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Complex Approach to Xenobiotics Hepatotoxicity Testing using a Microfluidic System

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We analyzed hepatotoxicity of three drugs: acetaminophen, metformin, and isoniazid. Spheroids of differentiated HepaRG cells cultured under microfluidic conditions were used as the model. Acute toxicity of substances was assessed by analyzing cell viability, while lactate concentration in the culture medium was used as the potential marker for evaluation of chronic exposure and non-lethal side effects of xenobiotics. The results were compared with mitochondrial activity and DNA fragmentation data. The efficiency and possibility of applying the integrated approach for assessment of drug hepatotoxicity are discussed.

Key Words: *HepaRG; hepatotoxicity; xenobiotics*

Drug-induced liver injury is the most common cause of liver dysfunction and the main cause for drug withdrawal from clinical trials and from the market.

Hepatotoxicity can be caused by both original drugs and their metabolites and induces cell death via necrosis or apoptosis. The most common approaches for analysis of cytotoxicity are MTT (evaluation of mitochondrial activity) and neutral red staining (evaluation of lysosome activity). However, cell viability is the late indicator of toxicity, and these methods only make possible evaluation of acute toxicity of the substance and cannot be applied for the studies of chronic exposure to xenobiotics and non-lethal side effects [7].

Apoptosis can be triggered by DNA breaks and leads to DNA fragmentation accompanied by the formation of free 3'-hydroxyl groups. TUNEL method makes possible tagging formed gaps and therefore is suitable for estimation of the functional status of cells and genotoxic effects of xenobiotic.

Changes in structural, biochemical, and metabolic components occur prior to cell death and can lead to severe functional impairment without affecting cell viability. Thus, along with viability evaluation, other markers of hepatotoxicity should be analyzed, *e.g.* lactate production during anaerobic metabolism of glucose. Accumulation of this metabolite as a result of glycolysis may be indicative of hypoxia, oxidative stress, and mitochondrial dysfunction [2]. Moreover, lactate biosensor enables real-time measurement of this parameter [10,12].

The purpose of this study was to develop a complex approach to the analysis of hepatotoxicity of xenobiotics (acetaminophen, metformin and isoniazid) based on the use of differentiated HepaRG cell spheroids cultured in microfluidic system as the model of the liver.

MATERIALS AND METHODS

HepaRG cells were cultured in William's E medium supplemented with L-glutamine (Gibco), 10% fetal calf serum (HyClone), 5 μg/ml of recombinant human insulin (Gibco), 5×10^{-5} M hydrocortisone hemisuccinate (Sigma), and 1% penicillin/streptomycin (Gibco) in a CO_2 incubator (37°C; 95% air, 5% CO_2; relative humidity 98%). Upon reaching confluent monolayer, 2% DMSO was added to the culture medium in order to induce differentiation. In 14 days after induction of differentiation, the spheroids consisting of 5000 cells were formed as described elsewhere [15]. The obtained spheroids were cultured in

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a microbioreactor with recirculating nutrient medium (BioClinicum) [11].

Incubation with xenobiotics (acetaminophen, metformin, and isoniazid; Sigma) was carried out for 48 h in serum-free nutrient medium: William's E medium (Gibco), 2 mM L-glutamine (Gibco), 1 mg/ml human blood serum albumin (Sigma), a nutritional supplement consisting of insulin, transferring, and selenium (5.5 mg/ml insulin, Gibco), non-essential amino acids (Gibco), 50 μM of hydrocortisone hemisuccinate (Sigma), and 1% penicillin/streptomycin (Gibco).

Cell viability was estimated by conversion of soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT) into purple-blue insoluble intracellular crystals of MTT-formazan by live cells as previously described [2]. Spheroids were incubated with 5 mg/ml MTT for 2 h in a CO_2-incubator. Formazan crystals were dissolved in DMSO for 20 min on a shaker in darkness. Optical density was measured at 530 nm. Cell viability was calculated as the ratio of optical densities of experimental to control sample and expressed in percents.

DNA breaks were detected by TUNEL cytofluorometry using Click-iT Plus TUNEL Assay (Life Technologies) according to manufacturer's protocol.

