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# Activity of Hepatic Enzymes of Isolated Hepatocytes under the Influence of Copper Acetate

Natalia N. Dremina, PhD; Irina S. Trukhan, PhD; Olesya V. Say; Irina A. Shurygina, PhD, ScD

*Irkutsk Scientific Center of Surgery and Traumatology*
*Irkutsk, the Russian Federation*

## Abstract

The article describes a biochemical method for characterizing the hepatocyte culture under the action of copper acetate (CA). Isolated primary hepatocyte culture was exposed to CA in different concentrations, and then the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and the amount of glucose, in the culture medium was determined. The result showed that the level of cell membrane damage and the release of ALT and AST into the extracellular space depend on the concentration of the acting substance, and, in addition, AST was found to be more sensitive to the toxic effects of CA. However, the activity of ALP proved to be an indicator of the copper ions' action on the enzyme but not of destructive processes in the hepatocyte culture. At the same time, a decrease in the glucose level may be used as a characteristic of the cell metabolic activity. Based on the results obtained, we can conclude that proposed analysis methods allow us to comprehensively characterize the processes occurring with cultured hepatocytes under the influence of CA.(**International Journal of Biomedicine. 2022;12(1):58-62.**)

**Key Words:** hepatocytes • alanine aminotransferase • aspartate aminotransferase • alkaline phosphatase

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## Abbreviations

**ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **ALP**, alkaline phosphatase; **CA**, copper acetate

## Introduction

The mammalian liver is the essential center in which numerous metabolic, regulatory, homeostatic, and detoxification processes are concentrated, making normal organism development possible. It is known that the functional features of this organ are provided by hepatocytes, highly differentiated cells that make up about 90% of the volume of the liver parenchyma.[1-4] Hepatocytes ensure the metabolism of endogenous compounds and xenobiotics by forming highly water-soluble metabolites that are subsequently excreted from the organism and they are also responsible for the synthesis and secretion of various proteins (albumin, fibrinogen, coagulation factors) and bile acids. Hepatocytes are also involved in maintaining the homeostasis of carbohydrates, lipids, and amino acids, and in the formation of urea. Because of their morphological and functional characteristics, these cells are of particular interest for cell technologies. Currently, the application areas of the human and animal hepatocyte cultures are quite extensive; however, the most popular uses of these cultures remain in the screening of pharmaceuticals, including for the so-called ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) studies of new compounds.[5] That is because the metabolism of liver cells is the main removal route of about 75% of drugs from the body and is mediated by the functioning of the hepatocyte enzymatic systems, which provide the reactions of functionalization and conjugation of xenobiotic compounds.[6] In this regard, hepatocyte cultures are still considered the most

*Corresponding author: *Natalya N. Dremina, PhD. Irkutsk Scientific Center of Surgery and Traumatology. Irkutsk, the Russian Federation, E-mail: drema76@mail.ru*

suitable model for analyzing the absorption kinetics of drugs and test compounds, assessing hepatic clearance, recognizing metabolites, and predicting drug interactions with each other.

Analysis of the transcriptome, proteome, and metabolome of cells exposed to the compounds under study makes it possible to predict the risks associated with the occurrence of hepatocellular affections; however, these methods are expensive, difficult to perform and require high-accuracy equipment. Therefore, the search for techniques characterized by high sensitivity, low expenses, and easy realization is still topical. That is why the possibility of using clinical diagnostic methods in biotechnological practice attracts attention. In particular, clinical analyses reflecting pathological changes in the liver functioning and hepatocyte damage of varying severity are of special interest. Such methods include tests widely used in medical practice for diagnosing liver disease and based on the determination of the activity of ALT, AST, and ALP in the blood serum.[7,8]

The purpose of this investigation was to estimate the suitability of biochemical methods to determine the activity of AST, ALT, ALP, and the amount of glucose in the culture medium for toxicity analysis when exposed to different concentrations of CA on hepatocytes.

The choice of the preparation was determined by the properties of this metal. On the one hand, copper is an essential element for organism development and takes part in many physiological processes that include metabolism, tissue respiration, and the synthesis of collagen and elastin; it is also responsible for the activity of enzymes, hormones, and vitamins. However, in excess, copper is an extremely toxic element. In the case of metabolic disorders, copper can accumulate in the organism, in particular in the liver, which results in severe diseases of the central nervous system and internal organs.[9]

## Materials and Methods

*Isolation of the primary culture of rat hepatocytes*

An acute experiment was carried out to isolate the primary culture of hepatocytes of Wistar rats weighing 200g. Animals were housed in accordance with the Good Laboratory Practice (GLP) rules. The experiments were performed in accordance with the norms for the humane treatment of animals regulated by the International Guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the Irkutsk Scientific Center of Surgery and Traumatology. All surgical interventions were performed under aseptic conditions. The surgery was performed under general intravenous anesthesia using 0.7 ml of a 5% solution of telazol in phosphate-buffered saline intramuscularly and subsequent chloroform inhalation until the loss of locomotor activity but with the maintenance of the cardiac muscle contractile activity.

