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Experimental works

Morphometric and ANOVA analysis of the loach embryo parameters under the PEG-carrier influence

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In the biomedical field, polyethylene glycol-based materials demonstrate remarkable positive potential as drug delivery systems, in wound healing, and tissue engineering. It's known that complex of polyethylene glycol and thiazole derivative has demonstrated high cytotoxicity against diverse tumor cell lines, such as melanoma, glioblastoma, hepatocarcinoma, and leukemia. However, studies on the impact of nanocarriers on sensitive test systems, such as cold-blooded animal embryos, also deserve attention. Our research on loach embryos established that 10 µmol/L of PEG-carrier initiated positive, significant changes in the survival of embryos and prelarvae, while 100 µmol/L caused embryo swelling. The aim of this work was to identify the morphometric parameters of cold-blooded animal embryos during early development and to analyze their changes under the influence of the PEG-carrier using ANOVA. The relative surface area and diameter of the roe, blastomers, embryos, and yolk were the most informative parameters**.** We observed that the *mPEG*-carrier at 10 µmol/L doesn't exhibit embryotoxic properties and even positively influences embryo survival. A significant increase in the relative surface area and diameter of blastomers and embryos (roe, embryo, yolk) was only observed under the influence of 100 µmol/L of the carrier. The share of *mPEG*-polymer's influence on the morphometric changes in embryos was significant (58.0–80.0 %) at the 3rd and 6th hours of development, with the exception of changes in the area of eggs/yolk. Two-factor analysis confirmed that changes in the relative area and diameter of the roe, embryo, yolk, and blastomers of loach are significantly caused by the *mPEG*-polymer addition factor (51.3 %, P≤0.05) to the incubation medium, and do not depend on the factor of development time. As known, an increase in the size and relative surface area of germ cells and embryos indicates the embryotoxic properties of substances, potentially leading to embryo death. Therefore, PEG-modification improves the biocompatibility of nanomaterials and can significantly reduce the toxicity of active components. However, the use of PEG-polymer requires strict control over concentration, structure, and administration regimen due to its clear dose-dependent toxicity, potential immune responses, and biodegradability issues.

Key words: morphometric parameters, loach, embryo, polyethylene glycol, polymeric carrier, ANOVA

Introduction

Polymeric carriers (nanomaterials) are constantly being modified to meet new application requirements in various fields, including drug/protein/nucleic acid delivery or the delivery of contrast probes for disease diagnosis and analysis [6, 8, 24, 39].

Polyethylene glycol (PEG) is a polyether compound and a versatile, water-soluble polymeric carrier with variable molecular weight. It's widely used as an additive in food and cosmetics [24, 34] and is approved by the USFDA [11]. In modern pharmacology, PEG is primarily used as a synthetic macromolecular polymer for the modification of small drug molecules, peptides, proteins, or nanodrug delivery systems to improve their water solubility, biocompatibility [15, 24, 44], and stability of molecules [39, 43].

Variable molecular weight PEG particles demonstrate anti-inflammatory, anti-apoptotic [4], immunosuppressive [39, 43], and stabilizing properties on subcellular organelles, plasma membranes, endothelial cells [4, 33], cardiomyoicytes [36] and postovulatory aging mouse oocytes [45]. In preclinical investigations, intravenous delivery of high-molecular-weight PEG (35 kDa) was shown to mitigate ischemia-reperfusion injury in the heart, liver [4, 42], and intestine [7], while also enhancing organ viability. The authors believe these positive effects are partly due to the activation of survival and compensation pathways (protein kinase B, adenosine monophosphate-activated protein kinase, and endothelial nitric oxide synthase) [34–35]. PEG also stabilizes the actin cytoskeleton in endothelial cells by preserving sarcolemma lipid raft architecture and structure [4, 33]. More recently, polymeric carriers have been recognized as gene carriers due to their low cytotoxicity and immunogenicity, moderate transfection efficiency, lack of size limitation, low cost, and rapid reproducibility [10, 27].

