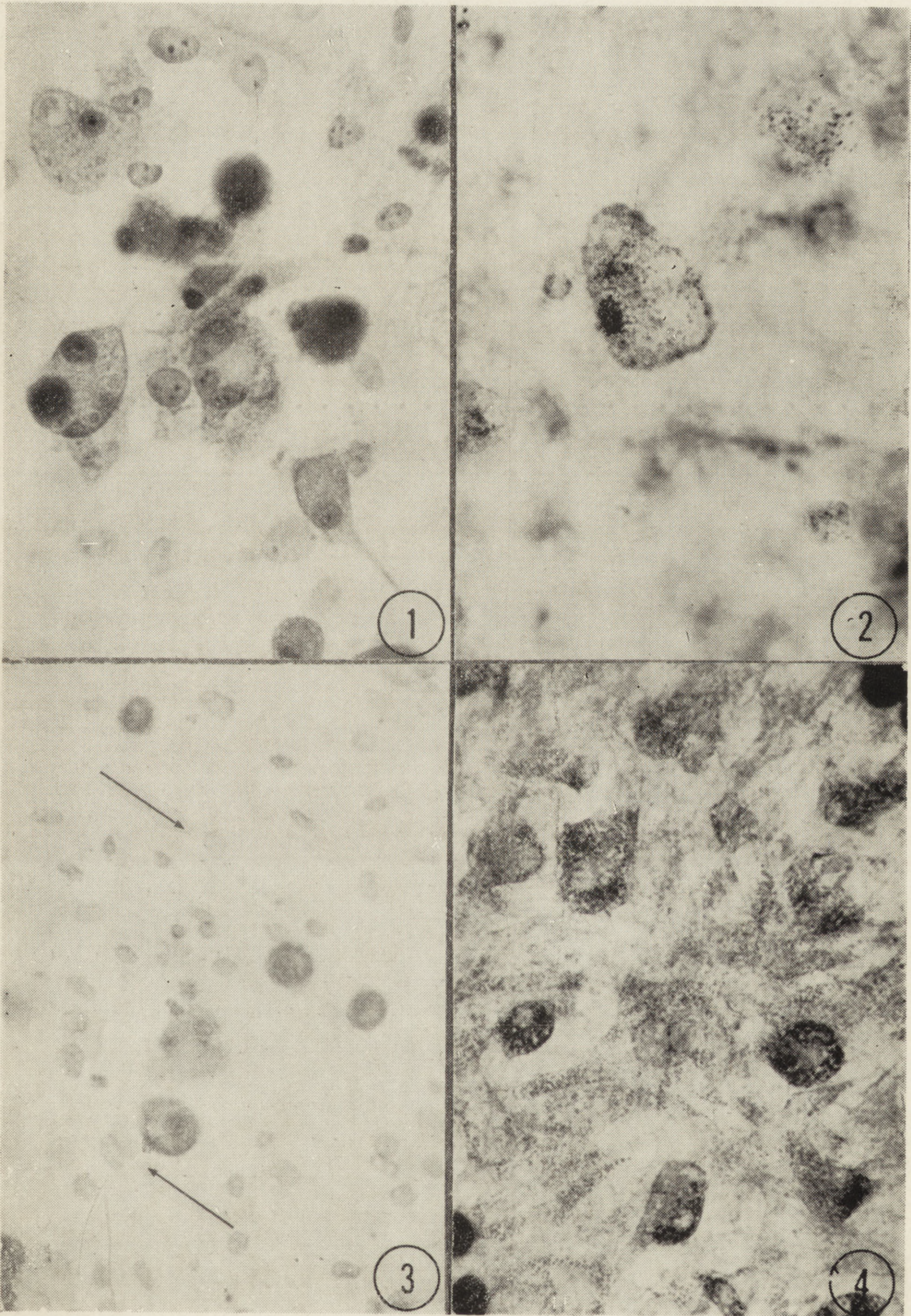

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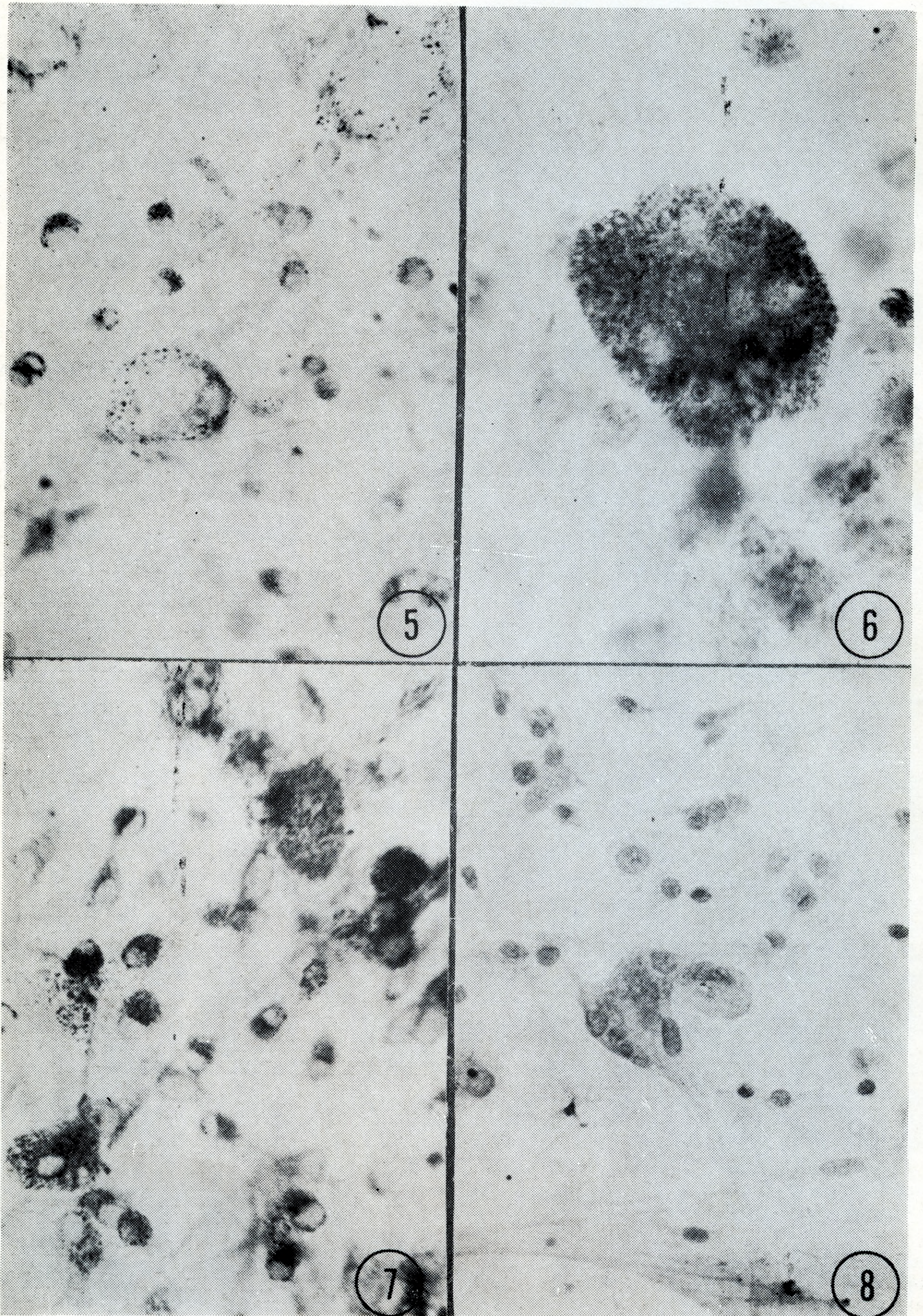

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The cultures maintained in medium containing sodium malonate with sodium glutamate (group IV) revealed morphological and histochemical changes, which were generally similar to those observed in group III. However, their intensity was less. Glial abnormalities were more significant in 1-week-old cultures than in the 3-week ones. Prolonged culturing in medium containing sodium glutamate resulted in a reduced intensity of cellular abnormalities. In 1-week-old cultures studied on the 3rd day of experiment Opalski cells occurred in 11 of 14 cultures, being numerous in only one and singly dispersed in the remaining ten. When studied on the 6th day, 7 cultures of 14 contained some Opalski cells, numerous in one and single in six. On the 9th day of experiment only 4 cultures of the total number of 14 revealed the presence of single hypertrophied giant cells. On the other hand 3-week-old cultures contained single Opalski cells only in 3 of the 18 cultures studied on the 3rd day of the experiment. Cultures examined on the 6th and 9th days of experiment contained no Opalski cells. At any period of observation their morphological and histochemical properties were identical with those observed in the former groups (Figs. 5—7).

