Determination of the Mutagenicity of 2-aminoanthracene
Using Chicken Hepatic S-9 Fraction

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Abstract: The abundance of potential genotoxins makes it necessary to improve microbial test systems that allow to identify these substances quickly. At the same time, the methodology of metabolic activation of promutagens in vitro is very important. Microsomal preparations of rodents, which are generally used for that, have significant disadvantages associated with the potential health risks of the inducers of the metabolism of microsomal cytochromes. For the metabolic activation of promutagens, we have developed a protocol of microsomal homogenate fraction (S-9) preparation. We propose to complement the set of methods for quality control of feeds for valuable and rare chicken breeds with the Ames test (Salmonella/microsome) using the activated chicken hepatic S-9 homogenate fraction.

Keywords: Ames Test (Salmonella/microsome), S-9 Fraction, Genotoxicity, Mutagenicity, Chicken

Introduction

Quality control of feed, mixed fodders and mixed fodder raw materials is an important requirement for increasing productivity in poultry farming (Guerre, 2016). All feed and feed supplements are tested according to standard parameters characterizing its nutritional value, toxicological status and microbiological safety (Komarova, 2013; Turitsyna and Donkova, 2010; Tutel’ian, 2004). However, the feed quality control does not provide the exhaustive analysis of the presence of potential genotoxins, which can result, when using these feeds, in increasing the genetic load in both birds and humans. It is known that both chemical plant protection products and other anthropogenic pollutants can accumulate and cause the development of fodder toxicoses in poultry (Alexandrov, 2000) and, subsequently falling into food, genotoxic (Dubinin, 1977; Kurinny, 1983; Abilev, Glaser, 2015; Kier and Kirkland, 2013), carcinogenic effect and consequently, are a serious threat to human health (Abilev, Glaser, 2015; Poirier, 2016; Lin et al., 2016; Roos et al., 2016).

For screening of potential mutagens for humans, microbial test systems are widely used (Biran et al., 2010). The basis for such an approach was provided by Bruce Ames, author of the Salmonella/microsome test. The Ames test is based on the detection of reversion to the wild type of mutated histidine cells (His-) on a media lacking in histidine (Ames et al., 1975). One of the biggest drawbacks of all bacterial test systems is the lack of the xenobiotics metabolic biodegradation system inherent in vertebrates, so the metabolic activation of the test sample is a mandatory stage of screening using bacterial tests. To capture the contribution of biotransformation to the mutagenicity of xenobiotics, a microsomal fraction of the liver (a supernatant after centrifugation of the liver homogenate at 9000 g, the so-called S-9 fraction) is used. Hepatic S-9 homogenate fraction contains microsomes and cytosol with the main metabolic enzymes of both I and II phases of the metabolism: cytochrome P450, flavin monooxygenase, aldehyde oxidase, glutathione transferase, monoamine oxidase, UDP (uridine 5-diphosphate glucuronosyl transferase) and others.

Typically, for metabolic activation of promutagens, a commercial preparation S-9, obtained from rat liver, is used. However, the metabolism of xenobiotics in birds and mammals is no identical (Hutchinson et al., 2014). In particular, in the 1980s it was demonstrated by the employees of faculty of Genetics at the Leningrad State University (now St. Petersburg State University) that the metabolic features of the chicken liver allow to obtain preparations with a high ability for metabolic activation of promutagens without the induction of birds by...
phenobarbital or arochlor (Pavlov et al., 1985). Thus, a
standard protocol for the metabolic activation of
promutagen using the chicken hepatic S-9 fraction can be
a useful addition to a set of genotoxicity control methods.

Materials and Methods

The research was carried out according to the
approved conditions at JV «Svetly», which is a structural
unit of CJSC «Agrofirma Vostok» (Volgograd region,
Russia), the sow farm of the second order for poultry
breeding «Highsex Brown». Parent herd of the «Highsex
Brown» cross (hatched on August 25, 2016) was
obtained from the Sverdlovsk PPR Ltd. (Sverdlovsk
Region). Each experimental bird was contained in the
cell battery Big Dutchman (Germany). The microclimate
parameters were set according to the recommendations of the
manufacturer of cross-country «Highsex Brown»
company «ISA Hendrix Genetics» (Holland). The birds
were fed with the standard mixed fodder manufactured at
the feed mill of the company. Feeding of the experimental
animals was performed under the above conditions.

Experiments on animals were conducted in
accordance with the principles of the European
Convention for the Protection of Vertebrate Animals,
used for experiments or for other scientific purposes.

Optimization of the Protocol for Obtaining the
Hepatic S-9 Fraction

Five preparations were used to optimize the protocol
for the production of the hepatic S-9 fraction.

Preparation №1 was obtained on the basis of rat
hepatic S-9 homogenate obtained according to a standard
procedure using Aroclore 1254 (Sigma-Aldrich)
(Vogel, 2006). The protein content in the preparation is
10 mg/ml (biuret method http://www.olvest-
d.ru/catalog/biohim-nabori/substraty/tovar-71.html). The
final product was stored at a temperature of -80°C,
indicated in the standard protocol (Ames et al., 1975).

The quality of the preparation was examined depending on the conditions of its storage. Storage of S-9 fractions is important for the screening of genotoxicants. There is an opinion that enzymes of microsomal fractions show instability under storage conditions at -20°C and above. For example, some denaturing agents, such as proteases, are active at -20°C (Hubbard et al., 1985). Therefore, the preparation №2 was prepared in a manner similar to that used for preparation №1, except that the finished product was stored at -20°C.

