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PHARMACOLOGICAL IN VITRO MODELS IN PRE-CLINICAL DRUG TESTING - EXAMPLE OF hSERT TRANSFECTED HUMAN EMBRYONIC KIDNEY CELLS

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Preclinical drug testing should be considered an important stage during examinations of its efficiency and safety in any likely indication observed. Purpose of the process is acquisition of substantial amount of particular drug-related data before approaching clinical trials in humans. Historical preclinical testing relied on available testing in microbe cultures and animal models. During recent decades laboratory techniques of human cell lines cultivation have been developed and improved. These provide unique possibility of drug acting mechanism testing in a simplified environment lacking basic homeostatic mechanisms. Some examples of these are measuring drug impact to biochemical transport, signaling or anabolic processes. Humane cell lines of embrional kidney 293 are an example of easy-to-grow and disseminate and quite endurable cell line. This methodological article notices some of the details of HEK293 cells cultivation and breading. We took transfection as an example of in vitro model creation for drug testing. Transfection refers to gene introduction into HEK293 cellular genome in order to achieve membrane expression of coded protein. In our case it would be human serotonin transporter. Article contains description of one particular methodological approach in measuring human serotonin transporter expression. The role and importance of serotonin pump in affective disorders genesis was already widely recognized. Aim of the paper was to emphasize feasibility of cell cultivation and its advantages in comparison with alternative traditional methods. *Acta Medica Medianae 2012;51(2):34-38.*

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Experimental in vitro models of pre-clinical drug testing

Any experimental substance with a noticeable therapeutic potential in a humane disease or disorder must pass the long-term preclinical drug development cycles. This practice is established on the long farmacovigilance world history in recognizing the severe drug side effects, registered and approved for the humane usage pretermely (1). Such compounds were withdrawn from the use leaving the serious consequences to the society affected by the adverse event and to a company which invested the great amount of time and resources into the new drug development as well (2- 4).

Fortunatelly for patients, the rapid development of applied organic chemistry, physiology and the basic pharmacotherapy during the last century and a half, provided us with animal models for certain diseases. Such are the examples of the induced malignant tumors, convulsive disorders or diabetes in rodents and lower primates. That way we learn to discover more about the efficacy of the observed indication and safety of a drug application before the first experiments on the humane subjects. Furthermore, towards the lower levels of hierarchical life the organization by Line`s biological classification in-vitro laboratory experiments with cultivated organisms followed (5). They could be very useful in examination the microbicide effect and resistence to antibiotics or, much later, mutagenic potential of various drug kinds.

However, a quite new phase in preclinical drug development was set by the possibility of extracorporeal mamal cell cultivation under "in-vitro" circumstances. Early supporters of this development noted the advantages of isolated observation of biological processes and drug impact upon them in the simplified artificial surroundings and the absence of most distracting homeostatic mechanisms. The very act of cultivating of eukaryotic cells "in-vitro", was developed during the last half of the century conducted by the idea that imitating the "in-vivo" conditions, cells could be

sustained and that their metabolism and the reaction towards the various external influence could be examined (6). The methodology of cultivation and reproduction of human cell lines, which later became the cultivation of the whole tissue, represents one of the basic postulates of the experimental biomedicine and the useful tool for the development of new diagnostic and therapeutic procedures. The present standards of a good laboratory practice and relative simplicity of the working technique with the cell cultures in vitro are the outcome of a long process of improvement of their growth medium structure and sterile working conditions (6-9).

Humane cell line of embryonic kidney 293

Most of the animal cells show the ability of survival, proliferation and manifestation of certain differentiation level in culture phenotype throughout several generations, if they are obtained with certain conditions considering humidity, temperature, nutrient presence, oxygenation, adequate pH environment and the absence of microorganisms. By the way of formation cell lines can be roughly divided into primary and continuous. The first one was derived from the mechanic excision and enzymatic digestion directly from the tissue in vivo, and have limited lifetime and they are relatively difficult to be kept in vitro (6, 7). Continuous lines among which is HEK 293 itself are most often the population of monoclonal origin and, although they are less differentiated than their primary relatives, they can be successfully immortalized by oncogenic viruses and actually transformed into tumor cells which can be indefinitely propagated and diffused in laboratory conditions. They are typical for their distinctive tendency to mutations and morphological phenotype changes (8-10).

HEK 293 cells are macromorphologically similar to fibroblasts and the accurate progenitor embrional kidney tissue is not known. Examining their genotype and nature and kinds of several dozens of proteins that are expressed in cytosol and on the membrane and are uniquely characteristic for the neural tissue, Gerry Shaw sets the hypothesis about their neural origin which haven`t met the consensus of experts (8, 9). We have chosen the line mentioned above as the one suitable for work, because of the feasible gene input for the target protein-transferor of biogenic amines, whose behavior can be afterwards observed, in vitro, under the influence of xenobiotic ligands under the circumstances that are simpler than those prevailing in nerve synapses, in vivo.