Alzheimer cells type II occurring both in 1- and 3-week-old cultures were less numerous than in group III, but they were present during the whole period of observation. Intermediate cellular forms between normal astrocytes and Opalski cells (Fig. 8) were more numerous than in group III. Small, round, process-lacking cells described in group III were not noted. The number of cells showing features of non-specific

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Fig. 5. One-week culture, 120 hrs in a medium containing sodium malonate and sodium glutamate. Succinate dehydrogenase. Opalski cell with trace enzyme activity. × 400.

*Ryc.*5. Hodowla 1-tygod., 120 godz. w medium z malonianem sodu i z glutaminianem sodu. Dehydrogenaza bursztynianowa. Komórka Opalskiego z osłabioną aktywścią enzymatyczną. Pow. 600 ×.

Fig. 6. One-week culture, 120 hrs in a medium containing sodium malonate and sodium glutamate. Glutamate dehydrogenase. Opalski cell with reduced enzyme activity. × 600.

Ryc. 6. Hodowla 1-tygod., 120 godz. w medium z dodatkiem malonianu sodu i glutaminianu sodu. Dehydrogenaza glutaminianowa. Komórka Opalskiego z osłabioną aktywnością enzymatyczną. Pow. 600 ×.

Fig 7. Three-week culture, 120 hrs in a medium containing sodium malonate and sodium glutamate. Glucose-6-phosphatase. Opalski cells with well manifested enzyme activity. × 400.

Ryc. 7. Hodowla 3-tygod., 120 godz. w medium z dodatkiem malonianu sodu i glutaminianu sodu. Dehydrogenaza glikozo-6-fosforanowa. Komórki Opalskiego z dobrze wyrażoną aktywnością enzymatyczną. Pow. 400×.

Fig. 8. One-week culture, 120 hrs in a medium containing sodium malonate and sodium glutamate. Transient forms between Opalski cells and normal astrocytes. Toluidine blue. × 400.

Ryc. 8. Hodowla 1-tygod., 120 godz. w medium z malonianem sodu i z glutaminianem sodu. Komórki przejściowe, między komórkami Opalskiego i prawidłowym astroglejem. Błękit toluidyny. Pow. 400 ×.

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degeneration was less than in group III. Numerous glial cells were entirely normal, this being true especially for the group of 3-week-old cultures.

Cultures of control groups, both those grown in a standard medium and those kept in medium containing sodium glutamate alone, were morphologically and histochemically identical and showed features typical for glia cultured *in vitro*.

DISCUSSION

Our observations indicate that glutamate, given to the glial cultures together with ammonia and malonate, the gliotoxic effect of which has been proven in our previous investigations, exerts a protective activity, diminishing the cytotoxic effect of both compounds. Contrary to alfa-oxoglutarate it does not inhibit this effect entirely.

Under the influence of glutamate the number of typical pathological glial forms and the intensity of non-specific glial degeneration are greately diminished, this effect being enhanced with prolonged culturing time in medium containing glutamate. Nevertheless, the cytopathological and cytochemical pattern of glial abnormalities remains unchanged as compared with that observed in cultures exposed to the activity of ammonia and malonate (Mossakowski et al., 1970; Renkawek et al., 1973). The only new morphological feature consists in the appearance of small, round, astrocytes lacking processes, and revealing histochemical properties of Opalski cells. Their identification without electron-microscopic studies does not seem possible. It may be supposed, however, that they are abortive forms of Opalski cells, the full development of which is inhibited by sodium glutamate action. It seems worth pointing out that they occur only in cultures grown in medium containing ammonium chloride together with glutamate. These were the cultures in which the number of typical intermediate forms between normal astrocytes and fully developed Opalski cells was less than in any other experimental group. They never occurred in the group cultured in medium containing both malonate and glutamate, in which, on the contrary, numerous intermediate cellular forms were present.

