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# CYTOTOXICITY REDUCTION OF RIFAMPICIN BY INCORPORATION INTO RED BLOOD CELL GHOSTS

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**ABSTRACT**

The problem of selective distribution of drugs within the human body has been always relevant. However, only now with development and introduction of targeted delivery methods into clinical practice we got closer to solving this issue. Drug carriers including autologous erythrocytes play a key role in addressing them. The objective of this study was to prove that the reduction of toxicity of rifampicin included in erythrocyte ghosts may be a result of not only of the change in pharmacokinetics and reduction of concentration in target organs, but also be caused by changes in level of cytokine production that occurs during absorption and digestion of erythrocyte ghosts, as well as interactions in the intracellular environment of macrophages. Hypotonic preswelling method was applied to encapsulate rifampicin into erythrocyte ghosts. A comparative study of cytotoxicity of free and encapsulated in erythrocyte ghosts rifampicin was conducted. The effect of free and encapsulated rifampicin on caspase 3 and 8 activities and the total content of glutathione were determined. Cytotoxicity of rifampicin encapsulated into erythrocyte ghosts is lower compared to free rifampicin. Rifampin possesses the ability to induce apoptosis in cell culture, but with inclusion of rifampicin erythrocytes, this ability decreases. The encapsulated form of rifampicin compared to the free drug, reduces the concentration of total glutathione in the cell culture to a lesser extent. Erythrocyte ghosts used as carriers for rifampicin have the ability to neutralize the cytotoxic effects of the antibiotic. Erythrocyte ghosts prevent the apoptosis-stimulating effect of rifampicin, when the antibiotic is introduced into the cell culture in the form of erythrocyte ghosts.

**Key Words**: Red blood cells, Rifampicin, Drug delivery system, Cytotoxicity, Apoptosis, Pharmacodynamics.

## INTRODUCTION

The problem of selective distribution of drugs within the human body has been always relevant. However, only now with development and introduction of targeted delivery methods into clinical practice we got closer to solving this issue. Modern pharmacology currently possesses two distinct trends. The first direction is founded on development of methods for targeted delivery of drugs. The second direction is based on development of new drug formulations to create drugs depots in human organism for controlled drug release, prolonged effect, improved therapeutic efficacy, and reduced toxicity [1, 2]. Drug carriers play a key role in addressing these challenges. They enable not only development of a depot form of the administered drug, but they also protect the drug from premature degradation and inactivation, prevent or reduce immune and allergic reactions, and provide targeted delivery to organs and tissues [3, 4]. Among other systems, autologous erythrocytes can act as carriers for targeted drug delivery. Application of blood cells as drug carriers for targeted transport is logically justified and seems to be one of the easiest and most affordable options for clinics. Erythrocytes, leukocytes, and platelets have already been used as carriers for drug delivery [5]. Human erythrocytes can be considered to be the most promising carriers. Not only are they easily isolated from blood, where they are present in large quantities, they also have sufficiently long lifetime. Furthermore, during aging they are exposed to

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natural biodegradation processes in an organism [6, 7]. Cellular drug carriers based on ghosts of autologous red blood cells are called "pharmacocytes" [8-10].

Pharmacokinetic properties of a drug encapsulated in pharmacocytes are predicted to change due to the fact that pharmacocytes like normal red blood cells are consumed by macrophages, and, as a result, the greatest amount of the drug loaded in pharmacocytes, accumulates in reticulo-endothelial system (RES) tissues or in places of cell accumulation that are capable of phagocytosis (abscesses, suppurative focus, and etc.) [11, 12]. It is assumed that the release of the drug from pharmacocytesis caused by its destruction during phagocytosis [13]. Consequently, this fact draws attention to antibiotics as prospective objects for inclusion into erythrocytes, which could potentially result in increased concentration of antibiotics in areas of accumulation of phagocytizing cells, e.g. in the area of pyoinflammatory focus [14]. Moreover, in this event it would be logical to accept the possibility of reduced toxic effects of the drugs encapsulated in pharmacocytes. This research article is an attempt to experimentally prove the possibility of reducing cytotoxic effect of the antibiotic encapsulated in pharmacocytes.