The primary function of modern polymeric carriers is to transport drugs to target organs and protect them from interactions with other molecules, potentially resulting in a loss of the drugs' pharmaceutical activity [6, 24]. To date, numerous polyethylene glycol-modified (PEGylated) delivery systems with unique characteristics and functions have been developed [8, 44]. However, only a few of them have received clinical approval for human use in humans due to their potential toxicity [30–31]. The poor safety and efficacy of these substances are significant reasons for the high failure rate of clinical trials for nanodrug delivery systems. These factors are not only related to the loaded and released drugs but also to the characteristics of the delivery systems themselves. Although PEG itself is considered non-immunogenic, there's increasing evidence that it can initiate an immunogenic response in an organism, for example, when conjugated to proteins and peptides [22]. Under these conditions, the combination of PEG with other biomolecules can lead to the synthesis of antibodies against PEG after administration, which is a significant obstacle to the use of PEGylated products. Despite extensive clinical experience, the most common effects of PEG exposure include hypersensitivity, toxic by-products, organ atrophy, incomplete biodegradation, and accumulation in the body [38, 41]. For instance, Patel and colleagues found diffuse signs of retinal degeneration and cataract formation in patients injected with PEG-400, along with a significant decrease in electroretinogram amplitude and retinal atrophy after PEG injection [35].

Presently, the most popular models for assessing the toxicity of newly synthesized substances and nanocarriers are mammals and cold-blooded animals. Mammalian models, particularly mice, can be used to assess whole-body and organ-specific toxicity. In particular, a thiazole derivative complexed with poly(PEGMA)-based polymer nanomicelles [12–13] has shown a high level of cytotoxicity against specific tumor cell lines compared to unconjugated thiazole and/or the classical chemotherapeutic drug doxorubicin [12].

Aquatic models, such as zebrafish (*Danio rerio*) [21, 25–26, 40] or loach (*Misgurnus fossilis/anguillicaudatus)* [16–17, 20], offer many excellent characteristics, including high sensitivity, ease of care, rapid embryonic development, and transparent body parts that can be observed using a light microscope. Our previous studies established that adding 10 μmol/L of a PEG-containing carrier to the incubation medium initiated positive, significant changes in the survival of loach embryos and prelarvae, in contrast to the effect of the PEG-carrier at a 100 μmol/L concentration [3, 5]. Therefore, the aim of this work was to identify the main morphometric parameters of loach embryos during early embryogenesis and analyze the changes in these parameters under the action of the PEG-carrier using analysis of variance (ANOVA) [19].

Materials and methods

Egg and embryo collection of *Misgurnus* and their breeding were conducted following established protocols [16–18, 20]. Toxicity assays were consistently carried out in Parafilm-sealed Petri dishes, each containing 100 embryos in Holtfreter's medium (mmol/L: 110 NaCl, 1.4 KCl, 1.8 $CaCl_2$, and 5 Tris-HCl, pH 7.4 [18, 20]), to minimize solvent evaporation. In the experiment, embryos were incubated in Holtfreter's medium supplemented with PEG-carrier to final concentrations of 10 and 100 μmol/L. Three female and three male loaches were used in the investigation. For experimental repetition, three dishes were prepared per pair of individuals, totaling 900 fertilized eggs. Embryo observations, for both control and experimental groups, were carried out up to the blastula stage (6 h after fertilization) using an *MBS-9* binocular microscope with a photographic attachment in real time. Embryos were photographed using a digital microscope *XS-3330 LED MICROmed*. The morphological development of embryos from both groups was assessed using the Fujimoto development tables [16], and morphological parameters (relative surface area and diameter of the egg,

blastomers, and embryo) were measured using the computer programs *ImageJ* and *Photoshop* (CC 2014v15).

The carrier poly(VEP-co-GMA)-*graft*-*mPEG* (here after referred to as *mPEG* (M_n = 750 Da [29]) was synthesized at the Department of Organic Chemistry (Lviv Polytechnic National University). The synthesis steps were described in detail in a previous publication [29]. A water dispersion of the polymeric carrier based on polyPEG was prepared by dissolving it in dimethyl sulfoxide (DMSO), and the resulting solution was subsequently transferred into water.

Statistical analysis of the obtained results was carried out using *MS Excel* 2016 and *Origin* 2018 programs. All data are presented as arithmetic mean (M) and confidence interval (CI). To determine statistically significant differences between the means of independent investigation groups, ANOVA was used. The hypothesis of data normality was tested using the Shapiro-Wilk's test [28].

