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Examining of Changes in Continuous GMK Cell Cultures Treated with Hydrocortisonum Hemisuccinatum, Propranolol and Diazepam

Badanie zmian w ciągłej hodowli komórek GMK pod wpływem Hydrocortisonum hemisuccinatum, Propranololu i Diazepamu

Culturing of mammalian cells in experimental *in vitro* conditions is at the present time a basic method of multidirectional research in medical and natural sciences, particularly in examining drug mechanisms, immunologic (7, 8, 11) and genetic (10, 12) investigations and in studies aimed at finding mechanisms of oncogenesis (1, 2, 6, 9).

In vitro cell cultures, particularly human and monkey cell cultures are useful for examination of the influence of chemical compounds (including drugs) upon cell growth, morphology and several intracellular metabolic pathways. The characteristic feature of such an experimental system, compared to the organism of the experimental animal, is that metabolic function of internal organs (such as liver, lungs, muscles) is eliminated, (3, 4, 6). The influence of drugs and chemical compounds upon intracellular pathways is more direct. This makes possible a deeper analysis of drug effect on a cell function. Indirectly it allows to come to conclusions of drug pathways and particularly drug influence upon the growth and function of an isolated cell. This method may be useful for examining the properties of the already known and newly synthesized drugs (5, 9).

Basing on the above-mentioned assumptions, the influence of

Hydrocortisonum hemisuccinatum, Propranolol and Diazepam upon a monolayer culture of cells of a constant monkey line GMK (green monkey kidney) was resolved to be examined.

MATERIAL AND METHODS

A typical cell culture was used. It was a passed constant line of culture obtained from kidneys of green monkeys (*Cercopithecus aethiops*), called GMK (green monkey kidney) line. It was provided by Wytwórnia Surowic i Szczepionek in Lublin.

The influence of the undermentioned drugs on the growth and morphology of cells was examined.

a) **Hydrocortisonum hemisuccinatum**. Hydrocortisone sodium succinate, sodic salt of hydrocortisone succinate. As a drug it is widely and multidirectionally administered, chiefly in connective tissue diseases, various types of shock, attacks of severe bronchial asthma and many other illnesses.

b) **Propranolol hydrochloride.** Proprasylytum. 1-isopropylamino-3-(1-naphtyloxy)-2--propranol hydrochloride. It acts on ß-adrenergic receptors of the heart muscle. Effects of its action are multidirectional.

c) **Diazepam.** Benzodiazepine derivative. Methylodiazepinone, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one. This is a sedative, anxiolytic and anticonvulsant drug.

Generally, the methods can be divided as follows: a) *in vitro* cell culture methods, b) drug dilution methods, c) methods of microscopic examination of control cell cultures and experimental cell cultures, d) establishing of drug dose causing cytotoxic effect, e) methods of basic staining on cover-glass, f) special methods of staining on cover glass, g) microscopic examination of stained preparations, h) photographing stained preparations, i) analysing microscopic and photographic cell images (4, 9).

The cell culture was prepared according to classical regulations, using methods and mediums appropriate for a GMK cell culture. Drugs were diluted successively in sterile conditions, beginning from original ampoule concentrations to end dilutions. The examined drug dilutions in medium added to a cell culture were as follows:

Hydrocortisonum hemisuccinatum was examined at the following concentrations: 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml, 3.905 µg/ml. The cytotoxic dose of 500 µg/ml was established.

Propranolol was examined at the following concentrations: 500 µg/ml, 250 µg/ml, 125µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62µg/ml, 7.81 µg/ml, 3.905 µg/ml. The cytotoxic dose of 500 µg/ml was established.

Diazepam was examined at the following concentrations: 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml, 3.905 µg/ml, 1.195 µg/ml,

0.976 μg/ml, 0.488 μg/ml, 0.244 μg/ml, 0.122 μg/ml, 0.061 μg/ml, 0.030 μg/ml, 0.015 μg/ml, 0.007 μg/ml, 0.003 μg/ml, 0.001 μg/ml, 0.0009 μg/ml. The cytotoxic dose of 500 μg/ml, was established.

The examined cell cultures were incubated for 12, 24 and 36 hrs. The cytotoxic drug dose was established after a 12-hr incubation by finding the damage of cell monolayer, dead cells and growth inhibition at a definite drug concentration.