At the same time, we believe it is possible to state the following hypothesis: the reduction of toxicity of the drug included in erythrocyte ghosts may be a result of not only of the change in pharmacokinetics and reduction of concentration in target organs, but also be caused by changes in the level of cytokine production that occurs during absorption and digestion of erythrocyte ghosts and as well as interactions in the intracellular environment of macrophages. Proof of this assumption is the focus of this work.

## MATERIALS AND METHODS

"Rifampicin-Ferane" (ZAO "Brinsalov-A", Russia, Series No. 500812) was investigated in current experimental work.

### Drug assay

In order to perform quantitative analysis rifampicin was extracted from blood serum with acetonitrile. Chromatographic separation, and optic density was detected at 254 nm. However, since chromogenic pharmacocytes do not influence rifampicin analysis in investigating concentrations, chromatographic separation was not performed.

For *in vitro* evaluations of the carrier cells we used spectrophotometric analysis to characterize rifampicin. The absorbance of rifampicin was measured at 237, 255, 334 and 475 nm [15]. To prepare samples for drug assay, 0.2 ml of wash loaded erythrocytes were diluted with 1.8 ml of acetonitrile to produce rifampicin containing solution. Later, samples were centrifuged at 7000×g for 10 min, and absorbance of supernatant was measured. Next, the concentration of rifampicin loaded in erythrocytes was determined, and a solution of free rifampicin of the same concentration was used as for comparison.

### Preparation of human erythrocytes

Blood samples were drawn by venipuncture from healthy volunteers aged from 20 to 35 years and transferred to pre-heparinized polypropylene test tubes. After centrifuging at 1000×g for 10 min, plasma and buffy coat were separated by aspiration, and the remaining packed erythrocytes were washed three times with phosphate-buffered saline (PBS; Sigma).

### Preparation of rabbit erythrocytes

Blood samples were obtained from the jugular vein of anesthetized rabbits. After centrifuging at 1000×g for 10 min, plasma and buffy coat were separated by aspiration, and the remaining packed erythrocytes were washed three times with phosphate-buffered saline (PBS; Sigma).

### Encapsulation of rifampicin in human erythrocytes by hypotonic preswelling method

A hypotonic preswelling method described and validated by Tajerzadeh and Hamidi (2000) [16, 17] was used for loading human and rabbit erythrocytes with rifampicin. For this, 1 ml of washed packed erythrocytes was transferred gently to a polypropylene test tube. 4 ml of a hypotonic solution of sodium chloride with osmolarity of 0.65 was added, and the resulting cell suspension was mixed gently. The swollen cells were then separated by centrifuging at 600×g for 5 min, and the supernatant was discarded. Rifampicin was added to the erythrocyte suspension to a final concentration of 1 mg/ml.

Then the erythrocytes were resealed by the addition of 4 ml of a hypertonic solution of sodium chloride with osmolarity of 1.1. The suspension was mixed gently by several inversions. The resulting mixture was incubated at 37 °C for 30 min. After centrifuging at 1000×g for 10 min, the resulting carrier erythrocytes were washed with PBS to remove the unenclosed during the loading process rifampicin, the released hemoglobin and other cell constituents.

It was important to use sham-encapsulated erythrocytes that were prepared similar to other pharmacocytes with one major change - aqueous solution of rifampicin was replaced by distilled water.

### Preparation of human peripheral blood leukocytes

Blood samples were drawn by venipuncture from healthy volunteers aged from 20 to 35 years and transferred to pre-heparinized polypropylene test tubes. 10 ml of heparinized blood was added to 500 ml of distilled water and was thoroughly mixed. After 30 seconds, 50 ml of hypertonic solution of sodium chloride with osmolarity of 10.0 was added. After centrifuging at 800×g for 10 min,

the leukocytes were washed two times with Henk's solution (Sigma). Cells were resuspended in complete medium (90% of medium 199, 10% of fetal bovine serum, supplemented with antibiotics – penicillin 10 000 IU/ml; streptomycin 10 ng/ml). The number of cells was identified in Gorjaev's chamber. Later, the number of cells was normalized to the desired value by complete medium.

Cell suspension was transferred to 24-well culture plates - 1.0 ml per each well. The plates were later incubated in a CO2-incubator at 37 °C for 30 min. Next, 0.1 ml of each of the study samples were added to each well: group 1 - rifampicin-encapsulated erythrocytes, group 2 - sham-encapsulated erythrocytes, group 3 - free rifampicin, group 4 – control (untreated cells). The plate was incubated in a CO2-incubator at 37 °C for 24 hours. Upon completion, survivability of the study samples were evaluated using MTT-test, caspase 3 and 8 expression was defined, concentration of glutathione was assessed. The resulting cell cultures were dyed with annexin and 6-carboxyfluorescein diacetate.