One-way analysis of variance (4 series of analyses for each parameter) was applied to assess the relative contribution of the influence of the *mPEG*-carrier (at 40, 60, 150, and 330 min) to changes in the studied morphometric parameters. This analysis also considered the influence of unmeasured factors and assessed the statistical significance of these effects. To establish the contributions of the time factor (Factor 1, development time) and the studied factor (Factor 2, *mPEG*-carrier) to changes in the morphometric parameters of embryos, 8 series of two-way analysis of variance were conducted. For all analysis series, N_t represents the number of gradations for the duration of embryo development (N_t = 4), and N_c represents the number of gradations for the other factor under study (N_c = 2) [19]. Using the *SPSS/Statistica* program, Levine's test was performed to assess the homogeneity of variances of several samples. P-values of ≤0.05 or lower were interpreted as statistically significant.

Ethical Considerations. The study was conducted in accordance with the general ethical principles of animal experiments established by the First National Congress on Bioethics (Kyiv, Ukraine, 2001) and was approved by the Ethics Committee of Ivan Franko National University of Lviv, Ukraine, at the beginning of the research (Protocol no. 51-06-2025 from 02.06.2025). Adult loaches were kept in a refrigerator, with daily environment changes and suitable lighting and temperature conditions.

Results and Discussion

Loach development

In the first stage of the research, the process of loach development from fertilization to asynchronous division of blastomers (6 h after fertilization, late blastula) under normal conditions was analyzed. Fig. 1 and 2 summarize the changes in the relative morphological parameter values (relative surface area and diameter) of loach embryos during the early stages of embryogenesis in the group of control. A significant increase in the blastomers relative area and the embryos themselves was found. It should be noted that changes in the relative surface area of roe and the embryo itself of the loach did not differ at the studied stages of embryogenesis, but significantly increased throughout early embryogenesis (see fig. 1B).

In the control group of embryos, the blastomers diameter exhibited a significant decrease (fig. 2A). Loach eggs mainly have a spherical, streamlined shape [3], which contributes most to their existence and survival in a constantly moving aquatic environment. A significant increase in egg diameter was found with a proportional increase in the size of the embryo itself (by 41.7±0.6 %; see fig. 2B) in the group of control.

It is known that egg size, like other characteristics such as the size of the embryonic yolk, is adaptive in nature. These morphometrically changes were accompanied by a slight decrease in the area of the embryonic yolk, even though the young fish organism switches to a mixed type of nutrition quite late after hatching [16, 18, 20]. Studies by Fujimoto and co-authors showed similarities between loach development from the cleavage stage to the gastrula stage [16] and that of some other fish, such as ice goby [1] and medaka [22].

This is due to a periodic an increase in embryonic cell count during the first 6 hours of development (at this stage of development, the embryo consists of more than 1,000 cells) [16, 18].

The mPEG-carrier effect
on the morphological parameters of loach embryos

A study by Bagday et al. [3] established a low embryotoxic effect of *poly-mPEG*-carrier on embryos and larvae of *M. fossilis* L. However, no anomalies or malformations were detected in loach larvae that survived exposure to the polymer carrier in a 10-day experiment [3, 5].

Therefore, a study was conducted to determine the concentration dependence of the newly synthesized carrier's effect on the morphometric parameters of embryos during the embryogenesis of cold-blooded animals exposed to 10 and 100 μmol/L of *mPEG*-carrier.

At the second stage of our research, we observed a minor, though significant, increase in area (Fig. 3A–B) and diameter (Fig. 4A–B) of blastomers and embryos themselves (including roes (19,1±0,2 %), embryo (relative area — 30.5±1.1 % and diameter — 10.3±0.7 %) and yolk) under the influence of 100 μmol/L *mPEG*-carrier at the studied stages of embryo development.

Additionally, at 150 and 330 min of development, a significant increase (9.2±1.3 %) in the relative area of embryos and embryonic yolk was found under the influence of 10 μmol/L *mPEG*-carrier. It should be noted that under the influence of the studied *mPEG*-polymer concentrations at 6 hours of development, a significant increase in the relative area of the embryonic yolk was found on average by 22.3±0.7 % (Fig. 3B). This confirms the high sensitivity of embryonic objects as test systems.

