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Review

Overview and justification of a toxicological experiment design for establishing risk assessment indicators

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Abstract

N-nitrosamines in food products are a serious health threat due to their carcinogenic and non-carcinogenic effects on the kidneys, spleen, GIT, and liver. Meat products are a major source of their exposure. Within a joint Russian-Vietnamese study, health risks for the Vietnam population were assessed using the methodology developed in the Eurasian Economic Union. As a result, unacceptable risk levels associated with N-nitrosodimethylamine (NDMA) in meat products were established for children under 6 years and adults 18-50 years old. However, the reference dose for NDMA established by the US EPA is based on limited data and requires experimental definition. The objective of this study is to overview the publications devoted to the study of the toxic effects of N-nitrosamines and to justify an experiment design for identifying and validating biomarkers of exposure and response upon N-nitrosamines' exposure. In conformity with the international standards OECD and GLP principles, a design is going to be created for a 180-day experimental study of NDMA chronic toxicity using Wistar rats. The number of animal groups exposed to NDMA is four, and one group is control. Oral exposure to NDMA will take place using gavage 7 days a week. The experiment involves monthly monitoring of biochemical indicators (AST, ALT, and GGT) and NDMA levels in blood. The created design is expected to help establish points of departure (reference level, benchmark dose level) for chronic exposure as well as provide significant data for risk assessment. Taking into account three established approaches used in experimental studies to refine risk assessment's parameters, the Benchmark dose (BMD) method was selected for this experiment's design.

Keywords: *N-nitrosamines, toxicological experiment, health risk assessment, hepatotoxicity, biomarkers.*

1. INTRODUCTION

N-nitrosamines, which may appear in food products, are potentially hazardous for human health. Many studies report them to have carcinogenic effects without any established safety thresholds [1–3]. Simultaneously, N-nitrosamines manifest some threshold toxic effects, in particular, hepatotoxicity [4–6].

Meat and meat products are a major source of N-nitrosamines ingestion into the body [7, 8]. Some countries including the Russian Federation have established safe standards for contents of such chemicals as N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) in animal products [9]. Health risks for the population of the Socialist Republic of Vietnam (SRV) associated with exposure to N-nitrosamines in food were assessed within the joint Russian-Vietnamese study (2023–2024) relying on the methodology, which is used in the Eurasian Economic Union (EAEU) member states [10]. The next stage in the research is to define more precisely and to verify parameters of the "Exposure – Response relationship" in order to obtain well-substantiated indicators for assessing health risks caused by oral exposure to N-nitrosamines in food products. The accomplished assessment of health risks for the population of the Socialist Republic of Vietnam (SRV)

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caused by oral exposure to N-nitrosamines (NDMA, N-nitrosodiphenylamine (NDPA)) established unacceptable risk levels for children (younger than 6 years) and adults (aged 18-50 years) upon oral exposure to NDMA in meat and meat products. In particular, in the age group of 18-50 years, the hazard quotients (HQ) reached 1.22 and 1.74 for grilled meat products and baked meat products respectively. These risk levels are considered alerting according to the Guideline R.2.1.10.3968-23 "Health Risk Assessment upon Exposure to Chemical Pollutants in the Environment" valid in the EAEU countries. At the same time, NDPA levels in meat and meat products did not create unacceptable health risks. The highest NDMA dose introduced with grilled meat products and exerting negative impacts on the body equaled 0.02 µg/kg of body weight [10].

According to the Guideline R.2.1.10.3968-23, the reference dose (RfD) for NDMA, equal to 0.008 µg/kg of body weight as established by the US EPA, is established for non-carcinogenic health risks [11]. This value was calculated based on LOAEL equal to 25 µg/kg/day, which was obtained in a 10-week experiment conducted by Anderson *et al.* using a single NDMA dose on 10 white mice. The total modifying factor 3000 was used to establish RfD; it included tenfold extrapolation 'animal – human' (MF=10), tenfold higher level for sensitive population groups (MF=10), tenfold coefficient for transition from LOAEL to NOAEL (MF=10), as well as an additional threefold coefficient (MF=3) due to absence of any data on neurotoxicity and reproductive toxicity obtained by multi-generation studies. Development pathologies including stillbirth, shorter pregnancy, higher neonatal mortality and a higher proportion of males in a litter were considered critical effects in the experiment [12, 13].

