Application of luminescence spectral assay for monitoring of intracellular metabolism in hepatocytes

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Abstract. The method of evaluation of intracellular metabolism level in chickens’ hepatocytes, using luminescence spectral analysis with 5-((4,6-Dichlorotriazin-2-yl)amino) fluorescein hydrochloride (DTAF) is proposed. The dynamics of proteins content in chickens’ hepatocytes under experimental escherichiosis and in intact poultry was established using this method. A gradual increase in the proteins content in hepatocytes of intact chickens was revealed from 1 to 30 days. The proteins content in hepatocytes of chickens with experimental escherichiosis was gradually increased during the monitoring period, to the exclusion of the sixth day indicators, which were kept at fourth day level (4th day – 2.15 ± 0.01; 6th day – 2.15 ± 0.02). Moreover, these indicators were less than similar indicators of the control group throughout the experiment.

1. Introduction

Proteins have a significant importance in the molecular organization of the cell’ functional mechanisms [1]. It is known, that protein molecules have characteristic absorption and luminescence spectra [2]. A variety of pathological conditions are accompanied by corresponding physicochemical changes in the protein molecule structure. It is reflected in the spectral characteristics, which allow us to trace subtle biochemical changes in various cells or tissues status [3], including in liver hepatocytes [4]. In such case, the constancy of the luminescent marker concentration is controlled by measuring the dye concentration by light absorption at the maximum of dye absorption spectrum, which ensures the results standardization.

Fluorescence assay methods, realized by means of two-wave microfluorimeters, which are a combination of a fluorescence microscope with a spectrum-analyzer complete with electronic recording and control modules have been developed to study an intracellular metabolism regulation [5]. This method allows us to study the physicochemical processes in the cell and its organelles.

Luminescent-spectral assay have found increasing use in multiple areas of science and technology [6]. The data about using of a luminescence spectral assay to rate the functional state of the gastrointestinal tract of farm animals and poultry at the cellular level is poorly presented in scientific information sources. The development of biological markers, capable of capturing the subtest
biochemical changes at the cellular level, is relevant in connection with the development of new generation feed and medicines.

The purpose of our research was to analyze the dynamics of proteins content in chickens’ hepatocytes under experimental escherichiosis and in intact poultry using luminescent spectral analysis [7].

2. Materials and methods
The active procion dye 5-[4,6-Dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF) was used to determine proteins, containing amino-, imino- and hydroxyl groups.

The staining method of histologic liver specimen for the detection of proteins in chickens’ hepatocytes included the following steps:

- double dewaxing with ortho-xylene;
- double processing with absolute alcohol exposure for 3 minutes;
- staining for 6 minutes with an alcohol solution of DTAF (10⁻⁴ M);
- rinsing with distilled water for 2 minutes;
- air drying at room temperature for 10 minutes;
- clarification in xylene for a few seconds;
- coating with a synthetic medium (distrene dibutyl phthalate xylene).

Comparison of luminescence spectra of stained with DTAF and unstained histologic specimens was carried out to exclude tissue luminescence due to formalin fixation of proteins, containing amino-, imino- and amido- groups. It was found that unstained histologic specimens had a blue-green fluorescence spectrum with the maximum luminescence intensity at 480 nm, while stained with DTAF histologic specimens had a bright green fluorescence spectrum, due to pronounced luminescence of represented by hepatocytes liver parenchyma, with the maximum luminescence intensity at 540 nm, which was contingent on the amino- and imino- groups of hepatocyte proteins covalently linked to the fluorochrome. Comparison of unstained and stained histologic specimens’ luminescence spectra indicates an effect of overlap of luminescence spectra of unstained specimens, which completely disappear in the luminescence spectra of stained specimens. This is due to the more pronounced intensity of luminescence of stained with DTAF histological specimens.

Maxima of luminescence spectra of unstained (480 nm) and stained (540 nm) histologic specimens have significant difference between them, which provide them individual registration.

The luminescence intensity at 540 nm was registered to calculate the proteins content (relative units) in the photometric area.

The absorption spectrum was recorded in a stained histologic specimen, the transmission spectrum was registered in an area next to the specimen, and then absorbency was calculated using the data.

The wavelength, when passing through the photometric area light was almost not absorbed by the fluorochrome and had the smallest value, been according to 450 nm. Therefore, the absorbency value at 450 nm was using when calculating the proteins content in relative units.

The luminescence spectrum of uranium glass with a thickness of ~ 1.5 mm at 540 nm was taken into account as a reference when calculating the proteins content.

Using the obtained data, the proteins content in the photometric area of the histologic specimen was calculated in relative units (RU) according to the following formula:

\[ I_B = \frac{I_n}{D_n} I_3, \]

where: \( I_B \) - proteins content (relative units); \( I_n \) - luminescence intensity of the hepatocytes’ photometric area at 540 nm; \( D_n \) - absorbency of the photometric area at 450 nm; \( I_3 \) - luminescence intensity of the uranium glass at 540 nm.

It should be noted that, within the same histologic specimen, all other things being equal, luminescence intensity of areas with signs of an autolytic process was lower than in unchanged tissue. Such sectors of the specimen were not taken into account when proteins content calculating. After photometry of three regions with the highest luminescence intensity, the largest of the three obtained
values was used to calculate the result. The proteins content was calculated in relative units, and the largest of the three obtained results was taken into account for task solution.