The primary culture of hepatocytes was obtained by perfusion of the liver through the portal vein. Heparin sodium 5000 IU/ml solution (1ml) in 0.9% sodium chloride was injected, followed by a solution of Krebs-Ringer's buffer with EDTA without calcium (pH 7.2–7.4, 37 °C) (5) with a volume of 210ml and with a perfusion rate of 30ml/min. Then the buffer was replaced with a 0.07% collagenase solution (Sigma-Aldrich) in Hank's Balanced Salt with calcium and magnesium (HBSS, Sigma-Aldrich) of 120ml at the temperature of 37°C. The liver was transferred into pre-cooled Hank's buffer (T=4°C), the liver capsule was dissected, the cells were filtered through a cell filter with a pore size of 70 μm (Corning, BioCoat) and washed with Hank's buffer followed by centrifugation at 50 g for 5 minutes at 4°C. Cells were resuspended in William's E medium (Gibco) with the addition of 10% fetal bovine serum (FBS, Sigma-Aldrich) and Primary Hepatocyte Maintenance Supplement (Gibco) that consist of dexamethasone and a cocktail solution containing GlutaMAX™, HEPES, penicillin-streptomycin, insulin, transferrin, selenium complex, BSA and linoleic acid introduced to the medium according to the manufacturer's instructions. The obtained hepatocyte culture was incubated at 37°C, 90% humidity, and 5% $CO_2$ in Biostation CT (Nikon). To investigate the toxicity of the chosen preparation, the cells were used for the experiment within 24 hours after isolation because we did not observe changes in the functioning of the enzymatic systems of hepatocytes during this period.[5,10]

Two hours after isolation, the nutrient medium was replaced, and a CuAc2 solution was added to the medium to a final concentration of 50 μg/ml or 200 μg/ml. After 6h and 24h, the nutrient medium was taken to determine the ALT, AST, ALP activity, and glucose concentration. The culture medium of hepatocytes without the addition of CuAc2 served as a control (the corresponding amount of the water was introduced to the culture).

*Biochemical research*

A biochemical study was carried out on a biochemical analyzer Mindray BS-380 (Mindray, China). The activity of ALT and AST and the amount glucose were determined by a standard method using a Vector Best kit (Russia, series 12, 14); the ALP activity was analyzed using a Mindray kit (China, series 140319007).

Statistical analysis was carried out in the R software environment. A non-parametric Kruskal-Wallis test was used for comparisons of median values among groups, followed by post-hoc analysis using Tukey test. Mann–Whitney–Wilcoxon test was used for pairwise comparison. A value of $P<0.05$ was considered significant.

## Results and Discussion

At the first stage, we obtained a primary culture of Wistar rat hepatocytes. After 2 hours, the culture medium was replaced with a fresh one, and the hepatocyte culture was introduced into the experiment.

The analysis of ALT activity 6 and 24 hours after the introduction of CuAc2 into the culture medium showed that incubation with both low (50 μg/ml) and high (200 μg/ml) concentrations of this metal salt led to no significant increase in enzyme activity in the medium, in comparison with the control group (data not demonstrated).

A similar result was obtained when analyzing the AST activity after 6 hours of exposure with CA in two experimental

groups (CuAc2 50 μg/ml and CuAc2 200 μg/ml) (Fig. 1A). However, a significant increase was observed 24 hours after exposure to the CuAc2 in the concentration of 200 μg/ml on hepatocytes (Fig. 1B).

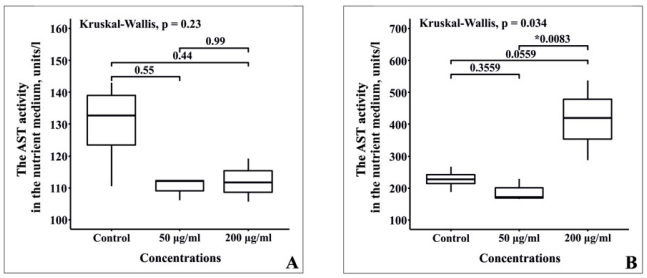


***Fig. 1.*** *Changes in AST activity after exposure to CuAc2 at concentrations of 50 μg/ml and 200 μg/ml on hepatocyte culture for 6 hours (A) and 24 hours (B) compared with the control group. Medians, first and third quartiles are shown.*

When comparing enzyme activity 6 hours and 24 hours after the addition of CA, an increase in the AST activity was found in two experimental groups and the control group (Fig. 2B), whereas significant ALT activity growth was observed only after incubation with CuAc2 at a concentration of 200 μg/ml (Fig. 2A). In this case, a rising enzyme activity may be associated with destructive processes attended by a breach of membrane integrity and the release of enzymes into the extracellular space.