Lactate concentration in the culture medum samples was measured spectrophotometrically using Lactate Dry-fast test-system (Sentinel Diagnostics) according to manufacturer's protocol with minor modifications.

The data were statistically analyzed using nonparametric Mann–Whitney *U* test. The differences were significant at $p<0.05$.

RESULTS

Apoptosis is accompanied by DNA fragmentation leading to the formation of free 3'-hydroxyl groups. TUNEL method makes possible tagging the formed gaps. After culturing of HepaRG spheroids with acetaminophen for 48 h in the microbioreactor, the genotoxic effect (detected by green fluorescence of Alexa Fluor 488 dye) appeared at a concentration of 1 mM (Fig 1). Increasing the xenobiotic concentration led to acceleration of deoxyuridine triphosphate incorporation into DNA (fluorescence intensity) in treated spheroids, and the number of apoptotic cells increased. Acetaminophen is a classical true direct hepatotoxin producing a predictable dose-dependent effect [8]. IC_{50} value according to MTT-test was 14.9±1.2 mM. Thus, the results obtained on HepaRG spheroids are consistent with the available data.

Enhanced glycolytic production of lactate is caused by hypoxia and oxidative stress. Therefore, elevated concentration of this metabolite in the medium is a marker of impaired energy metabolism in hepatocytes [5]. Low non-lethal acetaminophen concentrations (1 mM) led to an increase in lactate concentration in the culture medium. Increasing drug concentration was followed by gradual decrease in lactate

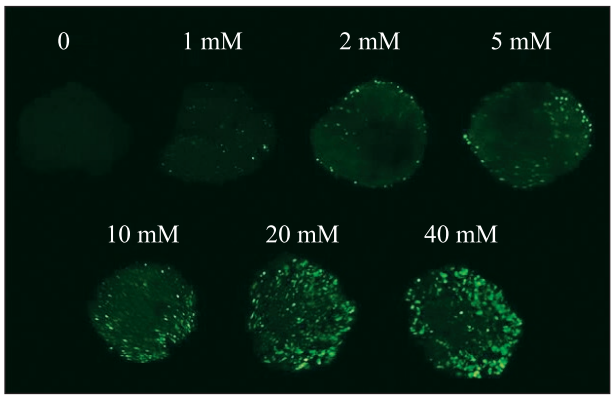

Fig. 1. Cytofluorometric analysis of DNA damage in HepaRG spheroids after 48-h culturing in the absence (0) and presence of 1, 2, 5, 10, 20, 40 mM acetaminophen. TUNEL analysis.

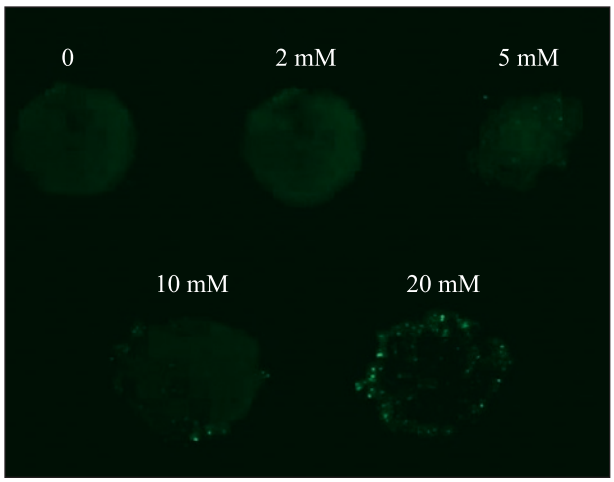

Fig. 2. Cytofluorometry of DNA damage in HepaRG spheroids after 48-h culturing in the absence (0) and presence of 2, 5, 10, 20 mM metformin. TUNEL analysis.