**Preparation of rabbit alveolar macrophages**

Alveolar macrophages were obtained from healthy rabbits of 3.2 kg in weight. Rabbits were anesthetized with thiopental sodium administered to the marginal ear vein at the dose of 40 mg/kg. Then, 5 ml of blood samples were withdrawn from the marginal ear vein and transferred to pre-heparinized polypropylene test tubes. This blood sample was used for loading rabbit erythrocytes with rifampicin by hypotonic hemolysis method.

Chest cavity was opened, the front end of the trachea was isolated from surrounding tissue, clamped and cut. The rear section of the trachea with the clip, the lungs and heart were removed entirely. The heart was carefully taken out without damaging the lungs.

The blood was washed away with warm saline solution (37 °C) and gentle soaking with a gauze pad. The clip was removed from trachea, and about 40–50 ml of warm Hank's solution (37 °C) was filled in until the lungs expanded. Hank's solution was filled directly into the main bronchi. Afterwards trachea was clamped again, lungs were gently massaged, and the resulting fluid was poured into sterile centrifuge tube. This procedure was performed twice.

The resulting fluid was centrifuged at 1000 RPM for 20 min (4 °C). Cells were washed two times with Henk's solution (Sigma). Afterwards, the cells were resuspended in complete medium (90% of medium 199, 10% of fetal bovine serum, supplemented with antibiotics – penicillin 10 000 IU/ml; streptomycin 10 ng/ml). The number of cells was identified in Gorjaev's chamber using Trypan blue dye. Next, the number of cells was normalized to the desired value (1 million/ml) by complete medium.

Cell suspension was transferred to 24-well culture plates - 1.0 ml per each well. The plates were later incubated in a CO2-incubator at 37 °C for 30 min. Next, 0.1 ml of each of the study samples were added to each well: group 1 - rifampicin-encapsulated erythrocytes, group 2 - sham-encapsulated erythrocytes, group 3 - free rifampicin, group 4 – control (untreated cells). The plate was incubated in a CO2-incubator at 37 °C for 24 hours.

**METHODOLOGICAL TESTS**

**Determination of viability and cytotoxic effect**

Peripheral blood leukocytes were used to determine the effect of rifampicin-encapsulated erythrocytes, sham-encapsulated erythrocytes and rifampicin solution on cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay ("In vitro toxicology Assay kit MTT based", Sigma). This colorimetric assay is based on conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells. For measurement of cell viability, cells were seeded at a density of 1 x 106 cells/mL in a 24-well plate and incubated for 24 h at 37°C and 5% CO2. Cells were treated with rifampicin-encapsulated erythrocytes (rifampicin consisting of phramacocytes concentration was found to be 20µg/ml of cellular culture), sham-encapsulated erythrocytes (it was added in the same amount as pharmacocytes) and rifampicin solution (concentration of 20µg/ml of cellular culture), and were incubated for 24 hours. MTT solution was then added and the mixture was incubated for 2 hours. Absorbance was measured at 570 nm and 690 nm. Data are reported as percent of control thus providing percent cell viability after 24-h exposure to the test agent. Viability was defined as the ratio (expressed as percent) of absorbance of treated cells to untreated cells.

**Assessment of Caspase-3 Assay**

Caspases are key mediators of cell death and are important to the process of apoptosis. Caspase 3 is a member of the CED-3 subfamily of caspases and is one of the critical enzymes of apoptosis. Caspase-3 activity assay was performed using Caspase 3 Assay Kit, Colorimetric (Sigma). Briefly, the peripheral blood leukocytes were treated with rifampicin-encapsulated (rifampicin consisting of phramacocytes concentration was found to be 20µg/ml of cellular culture), sham-encapsulated erythrocytes (it was added in the same amount as pharmacocytes) and rifampicin solution (concentration of 20µg/ml of cellular culture) for 24 hours. The assay was performed based on the manufacturer's protocol. The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase 3, resulting in the release of the p-nitroaniline moiety. p-Nitroaniline has a high absorbance at 405 nm. The concentration of the p-nitroaniline released from the

substrate was calculated from the absorbance values at 405 nmor from the calibration curve prepared with defined p-nitroaniline solutions.