In addition, available research data demonstrate a wider range of toxic effects produced by N-nitrosamines including NDMA [11, 14]. In particular, a few publications revealed that N-nitrosamines could affect not only development processes but the liver functioning as well [15, 16]. Some studies reported hepatotoxic effects of N-nitrosamines including hepatocyte destruction, portal development of venopathy and necrosis/hemorrhage, inflammatory infiltrates appearing in portal bile ducts [17, 18]. AST, ALT and GGT activities in serum are considered biomarkers of response to hepatotoxic effects produced by N-nitrosamines [6]; among them, GGT levels are specific for liver damage since they tend to grow even in case of insignificant and subclinical liver dysfunctions [4].

Given the existing uncertainties associated with the necessity to extrapolate short-term exposure effects on repeated exposure scenarios, it seems necessary to more precisely identify an employed reference dose for health risk assessment upon oral exposure to NDMA.

This necessitates a toxicological study in laboratory animals to refine the parameters for quantifying the non-carcinogenic risk of NDMA intake from food and to establish science-based risk assessment benchmarks (e.g., reference points, model parameters) applicable to various food types.

The objective of this study is to overview the publications devoted to the study of the toxic effects of N-nitrosamines and to justify an experiment design for identifying and validating biomarkers of exposure and response upon N-nitrosamines' exposure.

2. SELECTION OF BIOMARKERS FOR THE EXPERIMENT CONDUCTION

When developing an experiment design, it is advisable to be guided by international documents, which stipulate key organizational points in experimental studies, as well as by one's own scientific and research experience. The experimental design involved the selection of an animal model, the establishment of treatment and control groups, the determination of dose levels for the studied groups, and the definition of the study duration.

Following the analysis of the results reported in studies [11, 14-18], the hepatotoxic effect of NDMA is considered a key non-carcinogenic effect upon oral exposure to the chemical. Changes in liver function tests (levels of AST, ALT and GGT) are considered biomarkers of effect. These biomarkers are specific to hepatocyte injury [19]. This contrasts with markers of biliary impairment, such as total bilirubin, bile acids, and alkaline phosphatase [20, 21] (**Table 1**).

In addition, the content of NDMA in the blood is supposed to be considered as a biomarker of exposure, which corresponds to generally accepted approaches in conducting toxicological studies.

Table 1. *Comparative characterization of response biomarkers*

Serum biomarker	Localization in tissues	The type of damage characteristic of the biomarker	Damage specification	Comments
ALT (alanine aminotransferase)	Generally in liver	Increases with liver necrosis, as well as with the heart and skeletal muscle necrosis	Hepatocellular necrosis	Widely used to assess hepatocellular damage
AST (aspartate aminotransferase)	Heart, brain, skeletal muscles, liver	Increases with damage of the liver or extrahepatic tissues.	Hepatocellular necrosis	Less specific than ALT
Total bilirubin	Captured, conjugated in the liver and secreted into bile	A marker of hepatobiliary damage and liver function; also increases with hemolysis	Cholestasis, biliary tract; Liver function	A traditional biliary marker; combined with ALT, it is the best indicator of the severity of the disease in humans.
Alkaline phosphatase	General tissue localization	A marker of hepatobiliary damage	Cholestasis	A traditional biliary marker; associated with drug-induced cholestasis in humans
GGT (γ-glutamyl-transferase)	Kidney, liver, pancreas	A marker of hepatobiliary damage	Cholestasis, biliary tract	A traditional biliary marker; high sensitivity in humans
Bile acids	Bile ducts	Increase with liver damage and functional changes.	Liver function	The levels depend on the diet and fasting condition.