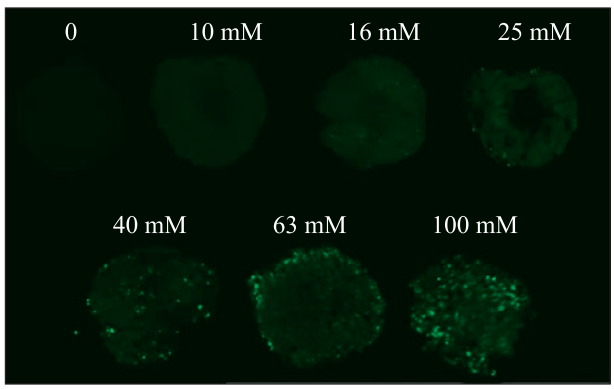

Fig. 3. Cytofluorometry of DNA damage in HepaRG spheroids after cafter 48-h culturing in the absence (0) and presence of 10, 16, 25, 40, 63, 100 mM of isoniazid. TUNEL analysis.

production; at concentrations >5 mM lactate content dropped below the basal level due to significant cell loss in the spheroid. Thus, mitochondrial damage leasing to glycolysis activation and enhanced lactate production can be detected at non-lethal concentrations of the xenobiotic. The use of lower concentrations also makes possible investigation of drug toxicity in case of repeated exposure and assessment of the reversibility of the observed effects [3].

In clinical practice, metformin is used as the antidiabetic drug. The mechanisms underlying the effects of metformin are not fully understood. It is known that metformin reduces blood glucose concentration by inhibiting gluconeogenesis in the liver. The drug is not metabolized in the liver. When HepaRG spheroids were incubated with metformin, fluorescence was observed at a concentration of 5 mM (Fig. 2). When its concentration was elevated to 10 mM, fluorescence intensity remained practically unchanged. IC_{50} value was 26.0±1.1 mM. At metformin concentration <10 mM, lactate production in the culture medium increased, and at higher concentrations it decreased, as in case of acetaminophen. Thus, the drug exhibits low toxicity against HepaRG cells in the structure of spheroids. Metformin is the only safe drug of the biguanide family. In the entire history of its application, only 20 cases of hepatotoxicity were described, which might be idiosyncratic or caused by interaction with other drugs.

Isoniazid is one of the main anti-tuberculosis drugs, but its use is associated with severe hepatotoxicity. Hydrazide, a product of isoniazid metabolism in the liver, plays an important role in the mechanism of its toxicity [1]. When HepaRG spheroids were incubated with isoniazid, fluorescence was observed at a sufficiently higher concentration, 25 mM (Fig. 3); IC_{50}=46.5±1.7 mM. Lactate concentration in the culture medium increased up to isoniazid concentration of 16 mM. Thus, hepatotoxicity of isoniazid for HepaRG spheroids manifested at a rather high concentration of the drug. It is known that hepatotoxicity of isoniazid is caused by idiosyncratic effect of the drug [9], *i.e.* the specific genotype and expression of metabolizing enzymes. Effects of such hepatotoxins is difficult to predict using *in vitro* systems.

Therefore, for adequate assessment of drug hepatotoxicity, several methods should be combined: assessment of cell viability and their functional status. Apparently, the integrated approach is particularly important in the study of cytotoxic drugs, similar by their structure and mechanism of action [13-14]. This makes possible identification of subtle differences in the manifestation of the pharmacological properties. Furthermore, the use of different methods of noninvasive evaluation of cell metabolism plays a key role in creating "organ-on-a-chip" platforms that are widely used in testing and development of new drugs [4,6,11]. In this paper, we used combination of MTT, TUNEL methods, and measurement of lactate concentration in the culture medium, while using the same cells sample, which reduces the time of the test and improves reproducibility of the results.