**Assessment of Caspase-8 Assay**

Caspase-8 activity assay was performed using Caspase 8 Assay Kit, Colorimetric (Sigma). Briefly, the peripheral blood leukocytes were treated with rifampicin-encapsulated erythrocytes (rifampicin consisting of phramacocytes concentration was found to be 20µg/ml of cellular culture), sham-encapsulated erythrocytes (it was added in the same amount as pharmacocytes) and rifampicin solution (concentration of 20µg/ml of cellular culture) for 24 hours. The assay was performed based on the manufacturer's protocol. The caspase 8 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Ile-Glu-Thr-Asp p-nitroaniline by caspase 8, resulting in the release of the p-nitroaniline moiety. p-Nitroaniline has a high absorbance at 405 nm. The concentration of the p-nitroaniline released from the substrate was calculated from the absorbance values at 405 nm or from the calibration curve prepared with defined p-nitroaniline solutions.

**Detection of apoptosis using a fluorescent conjugate of annexin V**

Double staining of cells was performed with Annexin V-Cy3 Apoptosis Detection Kit (Sigma). The cells were washed with PBS and suspended at a concentration of 1x106 cells/ml. The cells were stained with annexin V-Cy3.18 (1 µg/ml) and carboxyfluoresceindiacetate (100 µM) in binding buffer for 10 minutes at room temperature. After staining, the cells were washed with binding buffer and observed by fluorescence microscopy.

**Determination of total glutathione**

Reduced glutathione (GSH), a tripeptide (γ-glutamyl-cysteinylglycine), is the major free thiol in most living cells and is involved in many biological processes such as detoxification of xenobiotics, removal of hydroperoxides, and maintenance of the oxidation state of protein sulfhydryls. It is the key antioxidant in animal tissues.

Determination of total glutathione was performed using Glutathione Assay Kit (Sigma). The assay was performed based on the manufacturer's protocol.

**Determination of total glutathione**

All data were presented as mean±standard error of mean. All the samples were measured in triplicates. Student’s t-test (2-tailed distribution) was used to determine the significant differences between groups. The statistical significance was accepted at $p<0.05$. All statistical analyses were determined using Excel.

**RESULTS**

**Characterization of loaded erythrocytes – “pharmacocytes”.**

Drug content. The drug content was estimated in all batches of rifampicin resealed erythrocytes. The encapsulation potential of Rifampicin in human erythrocytes was found to be good. Rifampicin is introduced into the suspension at a concentration of erythrocyte ghosts 104 µg/ml, after 30 minutes of incubation in pharmacocytes mean concentration of rifampicin was 525,0 ± 53,0 µg / ml.

Shape and surface morphology. Photomicrograph (optical microscopy) of rifampicine loaded erythrocytes is shown in Figure. 1-3. Different magnifications (45× and 100×) were used while taking these photomicrographs. Average particle size rifampicine resealed erythrocytes was found to be similar to that of normal erythrocytes (7.2 µm). In Figure 4, the process of start and end of phagocytosis of pharmacocytes can be seen.

**Cytotoxic activity**

Cytotoxic activity of rifampicin-encapsulated erythrocytes was determined using MTT assays in the human peripheral blood leukocytes.

Free rifampicin and rifampicin-encapsulated erythrocytes induced cytotoxicity in both cases, as shown in Table 1. Rifampicin-encapsulated erythrocytes have lower cytotoxicity than free drug in the same concentration. In the group treated with sham-encapsulated erythrocytes cell viability was the same as in the untreated group.

On the first line data is reported as the average of three experiments. On the second line data is reported as percent of control thus providing percent cell viability after 24-h exposure to the test agents. Differences between treatment and non-treatment groups were analyzed by Student’s t-test (*$p<0.05$). Differences between free rifampicin and rifampicin-encapsulated erythrocytes groups were analyzed by Student’s t-test (+$p<0.05$).