The constant GMK cell line was maintained in a "mother" bottle by successive passing. Cells in the bottle were trypsinized; as a result the suspension of about 150 thousand cells in 1 ml was obtained and placed into test-tubes or bottles with cover glasses in.

After cover-glass surfaces had been cell-covered in 85—90% as a result of incubation, the culture medium was replaced by a solution containing the definite concentration of the examined drug.

After an appropriate incubation time (12, 24, 36 and 48 hrs) cells were examined under a light microscope and their morphology was evaluated. A part of the experiment — the cells on cover glasses were stained by means of the modified Giemsa method. The preparations were fixed, examined under microscope and photographed.

Staining by means of the modified Giemsa method: 1. Remove cover glasses with cell culture from test-tubes or bottles. 2. Rinse with PBS (buffered physiologic salt solution). 3. Air dry. 4. Fix using methanol (about 20 min). 5. Rinse with distilled water. 6. Air dry. 7. Stain using Giemsa stain for about 1 hr (stain preparation: to prepare stain add 50 drops of stain to 10 ml of distilled water). 8. Rinse with tap water. 9. Air dry, stick cover glass to glass slide using Canada balsam, examine under microscope.

RESULTS

HYDROCORTISONUM HEMISUCCINATUM

In the first group of experiments a cytotoxic dose of Hydrocortisonum hemisuccinatum for a monolayer continuous monkey-cell culture was established according to the microscopic evaluation of the morphology and growth of cells treated with the various concentrations of the drug in culture medium, (see Fig. 1). The cytotoxic dose of Hydrocortisonum hemisuccinatum was established compared to control culture. The dose was 62.5 μg/ml.

In the second group of experiments the influence of Hydrocortisonum hemisuccinatum upon intracellular alterations was examined, applying a special cover-glass culture.

The above-mentioned experiments were conducted as follows:

Successive drug dilutions were added to a 4-day cell culture; the culture was incubated in the temperature of 37° centigrade. Cover glasses were removed after definite time (12, 24, 26 and 48 hrs), fixed with methanol and stained by means of the modified Giemsa method. Cover glasses were sticked to glass slides using Canada balsam. Subsequently they were examined under microscope. Noticed changes were compared to corresponding control preparations.

Pathological changes in the cell monolayer were already evident after the incubation for 12 hrs. Where the highest drug concentrations had been used, cell growth and division were inhibited. The changes affected cell situation and shape; physiological and morphologic alterations in cytoplasm were also apparent. They progressed in time (12, 24, 36 and 48 hrs). These included alterations of nuclear outline, caryorrhexis, production of vacuoles, shrinkage and/or slight laceration of cytoplasm and cytolysis (see Fig. 2.).

PROPRANOLOL

In the first group of experiments the cytotoxic dose of Propranolol for monolayer continuous monkey-cell culture was established according to the microscopic evaluation of cell morphology and growth. According to the microscopic examination cytotoxic dose propranol was established. The dose was 62.5 μg/ml.

In the second group of experiments a methodological procedure analogical to Hydrocortisonum hemisuccinatum examination was followed.

DIAZEPAM

In the first group of experiments the cytotoxic dose of Diazepam for monolayer continuous monkey-cell culture was established, according to the microscopic evaluation of cell morphology and growth. According to the microscopic evaluation the cytotoxic dose of 3.905 μg/ml was established.

The microscopic review of cell cultures proved that there were pathological changes evident in cell monolayer; after the incubation for 12 hrs cell growth and division were inhibited in all the test-tubes. Changes affected cell situation and shape, and alterations in cytoplasm. In all

concentrations the alterations included: change of a nuclear outline, karyorrhexis, production of vacuoles, shrinkage and/or slight laceration of cytoplasm and cytolysis.

The second group of experiments was aimed at defining the influence of Diazepam on intracellular changes applying special cover-glass culture. The advantage of this method is that cells adhere and divide both at glass of test-tube and cover glass, which may be removed at any time, fixed, stained and prepared. Cells used for experiment should create a single and even layer, so-called monolayer.

DISCUSSION

HYDROCORTISONUM HEMISUCCINATUM

It was established that Hydrocortisonum hemisuccinatum in the concentration of above 62.5 μg/ml after 12 hrs reveals cytotoxic action. It mainly affects cytoplasm, then nucleus and cell membrane. The degenerative changes deepen in time proportionally to the increase of drug concentration.