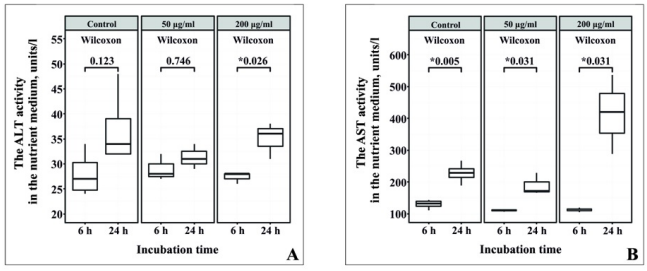


***Fig. 2.*** *Comparison of the ALT (A) and AST (B) activity in the control group and groups with adding CuAc2 at the concentrations of 50 μg/ml and 200 μg/ml after incubation for 6 or 24 hours. Medians, first and third quartiles are shown.*

Interesting results were obtained in the analysis of the ALP enzyme. It was found after 6 hours of incubation with CuAc2 at both tested concentrations, the enzyme activity was significantly lower than in the control group (Fig. 3A). A similar picture was observed after 24 hours of incubation with CuAc2 in different concentrations (Fig. 3B). This effect is probably caused by the replacement of zinc ions included in the structure of the enzyme with copper ions, as a result of which there is a decrease in the ALP activity.

When comparing glucose levels with the control group, a significant decrease of its amount was noted in both experimental groups 6 hours and 24 hours after the addition of CA solution (Fig. 4). The described change is associated with the high hepatocyte metabolic activity, which increases even more under the action of copper ions. In particular, it is known that copper is a cofactor of cytochrome c oxidase, one of the mitochondrial respiratory chain enzymes involved in the aerobic oxidation of various metabolites, including glucose. At the same time, during the following 18 hours of incubation with CA, no further decrease in the amount of glucose in the medium was observed, which may be associated with the accumulation of excess amounts of copper in the cells and the related toxic effect. In this case, the heterogeneity of the obtained primary culture can contribute to the maintenance of relatively constant glucose content as a result of balancing the intake of this carbohydrate by actively metabolizing hepatocytes and the death of cells that accumulate excess amounts of this element.

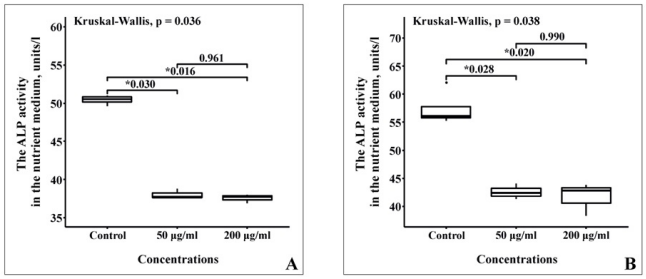


***Fig. 3.*** *Changes in ALP activity after exposure to CuAc2 at concentrations of 50 μg/ml and 200 μg/ml on hepatocyte culture for 6 hours (A) and 24 hours (B) compared with the control group. Medians, first and third quartiles are shown.*

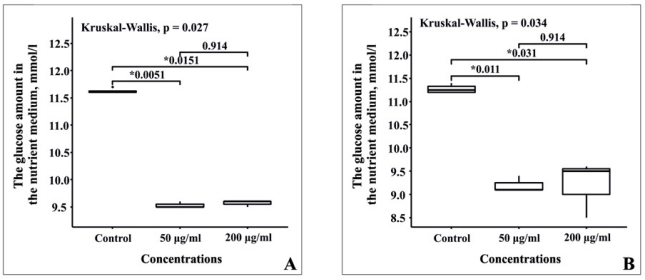


***Fig. 4.*** *Changes in glucose level after exposure to CuAc2 at concentrations of 50 μg/ml and 200 μg/ml on hepatocyte culture for 6 hours (A) and 24 hours (B) compared with the control group. Medians, first and third quartiles are shown.*