As shown in the table, free rifampicin at concentrations of 20 µg/ml and 24 hours incubation in the culture of leukocytes of human peripheral blood shows cytotoxic effect, the rate of cell viability decreases substantially, which is in principle not contrary to the reports on toxicology of rifampicin [18, 19, 20]. If rifampicin is present in the incubation medium in the form of pharmacocytes, the viability of cell culture is also reduced, but the degree of cell viability inhibition is significantly smaller than in case of free rifampicin not incorporated into pharmacocytes. There is a reason to believe that cytotoxic effect of rifampicin is balanced out to a certain extent by the ghosts of erythrocytes. This assumption can be made on the basis of the results of experiments on incubation of leukocytes with ghosts of erythrocytes: upon introduction of ghosts of erythrocytes

into the incubation medium of leukocytes, the vitality level of leukocytes increases.

**Effects on apoptosis**

To characterize the possible effect of pharmacocytes on apoptosis a study was conducted using quantitative test to determine the initiation of one enzyme - Caspase- 8 and one of the effector enzyme Caspase-3, as well as using a qualitative test for annexin V.

The peripheral blood leukocytes were treated with rifampicin-encapsulated erythrocytes, sham-encapsulated erythrocytes and rifampicin solution for 24 h. The apoptotic pathway was examined using Caspase 8 Assay Kit and Caspase 3 Assay Kit (Sigma) as described in the accompanying protocol. During preliminary experiments we could not determine the expression of Caspase-8 and Caspase-3 in the culture of leucocytes from human peripheral blood (5x106 /ml). Therefore, it was decided to use a different cellular system of a culture of rabbit alveolar macrophages (5x106 /ml). Qualitative reaction for annexin V was conducted in the culture of human leukocytes. The results are shown in tables 2 and 3.

As shown in tables 2 and 3, after 24 hours of incubation in culture free rifampicin induces expression of caspase 8 which is apoptosis initiating proteolytic enzyme and caspase 3 which is primary effector enzyme during apoptosis in the rabbit alveolar macrophages. Rifampicin encapsulated in erythrocytes, as compared with free rifampicin, expresses the appearance of caspases in cell culture to a lesser extent. In this case, under our experimental conditions, the actual ghosts of erythrocytes (sham-encapsulated erythrocytes) clearly hinder the appearance of both caspase 8 and caspase 3. This suggests that the erythrocyte ghosts prevent apoptosis -stimulating effect of rifampicin, if this antibiotic is introduced into cell culture in the form of pharmacocytes.

Additionally, response to annexin V was tested. Fluorescent conjugate of annexin V was used for detection of apoptosis. Non-induced cells were stained with CDFA. Apoptotic cells were stained both with CDFA and annexin V-Cy3.18, while necrotic cells were stained with annexin V-Cy3, but not with CDFA.

In the qualitative response to annexin V (Figure 5) the apoptosis marker is present in a greater extent in a culture of human leukocytes in the presence of free rifampicin, and to a much lesser extent in the culture of leukocytes that contains pharmacocytes.

Thus, it can be assumed that rifampicin has the ability to stimulate apoptosis in cell culture, but inclusion of rifampicin into erythrocytes (pharmacocytes) significantly reduces this ability (or levels out).

**Determination of total glutathione**

We have conducted experiments to analyze rifampicin-encapsulated erythrocytes' effect on glutathione synthesis in cell culture of alveolar macrophages. It was established that the level of total glutathione reduces when free rifampicin was incorporated into cell culture of alveolar macrophages, as shown in Table 4.

As shown in table 4, when incubated with alveolar macrophages rifampicin is able to reduce concentration level of glutathione in cells, which is one of the key factors of antioxidant defense. At the same time, the presence of erythrocyte ghosts in the cell incubation medium enhances antioxidant activity. If the option of incubation of cells with rifampicin encapsulated in erythrocytes is considered, it is seen that rifampicin in this form is not able to inhibit antioxidant activity. We can assume that erythrocyte ghosts prevent pro-oxidant activity of rifampicin.