3. EXPERIMENTAL ANIMAL CHOICE AND ITS JUSTIFICATION

The objective of the experiment was to establish the non-carcinogenic effects of NDMA in animals over a 180-day period. Chronic toxicity is usually studied on rodents for a period between 6 months and 2 years [22]. At the same time, the OECD Guide recommends a chronic experiment to last for not shorter than 12 days; however, both shorter and longer experiments are allowed [23].

The experimental animal model was selected due to OECD Guide on Chronic Toxicity Studies [24]. According to the guide, the preferred rodent species are the rat and the mouse, may be used because of a number of advantages. The rats are preferred due to several considerable advantages. Thus, relevance of an employed biological model is the most significant aspect. According to studies by Kogure *et.al.* [25] and Muzhikyan *et.al.* [26], the rats' liver has similar structure and performs functions similar to the human liver, especially, when it comes down to metabolism of drugs and toxicants. This ensures biological plausibility of experimental results and is the key criterion for selecting these animals for the present study.

Use of rats as model objects has been recognized by international regulatory authorities such as the Organization for Economic Cooperation and Development (OECD) [25]. The vast body of historical knowledge on rat biology offers a robust framework for interpreting new results, lending greater relevance through comparison with existing baselines [23]. Therefore, rats are the most optimal model for a toxicological study.

In addition, it is noteworthy to mention economic expediency of this model object since it is relatively cheap to buy and keep rats in comparison with bigger laboratory animals; this makes them the optimal choice for large-scale studies [28]. Furthermore, the rat's body size offers an optimal balance: it is large enough to permit standard experimental procedures and repeated biological sampling, yet small enough to allow for efficient housing in a vivarium setting [29].

Therefore, this combination of practical and economic advantages gives solid grounds for selecting rats as a basic model for biomedical research.

Prior to the experiment, rats' age will be 6 weeks; male weight (SD±m), 196.7±16.1 grams; female weight, up to 178.6±15.0 grams.

The experimental animals will be kept in conformity with the Good Laboratory Practice (GLP) principles [30] and the OECD Guide on Chronic Toxicity Studies [24]. The animals will be bought from a certified farm involved in animal breeding for toxicological and clinical studies and delivered to the vivarium. The rats will undergo 5-day quarantine under observation in a room isolated from other animal species. In compliance with Good Laboratory Practice (GLP), proper animal husbandry involved establishing and maintaining optimal housing, care, and handling conditions. This included comprehensive health management (e.g., quarantine, isolation of sick animals), acclimation to the test environment, unambiguous animal identification upon group assignment, maintaining clean enclosures, and minimizing external variables that could impact the study data [30]. In the planned experiment, 2 to 3 animals are to be kept in each cage. Each cage should be marked stating the group, which animals in it belong to, their body weight, age, sex, the number of animals in the cage, and duration of experiment.

The minimal number of animals in each group is established considering the Feder formula [31] and is equal to 5 animals. At the same time, the total number of animals necessary for the experiment (n=70, including 5 reserve animals), has been established by using Statistica v.11 software package. The number of animals in each group is 6-7 species of both sexes, which will ensure obtaining statistically authentic results for establishing health risk assessment indicators. One animal is reserved for each group for emergencies.

4. SELECTION OF DOSE FOR THE EXPERIMENT

According to OECD requirements, the minimal number of animal groups for determination of the dose–response relationship endpoints is three as well as controls [32].

Multiple chronic oral exposure studies have been conducted in rats to evaluate NDMA toxicology across different dose ranges and exposure durations. Chronic studies revealed the effects occurred in NDMA oral exposure. Mutagenic effects show a clear dose-response pattern, with studies demonstrating positive mutagenicity at doses 2 mg/kg body weight and more, typically resulting in 2-5 fold increases in liver mutant frequencies compared to controls (the range of group doses was from 0.36 to 2 mg/kg/day) [33]. The carcinogenic dose-response relationship has been established at much lower exposure levels, with the carcinogenic dose in rats 10 µg/kg/day (which was the only dose in the study) [34]. At the lowest end of the dose-response spectrum, even chronic exposure at regulatory benchmark levels shows measurable effects. Studies using the rat-equivalent dose of 7.2 ng/kg/day, corresponding to the human acceptable daily intake, demonstrate that chronic low-dose NDMA exposure impairs immune function and reproductive health, elevates urea levels, and reduces ALT levels, suggesting renal involvement and mild hepatocellular compromise despite the absence of overt toxicity in organ weights or gross pathology (the dose was the only used in the study) [35].