# Discussion

The ALT and AST enzymes are considered as specific markers of liver cell damage. The level of increased enzyme activity allows us to come to a conclusion about the degree of cytolysis processes, and it is generally recognized that the elevated ALT and AST activities are a consequence of damage to the hepatocyte cytoplasmic membranes, followed by the release of intracellular enzymes into the extracellular space. ALT activity can increase during the late phase of cellular damage when cytolysis occurs as a result of direct damage to hepatocytes. Although plasma membrane injury and protein leakage are considered the most common causes of elevated ALT levels, there is evidence that other

mechanisms may be involved.[11] Unlike ALT, the main part (over 80%) of AST is concentrated in the mitochondria, and the hepatic AST fraction primarily reflects the damage to the hepatocyte mitochondrial membrane.[7] It is also known that some substances exhibit a higher affinity for the inner mitochondrial membrane, entering the matrix region, promoting the release of AST, the mitochondrial function, and disruption of energy metabolism, which eventually leads to initiating apoptotic pathways.[12]

Another factor contributing to the increase in the level of aminotransferases in some cases is not cell damage but the induction of their expression. It is assumed that some substances are capable of increasing enzyme activities.[13] For example, in an experiment on mice, the growth of ALT and AST activity was noted after exposure to tetrachloromethane, and it was canceled after the use of protein synthesis inhibitors.

ALT and AST expression can also be regulated through IRE1α/c-Jun pathway signaling. It has been shown that inhibition of microsomal triglyceride transfer protein (MTP) leads to GPT/GOT1 (ALT and AST genes) transcription enhancement by increasing the regulation of the IRE1α/cJun pathway, which results in intensification of ALT and AST synthesis and release. Thus, the transcriptional regulation of the GPT/GOT1 genes is the main stress response mechanism leading to an increase in the level of transaminases, which may represent a normal reaction to stress for survival.[14] In general, although elevated levels of aminotransferase activity may be due to a number of reasons, prevalent causes, apparently, are damage to plasma membranes and cell death. This is evidenced by the dependence of the increased level of enzyme activity on the concentration of copper ions in the culture medium and the higher sensitivity of AST in comparison with ALT. The latter is explained by the fact that AST is presented in hepatocytes predominantly by the mitochondrial isoenzyme and respectively makes a greater contribution to the rise in activity caused by destructive processes in the culture especially affecting mitochondria.

ALP, which catalyzes the removal of phosphoric acid from its organic compounds, has optimal activity at pH of 8.6–10.1 and is located on the external surface of the cell membrane where it takes part in the transport of phosphorus. The enzyme is found in almost all organs, but its maximum amount is detected in the hepatobiliary system, bone tissue, intestines, placenta, and lactating mammary gland. For diagnostic purposes, the identification of bone and hepatic isoforms of ALP is most often carried out.[15] The activity of the hepatic phosphatase form is commonly enhanced due to damage or destruction of hepatocytes, as well as breaching of bile transport. The hepatocellular mechanism of increasing the ALP activity plays a crucial role in viral and autoimmune hepatitis, and toxic and medicinal damage to the liver. Along with pathological conditions, there is also a growth of physiological ALP activity in blood serum, which is noted during intensive development or pregnancy. Low ALP levels are observed in hypothyroidism, pernicious anemia, congenital hypophosphatasia, and zinc deficiency, as well as in the chronic form of Wilson–Konovalov disease.[16]

Zinc and magnesium are essential cofactors of the ALP enzyme. It has been demonstrated that when high concentrations of copper solution were added to the nutrient medium of hepatocytes, zinc was replaced by copper in the structure of ALP and the enzyme activity decreased.[15] Apparently, in our study, high concentrations of CA solution also influenced ALP activity.

## Conclusion

The results of the biochemical analysis of the culture medium reflect both the physiological and pathological state of hepatocytes under the action of a CA solution. Therefore, this method can be used in cell technologies to analyze the enzymatic activity of hepatocyte cultures. In particular, the determination of ALT and AST activity levels can be utilized to assess the degree of toxic effect, though AST, due to its features, was found to be more sensitive when exposed to a copper preparation. At the same time, the ALP activity level in this study did not serve as an indicator of the destructive processes in the hepatocyte culture, reflecting to a greater extent the interaction of the enzyme with copper ions. The analysis of the glucose content in the medium is applicable to estimating the metabolic activity of cells under the influence of the tested substance. In general, it can be concluded that the methods used allow us to assess the change in the balance between the death of hepatocytes and an increase in the energy metabolism, depending on the amount of copper ions in the medium.

## Competing Interests

The authors declare that they have no competing interests.

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