An example of experimental procedure of gene implementation for serotonin membrane transporter

As a chosen example of in vitro methodology for the examination of drug effect mechanism, serotonin membrane transporter expression was observed. The gene that codes the protein above by act of transfection is artificially inserted to the embryonic kidney cells. Transfection represents the implementation of an extraneous genetic material which most often codes the target protein by means of plasmid or viral vector (9-11). The molecule mentioned above is closely related to the depression pathogenesis, suicidal behavior, impulsive aggression and alcoholism during the last 20 years (12-18). The assumption that the level of expression and/or function of this molecule were changed increases the possibility of certain mental diseases appearance (15, 16, 19). It is important since, being situated on the presynaptic neuron membrane, it performs the sudden takeover of the serotonin released in exocitosis and that way controls its concentration in synaptic gap. Thus it affects the duration and the intensity of signal transmitted by binding of these endogenous ligands to post-synaptic 5HT receptors, seven types of them described currently (15, 16, 19-23).

The affinity of drug binding to SERT is the focus of many molecular biological studies. The reason for that is the fact that the drug effect mechanism of several key drug classes in neuro science is based on the pharmacological agonism or antagonism of natural biogenic amines role (22).

The main candidates in this group are antidepressants of selective inhibitor serotonin reuptake class SSRI (escitalopram, fluvoxamine), the later anti-psychotics (zyprasidon), obesity treatment drugs (sibutramine), migrene treatment drugs (risatriptane, naratriptane), certain antiemetics and prokynetics (metoclopramide, cetanserine, alosetron, cyproheptadine, tegaserode).

Methodology of rising, scattering and trans-fection on HEK293 cells

Consider the authentic, genotypic and morphologically standardized, continuous HEK293 line in deeply frozen condition (-190°C). The uniqueness of this line is reflected in the fact that it already contains the implemented and exprimated gene on the serotonin human transporter membrane (hSERT). The culture above is to be commercially obtained from a human cell line bank or from an academic source such as University Vanderbilt of Nashville, Tennessee, USA, which has a several year standardization and work with the line above experience ,so that it conceded its lines to other centres for the sake of further examination (12, 15, 19, 24).

The working procedure consists of the sudden line defrosting up to +37 °C which prevents the toxicity of cryo preservative by timely immersion into nutrient medium. The culture mentioned above is adherent, epithelial type, is sown on standard PVC Petri dishes of 75cm² (Falcon) with the necessary cladding with poly – D - lysine and is maintained on the basic DMEM

nutrient medium with the addition of 10 % of fetal bovine serum (FCS- GIBCO, Grand Island, NY). For passage, and further propagation of the cells during growth, it is sufficient to wash the cells with PBS buffer (NaCl 0.14mm, 2.7mm KCl, 10mm Na_2HPO_4, KH_2PO_4 2mm, pH 7.4), separate them from the base and make the suspension with commercial tripsine of a several minute duration and then change the medium for the chosen 10% of cells every 72 hours. The other conditions for keeping in the incubator considering PH of the environment, oxygenation, and resistance to contamination by microorganisms do not differ significantly from other human immortalized lines (5% CO_2, 37°C, pH 7.4).

After entering the phase of stable growth it is necessary to obtain the familiar solution concentration of examined hSERT ligands. Exposing the cell line to increasing concentrations and exposure time of the given compounds, we can measure the serotonin pump working tempo (with the known expression level) before and after the intervention (13, 14, 16, 20, 25).

Before every experiment, it is needed to visually examine the line vitality and provide the confluence (thickness) higher than 75%. The procedure is started with removing the old medium and rinsing the cells in Krebs-Ringer-Hepes buffer (KRH: 120 mM ; NaCl, 4.7 mM KCl, 10 mM Hepes, 1.2 mM MgSO4, 1.2 mM KH_2PO_4,5 mM Tris, 2.2 mM $CaCl_2$) at room temperature. In every Petri dish we add 475 µl of KRH buffer, with addition of 1.8g/L of glucose (gKRH). When we plan to add the drug, the cell will be pre-incubated with the desired agent durin 20 minutes at least (at 22°C). Before the planned intervention, in order to control it, we check the hSERT function. The measuring consists of following and the procedure is not significantly different whether we have presence or absence of experimental factor:

• Assuming that the synthesis of 5HT in the cytosol is negligible in the absence of an essential amino acid tryptophan in the medium (as a substrate from which forms mentioned transmitter).

• We observe the input of extracellulary added 5HT in a known concentration (10 µM of 5HT at the the cell population number 3.5-6*105 at 24 chamber flask), as only or at least dominant source of intracellular 5HT.