**Table 1. Toxicity of rifampicin-encapsulated erythrocytes obtained by hypotonic pre-swelling method, sham-encapsulated erythrocytes and rifampicin solution on human peripheral blood leukocytes (MTT-test)**

| The analyzed parameter | Rifampicin-encapsulated erythrocytes | Sham-encapsulated erythrocytes | Rifampicin | Control (untreated cells) |
|---|---|---|---|---|
| Absorbance | 0.092 ± 0.007*+ | 0.472 ± 0.008* | 0.064 ± 0.003* | 0.371 ± 0.011 |
| Relative values, % | 24.8 | 127.1 | 17.3 | 100.0 |

**Table 2. Influence of rifampicin-encapsulated erythrocytes obtained by hypotonic pre-swelling method, sham-encapsulated erythrocytes and rifampicin solution on Caspase 8 expression in rabbit alveolar macrophages**

| The analyzed parameter | Rifampicin-encapsulated erythrocytes | Sham-encapsulated erythrocytes | Rifampicin | Control (untreated cells) |
|---|---|---|---|---|
| pNA, nmol/$10^6$/1h | 0.0493 ± 0.0211 | Not defined | 0.1114 ± 0.0218 | 0.0267 ± 0.0120 |

**Table 3. Influence of rifampicin-encapsulated erythrocytes obtained by hypotonic pre-swelling method, sham-encapsulated erythrocytes and rifampicin solution on Caspase 3 expression in rabbit alveolar macrophages**

| The analyzed parameter | Rifampicin-encapsulated erythrocytes | Sham-encapsulated erythrocytes | Rifampicin | Control (untreated cells) |
|---|---|---|---|---|
| pNA, nmol/$10^6$/1h | 0.2459 ± 0.0351 | Not defined | 0.4321 ± 0.0948 | Not defined |

**Table 4. Effect of rifampicin-encapsulated erythrocytes, sham-encapsulated erythrocytes and rifampicin solution on total glutathione level in rabbit alveolar macrophages**

| The analyzed parameter | Rifampicin-encapsulated erythrocytes | Sham-encapsulated erythrocytes | Rifampicin | Control (untreated cells) |
|---|---|---|---|---|
| Total glutathione level, nmoles GSH per ml of sample | 0.572 ± 0.008 | 0.740 ± 0.065 | 0.472 ± 0.040 | 0.645 ± 0.105 |

**Figure 1. Intact erythrocytes after washing three times with PBS**

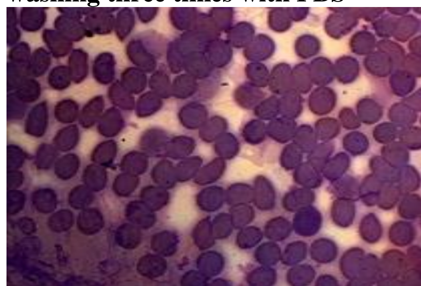


**Figure 2. Erythrocytes after adding a hypotonic solution and centrifugation**

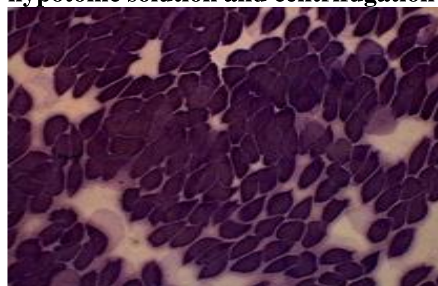


**Figure 3. Rifampicin-encapsulated erythrocytes**

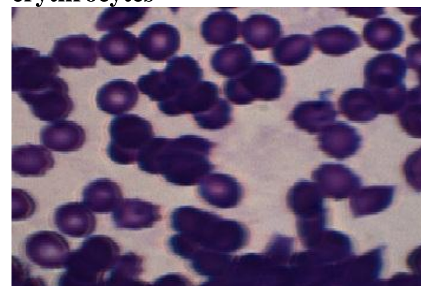
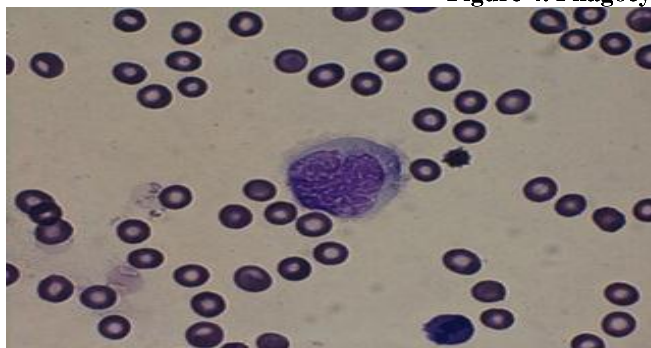