Preparation №3 was prepared according to the
following procedure. The birds were decapitated, the
blood drained, the carcasses immediately opened, the
liver was removed, washed with sterile 0.1 M KCl and
placed on ice. The liver was then homogenized in a steel
mechanical homogenizer (MPW-302, Poland) for 2 min
at 2000 rpm (pre-homogenization) followed by
homogenization in a Potter homogenizer with teflon
pestle in 9 volumes of 0.15 M NaCl. All homogenization
operations were also maid on ice. Isolation of the
microsomal fraction was carried out by differential
centrifugation according to a standard procedure (Abilev
and Glaser, 2015). The protein content of the preparation
was adjusted to 10 mg/mL, diluted with a homogenization
buffer. The final product was stored at -20°C.

Preparation №4 was prepared in a manner similar to
that used for preparation №3, except that the final
product was stored at -80°C.

Preparation №5 was prepared in a manner similar to
that for preparation №3, except that 3% of glycerol was
added to the homogenization buffer and the final product
was stored at -20°C with glycerol added to a
concentration of 30%.

As a standard promutagen requiring metabolic
activation, a solution of 2-aminoanthracene (Serva) in
DMSO (Sigma-Aldrich) was used at concentrations of 5,
10 and 50 µg/mL. As a negative control, DMSO was
added to the plate.

Before use, the preparations were stored for 30 days
under the above conditions.

Results

The results of testing the quality of microsomal
fractions are shown in Table 1. The activity of
preparations №1 and №2, based on the microsomal
fraction of the rat, is sufficient to detect the mutagenicity
of all treatment options, except for the effect of a
minimum dose of 2-aminoanthracene on strain TA98.

The study of the quality of the preparation of rat hepatic
S-9 fractions showed no significant differences in the
activity of the preparations stored at -80°C and -20°C. The
minimal activating ability was shown by the preparation
№3 from the chicken liver, prepared without a
cryoprotectant. The Ames test using TA100 and TA98
strains and this preparation does not allow detection of
the mutagenic effect of 2-aminoanthracene in a dose of 1
µg per plate. Preparations №4 and №5 are effective for
all combinations of doses and strains. The use of these
preparations allows for greater sensitivity compared to
the standard protocol based on the preparation №1.
Table 1: Comparison of the effectiveness of metabolic activation using rat hepatic S-9 homogenate and bird hepatic S-9 homogenate

| Strain of Salmonella typhimurium | Test specimens | Preparation №1 | Preparation №2 | Preparation №3 | Preparation №4 | Preparation №5 |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| TA98                             | DMSO (Control) | 26±5           | 22±5           | 19±5           | 24±3           | 25±5           |
|                                 | 2-aminoanthracene (1 µg /plate) | 32±6           | 30±5           | 28±8           | 150±21*        | 139±10*        |
|                                 | 2-aminoanthracene (20 µg /plate) | 864±92*        | 855±88*        | 255±35*        | 987±115*       | 1010±93*       |
|                                 | 2-aminoanthracene (50 µg /plate) | 1680±239*      | 1564±202*      | 2100±156*      | 2040±234*      | 2110±190*      |
| TA100                            | DMSO (Control) | 209±14         | 199±15         | 210±17         | 176±19         | 206±14         |
|                                 | 2-aminoanthracene (1 µg /plate) | 315±19*        | 302±17*        | 205±44         | 357±34*        | 299±34*        |
|                                 | 2-aminoanthracene (20 µg /plate) | 508±64*        | 493±58*        | 990±219*       | 2670±178*      | 2580±194*      |
|                                 | 2-aminoanthracene (50 µg /plate) | 2960±119*      | 2982±127*      | 3010±190*      | 3050±179*      | 3200±216*      |

* - a statistically significant mutagenic effect (t-test, p<0.05)

Discussion

It should be noted that the priority for the use of the liver of chickens for the activation of promutagens belongs to Russian scientists (Pavlov et al., 1985). An investigation of the frequency of induced mutations in a system of indicator microorganisms/cell extracts of animals and birds liver (Salmonella/microsome test) was conducted to analyze the mechanisms of mutagenesis. In the study of the metabolic activation of 2-aminofluorene, the higher activity of chicken liver microsomes was first demonstrated in comparison with liver preparations of mice (Pavlov et al., 1985), even after preliminary induction of the latter with a mixture of polychlorinated biphenyls, which increases the activity of cytochromes P-450 family enzymes by tenfold (Benford et al., 1988).

Conclusion

Thus, it can be concluded that although the chicken hepatic S-9 homogenate preparation was prepared and stored according to the standard procedure allows one to detect the genetic activity of the promutagen used in principle, it has an increased demand for the storage temperature (Table 1). However, the introduction of the cryoprotectant (glycerol) into the homogenization buffer and the storage buffer has made it possible to compensate for this deficiency.

Our research showed that minor changes in the test protocol make it possible to obtain chicken hepatic S-9 homogenate preparations, which are less demanding for storage conditions, that makes the process of its estimation more cost-effective and also provide a higher sensitivity to the mutagenic effect of 2-aminoanthracene in the Ames test.

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Author’s Contributions

Alexey V. Tutel’ian and Aleksander V. Usatov: Designed and performed experiments and wrote the paper. Eugenia V. Prazdnova and Vladimir A. Chistyakov: Developed analytical tools and analyzed data. Aleksander I. Klimenko and Maria A. Kolosova: Collected and analyzed data.

Ethics

This article is original and contains unpublished materials. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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