The differences in the intensity of morphological changes between cultures grown in medium containing ammonium chloride with sodium glutamate and those with sodium malonate and sodium glutamate (groups III and IV) may indicate a weaker protective effect of glutamate in the case of ammonia. Our previous observations (Mossakowski et al., 1970, 1972) have already shown that ammonia exerted the strongest cytotoxic effect of all substances examined. In the light of these obser-

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vations, the lack of Alzheimer cells type II in 1-week-old cultures, grown in medium containing both ammonia and glutamate, and their appearance not before 48 hrs of experiment in 3-week-old ones remain unexplained, especially when we compare this with their presence in both age groups of cultures containing in their media malonate together with glutamate. Alzheimer cells type II are the most typical and common cellular elements of hepatogenic gliopathy, occurring both in human and experimental pathology, accompanied by hyperammonemia (Mossakowski, 1966; Bruton et al., 1970; Cavanagh, Ma Hta Kyn, 1971; Norenberg, 1974).

All types of cellular abnormalities are much more advanced in 1-week-old cultures than in those 3-week-old. This indicates a greater sensitivity of intensively developing and maturing cells, prevailing in younger cultures, to the noxious action of the substances applied. This seems to be a general property of tissue cultured *in vitro*, as indicated in several other studies (Kraśnicka, Renkawek, 1972; Hoppe, 1974).

The present observations seem to support our previous supposition that disturbances in ammonia detoxication are responsible for the occurrence of tissue abnormalities typical for Wilsonian and hepatogenic gliopathy. Ragardless of the nature of the damaging factor — ammonia, on the one hand, and malonate, on the other, glutamate exerts an evident protective action against damage to glia. However, glutamate seems to be a weaker protective factor as compared with alfa-oxoglutarate. This difference, although difficult to explain, may depend on the fact, that in the process of ammonia detoxication into glutamine, alfa-oxoglutarate binds two molecules of ammonia, while glutamate only one.

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WPŁYW GLUTAMINIANU SODU NA OBRAZ MORFOLOGICZNY I HISTOCHEMICZNY GLIOPATII WYWOŁANEJ PRZEZ AMONIAK I MALONIAN SODU W WARUNKACH HODOWLI *IN VITRO*

Streszczenie

Przedmiotem badań była ocena wpływu glutaminianu sodu na obraz morfologiczny i histoenzymatyczny glopatii wywołanej przy użyciu egzogennego amoniaku i malonianu sodu w warunkach hodowli tkankowej.

Do hodowli glejowych 1- i 3-tygodniowych podawano medium zawierające odpowiednio chlorek amonu i malonian sodu z glutaminianem sodu o ekwiwalentnym stężeniu molarnym .Obserwacje prowadzono w okresie od 24 godz. do 9 dni przetrzymywania hodowli w odpowiednim medium.

Stwierdzono, że dodatek egzogennego glutaminianu sodu zmniejsza efekt uszkadzający zarówno amoniaku, jak i malonianu sodu, ale nie znosi go całkowicie Wzorzec uszkodzeń komórkowych był niezmieniony. Efekt łagodzący uszkodzenia glejowe był większy w przypadku malonianu niż amoniaku. Sam glutaminian sodu nie wywoływał uszkodzeń tkankowych.

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ВЛИЯНИЕ ГЛЮТАМАТА НАТРИЯ НА МОРФОЛОГИЧЕСКУЮ И ГИСТОХИМИЧЕСКУЮ КАРТИНУ ГЛИОПАТИИ, ВЫЗВАННОЙ АММИАКОМ И МАЛОНАТОМ НАТРИЯ В УСЛОВИЯХ КУЛЬТУРЫ *IN VITRO*

Резюме

Целью работы была оценка влияния глютамата натрия на морфологическую и гистохемическую картину глиопатии, вызванной путем использования экзогенного аммиака и малоната натрия в условиях тканевой культуры.

В 1- и 3-недельные глияльные культуры вносили среды, содержащие соответственно хлористый аммоний и малонат натрия с глютаматом натрия в эквимолярной концентрации. Наблюдения проводили в период от 24 часов до 9 дней после выдерживания культуры в соответствующей питательной среде.

Было обнаружено, что добавка экзогенного глютамата натрия уменьшает отрицательное воздействие самого аммиака и малоната натрия, однако, в отличие от α-оксоглютарата не снимает его полностью. Картина клеточных повреждений оставалась неизмененной. Эффект, смягчающий глияльные повреждения, был в случае малоната большим, чем аммиака. Один глютамат натрия не вызывал тканевого повреждения.

Проведенные исследования подтверждают гипотезу, что решающим звеном патогенетических глияльных повреждений является нарушение детоксикации аммиака.

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