**Figure 4. Phagocytosis of pharmacocytes**



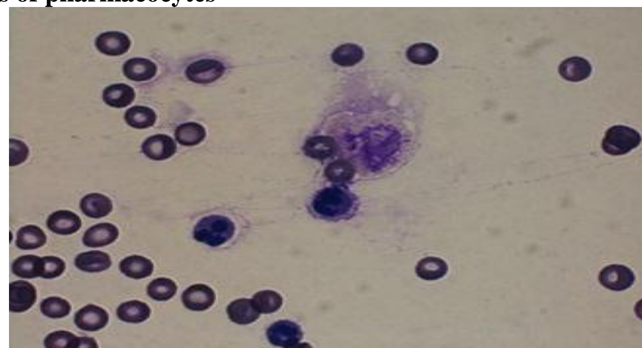
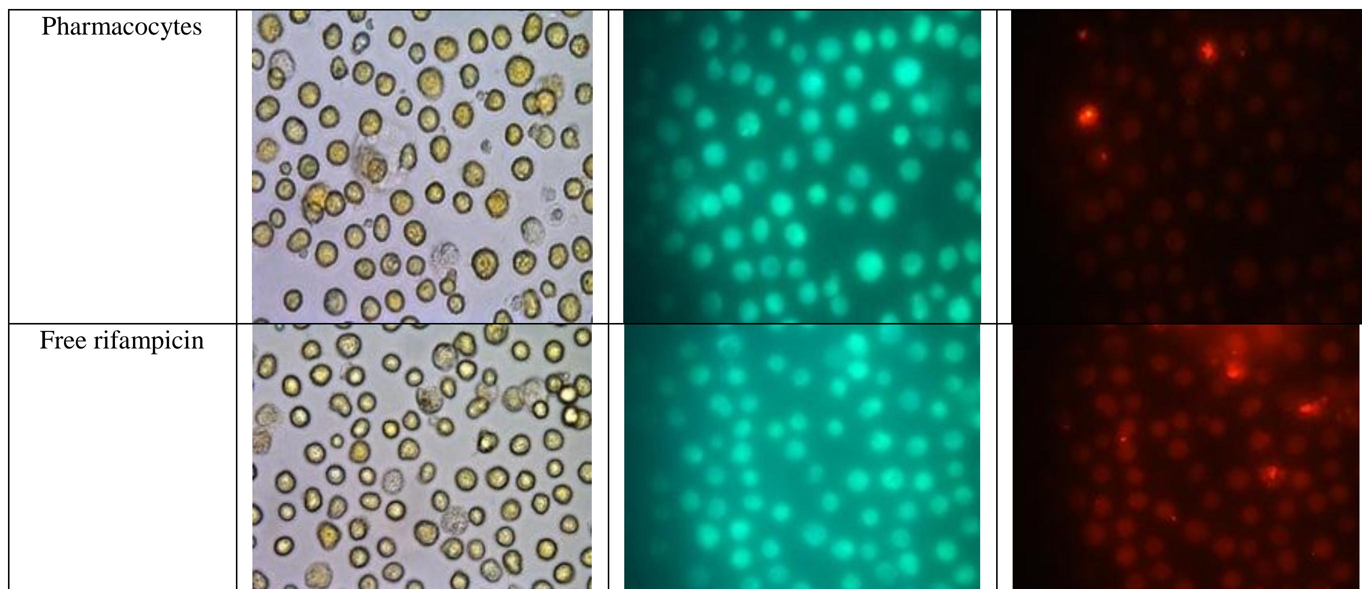


**Figure 5. Detection of apoptosis using a fluorescent conjugate of annexin V**

| Group | Light microscopy | Erythrocytes stained with annexin | Erythrocytes stained with 6-carboxfluorescein diacetate |
|---|---|---|---|
| Non-treated cells | | | |
| Sham-erythrocytes | | | |




**DISCUSSION AND CONCLUSION**

Rifampicin is an antibiotic which is widely and successfully used in long-term therapy of tuberculosis. Toxicity of rifampicin is described in a considerable detail. However, issues regarding the effect of rifampicin on proliferation, differentiation and death of somatic host cells are still poorly understood. Only individual data exists regarding rifampicin [21] that indicate toxicity of this antibiotic to epithelial cells, as well as its ability to cause irreversible unit of cell proliferative activity in step G1/S cell cycle and induce apoptosis in cell culture PEKE (pig embryo kidney epithelium).