Changes in cytoplasm were noticed. It was lacerated, thread-like, reticular, lace-like and distended. Cytoplasmatic vacuoles appeared. Nuclei were irregular, with granules, vacuolated, stained more distinctly than the ones in control culture.

PROPRANOLOL

It was established that Propranolol in the concentration of above 62.5 μg/ml after 12 hrs reveals cytotoxic action. It chiefly affects cytoplasm, then nucleus and cell membrane. The degenerative changes deepen in time proportionally to the increasing drug concentration.

The influence of Propranolol on monkey kidney cells is considerable; particularly large changes may be observed in cytoplasm. Large degenerative changes appear at higher drug concentrations. Theoretical calculations indicate that drug administered according to prescriptions should not be harmful to mammalian cells (see Fig. 3).

DIAZEPAM

Observations indicated that the first symptoms of pathology — disturbances of cell monolayer continuity — appeared after 12 hrs at the concentration of 3.905 μg/ml.

At the concentration of 3.905 μg/ml after 24 and 36 hrs cytoplasm shrinkage and a small number of atypic cells may be observed. After 48-hr time in the above-mentioned concentration the changes are very evident and they affect most of the cells.

At higher concentrations the changes increase. Granules and intranuclear inclusion bodies appear in cell nuclei. It is possible that the drug may be harmful to mammal organism cells. Both the conducted experiments and theoretical calculations indicate that the drug must be administered in low doses (see Fig. 4).

CONCLUSIONS

1. Studies of toxicity of the three drugs: Hydrocortisonum hemisuccinatum, Propranolol and Diazepam were conducted.

2. Hydrocortisonum hemisuccinatum revealed its toxic action at the dose of 62.5 μg/ml after 12-hr time since its application to cell culture. The dose indirectly gives evidence that the drug is safe and it should not act harmfully to mammal organism cells.

3. Propranolol revealed its toxic action at the dose od 62.5 μg/ml after 12-hr time since its application to cell culture. Such a high dose indirectly gives evidence that the drug is safe and it should not act harmfully to mammalian organism cells.

4. Diazepam revealed its toxic action at the dose of 3.905 μg/ml after 12-hr time since its application to cell culture. Such a low dose indirectly gives evidence that the drug should be administered in low doses to prevent mammalian organism cells from damage.

5. The observed cytotoxic effect of the three above-mentioned drugs originated in cytoplasm by vacuolation and damage of cytoplasm: subsequently cell nuclei altered morphologically.

6. Culturing of mammalian cells in experimental *in vitro* conditions is at the present time a basic method of multidirectional research in medical