In conformity with the experiment protocol, the animals will be randomly divided into five groups using stratified randomized distribution per body weight. The test groups will be exposed to different NDMA doses:

+ Test Group 1 will be given NDMA solution in a dose equal to 0.001 LD50 (40 µg/kg of body weight); this dose does not cause animal death and helps create models to describe the "Exposure – Biomarker of Response" relationship;

+ Test Group 2 will be given NDMA solution in a dose equal to LOAEL (2 µg/kg of body weight) [6];

+ Test Group 3 will be given NDMA solution in a dose equal to 0.00001 LD50 (0.4 µg/kg of body weight); this dose provides a step for creating models to describe the "exposure – biomarker of response" relationship;

+ Test Group 4 will be given NDMA solution in the maximum dose established by accomplishing health risk assessment and creating a negative health effect in adults upon consumption of meat and meat products (0.02 μg/kg of body weight) [10];

+ Group 5 will be Control Group given distilled water (1 mL).

The doses in the groups were chosen to cause the expected effect (biochemistry indicators increasing) but not to cause death of laboratory animals.

The experimental design, including group allocation and dose levels, was informed by previous research and regulatory requirements. The number of groups (and, consequently, NDMA doses) has been established in this study following guidelines provided in research literature. Thus, according to Sewell et.al., at least three test groups should be made; however, their exact number is determined by a range of doses selected for an experiment [36]. Buchanan *et al.* note that when studying toxicokinetics of a chemical, at least three doses should be used prior to assessing its toxicological properties: approximately 0.1 LD_{50}, 0.01 LD_{50} and 0.001 LD_{50}. Although three doses might be sufficient for demonstrating a dose-dependent effect, use of additional doses (both higher and lower ones) makes it possible to more precisely define LOAEL of an analyzed chemical [37, 38]. This study utilized five dose groups (including control), which allows for a more robust determination of the dose-response relationship and increases the reliability of NDMA toxicological parameter assessment.

NDMA solutions are to be administered into the laboratory animals daily on an empty stomach in the morning after weighing; doses will correspond to the experimental groups. Daily, after an overnight fast and body weight recording, the animals were administered an NDMA solution by oral gavage. The dose was calculated individually for each animal based on its most recent body weight and experimental group assignment. Blood samples will be taken once a month during the whole experiment to analyze biomarkers of exposure and effect. Biomarkers of exposure (AST, ALT and GGT) will be identified in blood using colorimetric techniques. Biomarkers of exposure (blood NDMA levels) will be established by using gas chromatography – tandem mass spectrometry (GS – MS/MS).

Due to the use of healthy inbreed Wistar rats from special farm, no criteria for exclusion were applied. However, stopping rules were death of more than 50% of animals in each group before the end of the experiment.

5. THE CHOICE OF APPROACH TO REFINE RISK ASSESSMENT'S PARAMETERS

Three established approaches are commonly used to refine parameters for risk assessment: the Benchmark Dose (BMD) method, the No-Observed-Adverse-Effect Level (NOAEL) method, and the use of Physiologically Based Pharmacokinetic (PBPK) modeling. Each of these approaches has its own advantages and disadvantages.