• It is necessary to add 50 µM of pargyline and 50 µM of ascorbic acid in order to prevent the intracellular degradation of serotonin input (5HT).

• After the availability of 5HT to the cells during one minute, it is needed to rinse the culture two times with cold KRH buffer and change the medium for a new one so that the further input would be interrupted.

Here we use another approximation in order to simplify the model of serotonin pump function examination, and it assumes that its motion is approximately one-way influx outwards to inwards, i.e. we neglect the biogenic amines efflux outer cells which de facto happens to some extent.

After the mentioned procedure we approach to the analysis of speed and scope of net takeover of 5HT by means of fluorescent essay (Neurotransmitter Transporter Uptake Assay Kit - R8174, MDS)

The results obtained can be incorporated into the simple mathematical model which allows us to quantitatively interpret the impact of ligand presence on the pump function.

Expected response in the hypothetical experiment of this kind

After the exposure of HEK293 with the experimental humane SERT on the membrane to the compounds with the proven affinity to binding to transporter mentioned, the following outcomes are assumed for the biogenic amines:

• Antidepressant agents of SSRI group, in accordance with previously confirmed basic mechanism of their affection in depression pathogenesis, in our example, significantly inhibit the SERT function (40%) and lower the tempo of 5HT reuptake and therefore probably leave the higher concentration of this signal molecule in the synaptic gaps zone in human brain in vivo.

• Different tested antiemetics and prokinetics with the hypothetic basic mechanism of their action by means of a few types of postsynaptic 5HT receptors upon the neuroendocrin digestive tract cells do not disturb to higher extent the action of the 5HT transporter so that, it does not represent the significant mechanism of their action in indications which they are according to the majority opinion registered for in some countries.

• The influence of antimigraine substances of later generation, of confirmed efficiency, does not inhibit the hSERT action either, and therefore, the mentioned substances require different theoretical and experimental foundation of their insufficiently known mechanism of action in vascular headache genesis.

Future projections of cultivating methodology

The example of routine working methodology with one kind of human cell line and transporter of one endogenic ligand considered to be crucial in many neural diseases genesis was described. In the time of increasing interest for stem cell lines this is only one of many examples of practical application of simple models in pre-clinical drug examination. The basic advantage of this cell and tissue cultivating is their human origin comparing to the work with complex animal models. A distinctive specificity complicates the generalization and application of the noticed results upon the human population. The other advantages are the safety of early estimation of observed agent relative efficiency in the given indication and timely

detection of some forms of drug toxicity such as mutagenicity and cytotoxicity that is induction of apoptosis. The disadvantages of this methodology are drastic simplicity considering in vivo conditions and the absence of homeostatic mechanisms which contrast the drug effect. However, it is up to the time and the whole tissue specialization, and maybe even the whole organs, to show the permanent position of this methodology in the development of clinical usage drugs.

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FARMAKOLOŠKI IN VITRO MODELI ZA PREDKLINIČKO ISPITIVANJE LEKOVA - PRIMER hSERT TRANSFICIRANIH HUMANIH EMBRIONALNIH ĆELIJA BUBREGA

Mihajlo Jakovljević i Olivera Milovanović

Predkliničko testiranje lekova je važan segment ispitivanja efikasnosti i bezbednosti leka u pretpostavljenoj indikaciji. Njegova svrha je u prikupljanju značajnog kvantuma informacija o leku pre ulaska u kliničku fazu ispitivanja na humanim subjektima. Pored ispitivanja na mikroorganizmima i animalnim modelima poslednjih decenija je usvojena tehnologija rada sa kultivisanim ćelijskim linijama humanog porekla. One pružaju jedinstvenu mogućnost ispitivanja uticaja leka na biohemijske transportne, signalizacione i anaboličke procese u pojednostavljenom okruženju sa drastično umanjenim uticajem homeostatskih mehanizama. Humane embrionalne ćelije bubreg su primer veoma zahvalne za kultivaciju i relativno otporne ćelijske linije. Metodološki rad koji je pred Vama iznosi detalje njihovog uzgajanja i rasejavanja. Kao primer stvaranja in vitro modela za testiranje lekova navodimo postupak transfekcije. On se odnosi na unošenje gena za membransku ekspresiju humanog transportera za serotonin na HEK293 ćelijama. Opisan je standardni metodološki pristup merenja ekspresije pomenutog transportera, učinka transfekcije. Uloga i značaj rada ”serotoninske pumpe” su već dobro opisani u genezi poremećaja raspoloženja.

Cilj članka je da ukaže na izvodljivost i prednosti rada sa humanim ćelijskim linijama umesto alternativnih prokariotskih i animalnih modela. *Acta Medica Medianae 2012;51(2):34-38.*

***Ključne reči**: humane ćelijske linije, kultivacija, transfekcija, HEK293, hSERT*