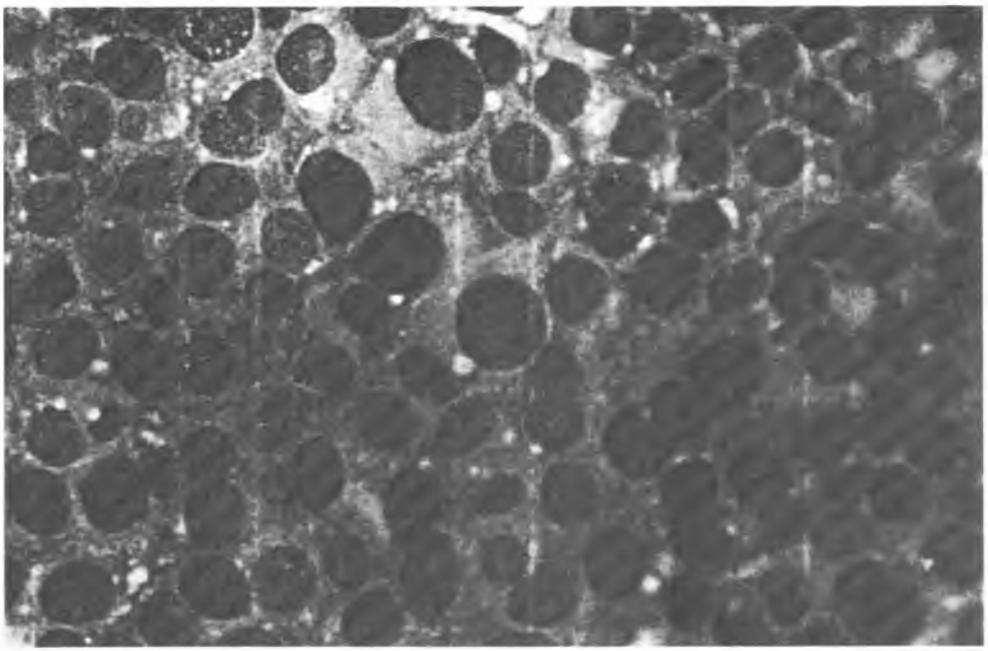

Fig. 1

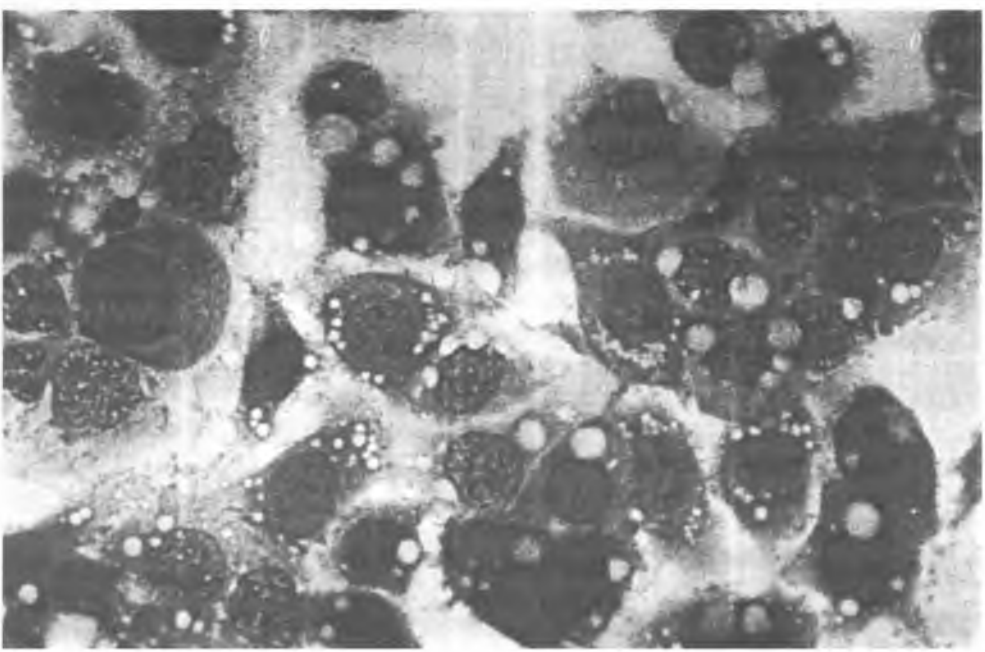

Fig. 2

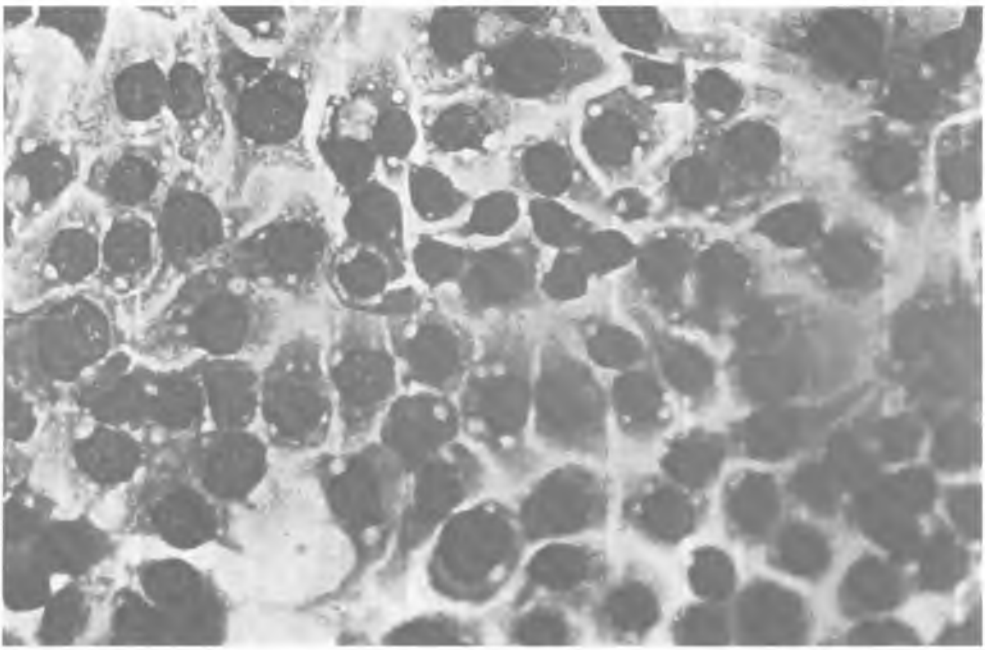

Fig. 3

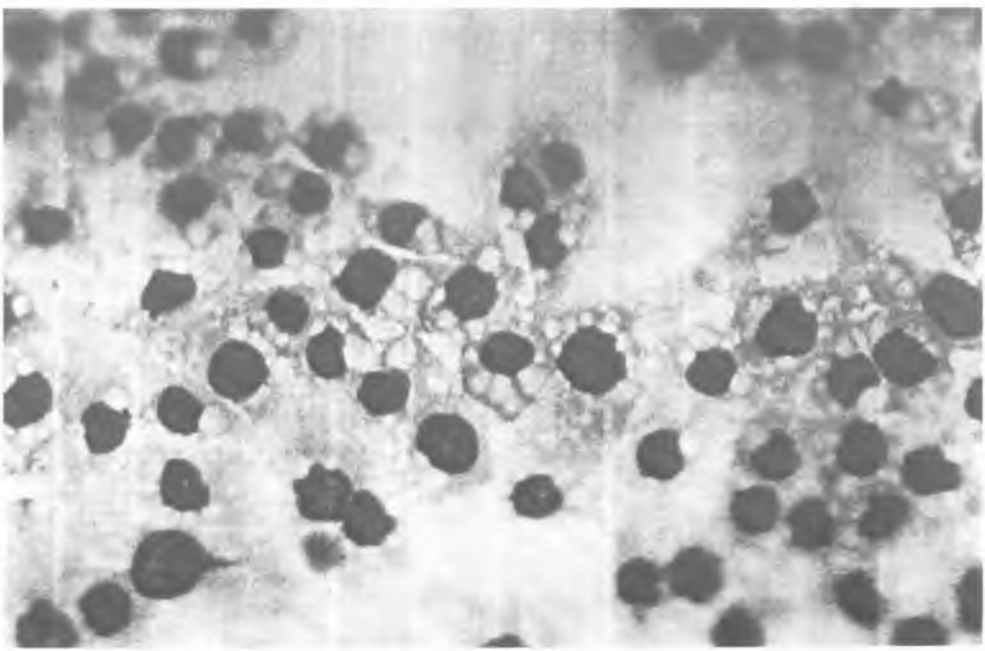

Fig. 4

and natural sciences, particularly in examining viruses and drug mechanisms, immunologic and genetic investigations and in studies aimed at finding mechanisms of oncogenesis.

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STRESZCZENIE

Badano zmiany cytopatologiczne w jednowarstwowej hodowli linii ciągłej komórek nerek małp (GMK), zachodzące pod wpływem trzech wybranych leków. Ustalono cytotoksyczne dawki poszczególnych leków. Opisano i przedstawiono na fotografiach zmiany w strukturach morfologicznych komórek, zachodzące po określonym czasie działania leku. Uznano, że hodowla komórek *in vitro* może być przydatna w badaniu toksyczności leków na organizm człowieka.

EXPLANATION TO FIGURES

Fig. 1. Breeding control of green monkey kidney cells after 36 hrs of breeding. Magn. 1600×.

Fig. 2. Degeneration of green monkey kidney cells after 24 hrs of Hydrocortisonum hemisuccinatum treatment (concentration: 31.25 μg/ml). Magn. 1600×.

Fig. 3. Degeneration of GMK cells after 24 hrs of Propranolol treatment (concentration: 3.905 μg/ml.) Magn. 800×.

Fig. 4. Degeneration of GMK cells after 48 hrs of Diazepam treatment (concentration: 250 μg/ml). Magn. 1600×.