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REFERENCES

1. U. A. Boelsterli and K. K. Lee, Mechanisms of isoniazid-induced idiosyncratic liver injury: emerging role of mitochondrial stress. *J. Gastroenterol. Hepatol*., **29**, No. 4, 678-687 (2014).
2. O. Burmistrova, M. F. Simoes,, Rijo, J. Quintana, J. Bermejo, and F. Estevez, Antiproliferative activity of abietane diterpenoids against human tumor cells. *J. Nat. Prod*., **76**, No. 8, 1413-1423 (2013).
3. M. J. Gomez-Lechon, A. Lahoz, L. Gombau, J. V. Castell, and M. T. Donato, In vitro evaluation of potential hepatotoxicity induced by drugs. *Curr. Pharm. Des*., **16**, No. 17, 1963-1977 (2010).
4. G. Langley, C., Austin, A. K. Balapure, L. S. Birnbaum, J. R. Bucher, J. Fentem, S. C. Fitzpatrick, J. R. Fowle, R. J. Kavlock, H. Kitano, B. A. Lidbury, A. R. Muotri, S. Q. Peng, D. Sakharov, T. Seidle, T. Trez, A. Tonevitsky, A. van de Stolpe, M. Whelan, and C. Willett, Lessons from toxicology: developing a 21st-century paradigm for medical research. *Environ. Health Perspect*., **123**, No. 11, A268-A272 (2015).
5. A. Limonciel, L. Aschauer, A. Wilmes, S. Prajczer, M. O. Leonard, W. Pfaller, and, Jennings, Lactate is an ideal noninvasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes. *Toxicol. In Vitro*, **25**, No. 8, 1855-1862 (2011).
6. E. M. Materne, A., Ramme, A., Terrasso, M. Serra,, M. Alves, C. Brito, D. A. Sakharov, A. G. Tonevitsky, R. Lauster, and U. Marx, A multi-organ chip co-culture of neurospheres and liver equivalents for long-term substance testing. *J. Biotechnol*., **205**, 36-46 (2015).
7. P. J. O'Brien, W. Irwin, D. Diaz, E. Howard-Cofield, C. M. Krejsa, M. R. Slaughter, B. Gao, N. Kaludercic, A. Angeline,, Bernardi,, Brain, and C. Hougham, High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Arch. Toxicol*., **80**, No. 9, 580-604 (2006).
8. M. Pirmohamed, A. M. Breckenridge, N. R. Kitteringham, and B. K. Park, Adverse drug reactions. *BMJ*, **316**, 1295-1298 (1998).
9. V. Ramappa, G., Aithal, Hepatotoxicity related to anti-tuberculosis drugs: mechanisms and management. *J. Clin. Exp. Hepatol*., **3**, No. 1, 37-49 (2013).
10. D. A. Sakharov, M. U. Shkurnikov, M. Y. Vagin, E. I. Yashina, A. A. Karyakin, and A. G. Tonevitsky, Relationship between lactate concentrations in active muscle sweat and whole blood. *Bull. Exp. Biol. Med*., **150**, No. 1, 83-85 (2010).

11. T. R. Samatov, M. U. Shkurnikov, S. A. Tonevitskaya, and A. G. Tonevitsky, Modelling the metastatic cascade by in vitro microfluidic platforms. *Prog. Histochem. Cytochem*., **49**, No. 4, 21-29 (2015).
12. M. U. Shkurnikov, A. E. Donnikov, E. B. Akimov, D. A. Sakharov, and A. G. Tonevitsky, Free testosterone as marker of adaptation to medium-intensive exercise. *Bull. Exp. Biol. Med*., **146**, No. 3, 354-357 (2008).
13. A. G. Tonevitsky, I. I. Agapov, A. T. Shamshiev, D. E. Temyakov,, Pohl, and M., Kirpichnikov, Immunotoxins containing A-chain of mistletoe lectin I are more active than immunotoxins with ricin A-chain. *FEBS Lett*., **392**, No. 2, 166-168 (1996).
14. A. G. Tonevitsky, A. Yu. Toptygin, U. Pfuller, T. L. Bushueva, G. V. Ershova, M. Gelbin, K. Pfuller, I. I. Agapov, and H. Franz, Immunotoxin with mistletoe lectin I A-chain and ricin A-chain directed against CD5 antigen of human T-lymphocytes; comparison of efficiency and specificity. *Int. J. Immunopharmacol*., **13**, No. 7, 1037-1041 (1991).
15. I. Wagner, E. M. Materne, S. Brincker, U. Sussbier, C. Fradrich, M. Busek, F. Sonntag, D. A. Sakharov, E. V. Trushkin, A. G. Tonevitsky, R. Lauster, and U. Marx, A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture. *Lab Chip*., **13**, No. 18, 3538-3547 (2013).