The data obtained in our experiment confirms presence of cytotoxic effect of rifampicin and the probability of stimulation of apoptosis by caspase - dependent pathway. There is some evidence related to cytotoxic effect of rifampicin in cell culture with the possibility of inhibition of enzymes of antioxidant defense. Suppression of glutathione levels, which is one of the factors of antioxidant cell defense, in the presence of rifampicin was observed by us and it is not contrary to reports of a possible stimulation of oxidative stress in cells under the influence of rifampicin. So, there is a data on rifampicin stimulated apoptosis of hepatocytes due to increase of oxidative stress level in the mitochondrial fraction of hepatocytes [22].

Thus, cytotoxic effect of rifampicin in the culture of human leukocytes and in culture of rabbit alveolar macrophages can be established. At the same time, the results obtained under our experimental conditions suggest the possibility of reducing cytotoxicity upon encapsulation of rifampicin within erythrocyte carriers and creating pharmacocytes.

Pharmacocytes generated from autologous erythrocytes are intended for further introduction human clinical applications into the human body. We agree with other researchers [23, 24] that pharmacocytes can be used as a system for targeted delivery of drugs. Possibly, pharmacocytes can provide increased concentrations of antibiotics in the foci of purulent inflammation, where commonly the most of the cells?? the increased numbers of activated macrophages capable for phagocytosis are concentrated. This phenomenon of targeted drug delivery to the activated macrophages selective distribution is the basis to improve the effectiveness of antibiotics by using pharmacocytes. To confirm this hypothesis a minimum pharmacokinetic data is required. Selectivity of drug distribution in the body cannot be a predictor of increased efficiency only, but also a reduced toxicity. In our study we did not study pharmacokinetics, although theoretically agree with the assumption that the probability of reducing toxicity of drugs when they are placed in pharmacocytes by selective distribution [25, 26, 27]. However, the results of our experiments suggest that except pharmacokinetic explanation of reduced toxicity of antibiotics when administered as part of pharmacocytes there may be explanations related to the nature of pharmacocytes. According to MTT assay we were able to show that erythrocyte ghosts (carriers of rifampicin) have the ability to neutralize cytotoxicity of the drug substance against cell culture of human leukocytes and alveolar macrophages of rabbit and to some extent prevent apoptosis in quantitative assays with caspases 3 and 8 and in qualitative test of annexin. Presumably, protective effect of erythrocyte ghosts can be related to these effects and may reduce one of the cellular factors of antioxidant defense - glutathione.

Thus, ghosts of autologous erythrocytes used to create transport carriers pharmacocytes have several properties that reduce cytotoxicity of the antibiotic rifampicin placed inside pharmacocytes. This property can be expected to be important in the manifestation of toxicity

reduction of antibiotic in a daily clinical practice by using pharmacocytes in clinical settings.

Our data is consistent with the findings of other researchers who believe that rifampicin modulates immune response, although the mechanisms by which it exerts these effects are not yet fully understood. There is some evidence that rifampicin could exert its effect on the immune system by regulating apoptosis at least partially through glucocorticoid receptor [28, 29].

Intracellular mechanisms leading to rifampicin - induced cell death are not yet clear, but there is evidence [30, 31] that the induced drug expressed pathological changes in the ultrastructure of mitochondria and causes excessive production of reactive oxygen species. These data suggest initiation of mitochondrial apoptosis pathway. Our data on reducing level of glutathione antioxidant factor under the influence of rifampicin may be a confirmation of this.

In accordance with the received data, it can be stated that free rifampicin induces macrophage apoptosis, whereas erythrocyte membranes (erythrocyte ghosts) show the opposite effect. Effect of rifampicin deposited in erythrocyte ghosts on apoptosis and the level of activity of antioxidant enzyme glutathione is the combined effect of rifampicin and erythrocyte membranes. The cause of correction of pro-apoptotic effect of rifampicin upon inclusion into ghosts of erythrocytes can be the impact of the actual components of erythrocyte membranes.

Thus, ghosts of autologous erythrocytes used to create pharmacocytes, transport carriers, have several properties that can significantly reduce cytotoxicity of the antibiotic rifampicin included into pharmacocytes. These properties can be expected to be important for clinical practice in order to reduce the manifestation drug toxicity and to avoid life-threatening complications. in clinical use of pharmacocytes in clinical settings.

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