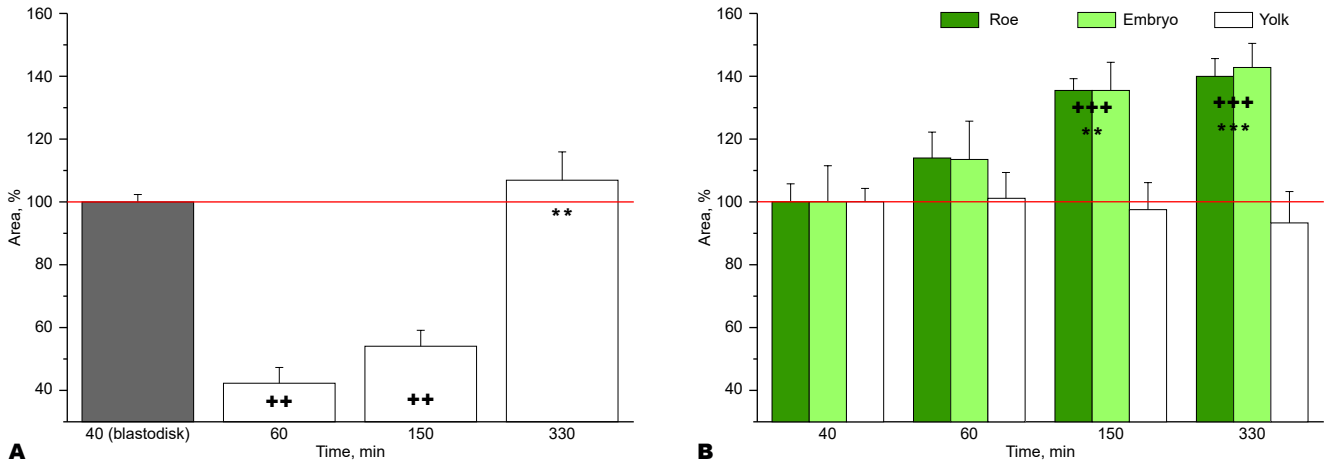

Fig. 1. Alterations in blastomers (A) and embryos (B) relative surface area during synchronous divisions (M±CI, n=10)
Note. Here and throughout, the value of the studied parameters at 40 min after fertilization is taken as 100 % (red horizontal line).
* — indicates significance compared to the indicators at stage 2 blastomers (P<0.05; P<0.001);
+ — indicates significance compared to the indicators at 40 min after fertilization (P<0.001).

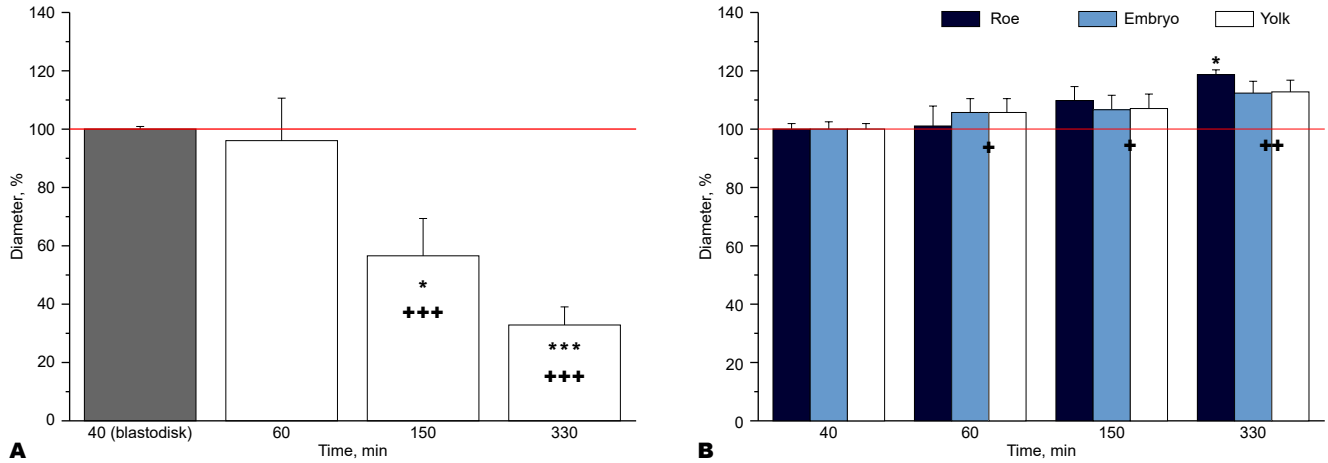

Fig. 2. Changes in the blastomers (A) and embryos (B) diameter during synchronous divisions (M±CI, n=10)
Note. Designations are as in fig. 1.