For instance, the NOAEL approach does not account for the variability and uncertainty inherent in experimental results, which are influenced by study design characteristics. Furthermore, NOAELs do not correspond to consistent response levels, making comparisons across studies, chemicals, or endpoints problematic. When calculating an acceptable daily intake (ADI), the use of a NOAEL introduces uncertainties related to the scope of the available studies, the animal species used in toxicological analyses, the design of epidemiological studies, and various toxicological parameters. Consequently, the magnitude of the uncertainty factor is determined by considering the potential impact of these variables on the reliability of the assessment [**39**]. The Benchmark Dose (BMD) approach, in turn, accounts for sample size and the shape of the dose-response curve. It mitigates much of the model dependency often associated with extrapolating modeling data to low doses. The BMD approach is recommended by the EFSA guidance [40]; however, it is technically more demanding than the NOAEL approach. The use of PBPK models, in turn, faces challenges related to structural and practical parameter identifiability. This is due to the high complexity of the models and the limited availability of clinical data, which leads to ambiguous estimates and complicates the extrapolation of results. Furthermore, high correlation among physiological parameters, the failure to account for uncertainty and variability in estimates, and the computational complexity of Bayesian methods significantly constrain the reliability and practical utility of these models for predictive purposes. These fundamental limitations necessitate careful study design, sensitivity analysis, and the application of model reduction techniques to ensure the physiological plausibility of the outcomes [41].

The information about the approaches used in toxicology summarized in **Table 2**.

Table 2. *Comparison of different approaches used in toxicology*

Approach	Advantages (Pros)	Disadvantages (Cons / Challenges)
NOAEL (No-Observed-Adverse-Effect Level)	Technically less demanding and simpler to implement.	• Does not account for the variability and uncertainty inherent in experimental data. • Heavily influenced by study design characteristics (e.g., sample size, dose spacing). • Does not correspond to a consistent response level, complicating comparisons across studies, chemicals, or endpoints. • Introduces uncertainties when used for deriving health-based guidance values (e.g., ADI), related to the scope of available studies, choice of animal species, and other toxicological parameters.
BMD (Benchmark Dose)	• Accounts for sample size and the shape of the dose-response curve. • Mitigates much of the model dependency associated with low-dose extrapolation. • Recommended by major regulatory bodies (e.g., EFSA). • Provides a more consistent and quantitative basis for risk assessment compared to NOAEL.	• Technically more demanding than the NOAEL approach. • Requires a higher level of statistical expertise and robust dataset for modeling.
PBPK Modeling (Physiologically Based Pharmacokinetic)	• Provides a mechanistic, physiology-based framework for extrapolation (e.g., across species, routes, or doses). • Combines "bottom-up" (physiological) and "top-down" (clinical data) approaches.	• Faces challenges with structural and practical parameter identifiability due to model complexity and limited clinical data. • High correlation among physiological parameters and failure to adequately account for uncertainty/variability can constrain reliability. • Computational complexity, especially of Bayesian methods for uncertainty analysis. • Requires careful study design, sensitivity analysis, and model reduction techniques to ensure physiological plausibility.

Since the objective of the chronic experiment is to determine the benchmark dose level of NDMA, the BMD approach was selected as recommended by international guidance and provided a more consistent and quantitative basis for risk assessment.

6. CONCLUSION

Thus, the overview of studies of N-nitrosamines' toxicity revealed the hepatotoxic effects of NDMA as well as mutagenic and carcinogenic. The overview results will be used for the experimental design justification.

During the justification of the design for the toxicological experiment it was established that the experiment should conform to basic postulates stated in international regulatory documents supported by scientific research. The basic postulates include the choice of animal models (Wistar rats were used as an animal model), the animals maintenance, the establishment of number of animals in group (13 animals in each group), dividing the animals in groups and selecting doses in the experiment (the doses were chosen to induce the expected

(biochemistry indicators increasing) but not to cause death of laboratory animals). Additionally, the BMD approach was chosen for the experiment since its objective to establish benchmark dose level of NDMA.

The study results will yield statistically robust data, including a Point of Departure (POD) based on the threshold principle for non-carcinogenic effects and model parameters for quantitative risk assessment at NDMA exposure levels above reference values. These parameters will then enable a data-driven health risk assessment for the general population based on actual exposure data.

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