The object of the study was Hisex brown chickens (cockerels). The chickens were divided into 2 groups according to the analogy principle: experimental (165 chickens) and control (165 chickens). The division of chickens into groups was carried out randomly by the method of “Random numbers”, body weight was used as a criterion (±10%).

Infection of chickens with 24-hour culture of *Escherichia coli* serotype 078 was carried out by oral inoculation of 2-day-old chickens with 0.2 ml of 2×10^8 colony forming units (CFU) / ml of *E. coli*. The control group chickens were inoculated with saline by oral route.

The chickens were decapitated on the 1–4, 6–8, 10, 15, 21, 30 days (n=15 each group) with subsequent dissection and registration of the results. Pieces of liver 0.5 x 1 cm were fixed in a 10% aqueous solution of neutral formalin for 7-10 days (after a day, the formalin solution was replaced with freshly prepared). Then the samples were removed from the fixing solution and washed with running water for a day. Dehydration of samples and paraffin embedding were performed according to standard methods. Slices of 4-7 μm were made using a sledge microtome “Mikrom” HM450 (Germany) and placed on chemically pure glass slides. Then, after dewaxing, they were stained with DTAF, followed by fluorescence microscopy with registration of luminescence and absorption spectra, using a universal color analyzer the LOMO MSFU-K microscope-spectrophotometer (Russia). Measurements were made using a standard monochromator with a halogen lamp KGM 9V 70W and mercury vapor lamp HBO 100 W / 2 as light sources. Measurement step was 0.5 nm, and a scan point diameter was 10^-4 mm at 480x magnification (12x40).

3. Results and discussions

During luminescence microscopic analysis of histologic specimens of the control and experimental groups of chickens, sectors with saturated bright green fluorescence of varying intensity were observed against the background of weak blue-green tissue fluorescence. The most pronounced fluorescence intensity was noted in histologic liver specimens of the control group chickens.

Using the obtained data, the proteins content in the analyzed area of the histologic specimens was calculated according to the above procedure. The research results are presented in table 1, and the identified trends are illustrated in figure 1.

**Table 1.** The proteins content in chickens hepatocytes of the control and experimental groups.

| Age, days | Proteins content, RU | Control group (n=165) | Experimental group (n=165) |
|----------|---------------------|-----------------------|---------------------------|
| 1        | 1.27±0.01           | 1.27±0.01             |                           |
| 2        | 1.47±0.02           | 1.47±0.01             |                           |
| 3        | 2.18±0.03           | 1.94±0.04***          |                           |
| 4        | 2.46±0.02           | 2.15±0.01***          |                           |
| 6        | 2.74±0.04           | 2.15±0.02***          |                           |
| 7        | 2.87±0.03           | 2.27±0.05***          |                           |
| 8        | 2.88±0.04           | 2.53±0.03***          |                           |
| 10       | 2.89±0.04           | 2.72±0.02***          |                           |
| 15       | 3.18±0.12           | 2.86±0.03***          |                           |
| 21       | 3.48±0.16           | 2.99±0.05***          |                           |
| 30       | 4.19±0.09           | 3.15±0.01***          |                           |

* the difference in this indicator is statistically significant between the experimental and control groups (* - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001).
Figure 1. The trends of proteins content in chickens hepatocytes of the control and experimental groups.

As it follows from the presented in table 1 data and the reflected in figure 1 trends, the proteins content in hepatocytes of the control group chickens increases from 1.27 ± 0.01 to 4.19 ± 0.09 RU or by 229.9% during the experiment, from 1 to 30 days.

The proteins content in hepatocytes of chickens with experimental escherichiosis statistically decreased relative to the control group data on the 3rd day of life (on the 1st day after infection). While, the protein content indicators gradually increased throughout the experiment from 1.27 ± 0.01 to 3.15 ± 0.01 RU, to the exclusion of the 6th day of life (2.15 ± 0.02 RU), when the indicator remained at the 4th day level (2.15 ± 0.01 RU). Over the period from 1 to 30 days, the quantitative protein content in hepatocytes increased by 148.0%. However, the I_B values in the experimental group chickens were less than in the control group poultry throughout the experiment. By the 30th day, this indicator values were less than the same of the control group by 33.0%.

4. Conclusion
Fluorescent dye 5-[(4,6-Dichlorotriazin-2-yl)amino] fluorescein hydrochloride (DTAF) is often used to visualize microscale deformations of different tissues matrix [9].

The results allow us to conclude that the developed luminescence spectral assay with DTAF is capable of revealing the proteins distribution features in histologic specimens of the chickens liver and determining their quantitative content in normal and pathological conditions. The protein content in hepatocytes is characterized by a gradual increase in intact poultry, and a decrease in chickens with intestinal pathology (escherichiosis).

The indicator of quantitative protein content in hepatocytes, detected by the luminescence spectral assay with DCTAF, can be considered as a biological marker of the poultry gastrointestinal tract status in addition to already known biomarkers. Our development result is essential for safety assessments of new drugs, for example, and could be used to predict drugs metabolism and, thus, provide a new tool for protecting from possible adverse drug events [10].

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