Such changes in embryo morphometric parameters were not accompanied by embryo death in survival experiments [4], which confirms the low embryotoxicity of 10 µmol/L poly-*mPEG*. It's worth noting that *mPEG*-based nanomicelles demonstrated a low level of cytotoxicity against specific breast tumor cell lines compared to unconjugated thiazole and/or the classical chemotherapeutic drug doxorubicin [12]. The IC_{50} of the cytotoxic effect on MDA-MB-231 cells was 26.5±2.9 µmol/L for the thiazole derivative, 6.9±0.4 µmol/L for the thiazole-PEG complex, and poly(PEGMA) demonstrated low toxicity (IC_{50}>50 µmol/L) [12].

Consequently, our sensitivity findings for loach embryo cells partially align with the IC_{50} coefficient for poly(PEGMA) [13] regarding its cytotoxicity on the tumor cell line. While the *mPEG*-carrier at 10 µmol/L concentration did not exhibit embryotoxic properties, the same cannot be said for the results with the 100 µmol/L carrier.

As a result of the calculations, a significant increase in blastomers and embryo relative surface area and diameter was found under the influence of 100 µmol/L *mPEG*-carrier. This may indicate swelling of the embryos and impaired water-salt metabolism, which can cause early embryo death. Although polyethylene glycol modification (PEGylation) reduces the concentration of carbon dots (CD) in the heart, the concentration remains elevated compared to most other nanomaterial types [6, 37].

Nevertheless, intermediate concentrations of NH_2–PEG (80 µg/ml) were used in experiments to determine PEGylation's toxicity on a mouse heart model and its effect on heart development in a zebrafish model [6]. Administration of PEG-CD to mice caused a statistically insignificant decrease in serum creatine kinase and lactate dehydrogenase levels detected on day 7, which returned to their respective control levels on day 21. Neither PEG-CD caused significant changes in cardiac cell morphology, nor did it lead to a significant elevation in heart rate or other ECG parameters. However, PEG-CD administration did cause minor changes in cardiac development, which aligned with the findings from *in situ* hybridization experiments [6].

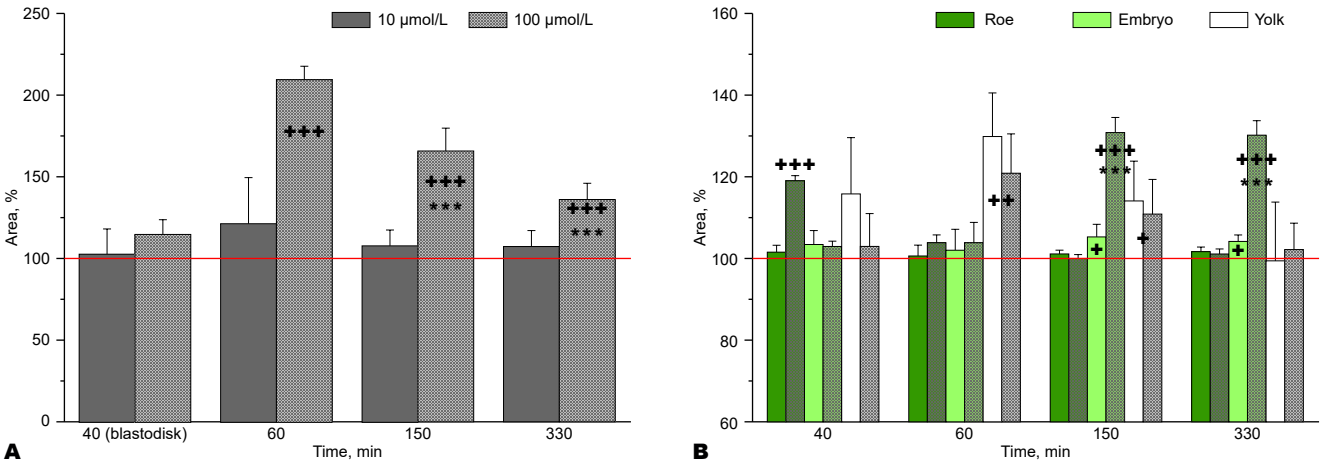

Fig. 3. Change in the blastomers (A) and embryos (B) relative surface area during synchronous divisions of embryonic cells under the influence of *mPEG*-carrier, 10 μmol/L and 100 μmol/L (M±CI, n=10)
Note. Throughout, the value of the studied parameters in the control is taken as 100 % (red horizontal line).
* — indicates significance compared to the indicators at stage 2 blastomers (P<0.05; P<0.001);
+ — indicates significance compared to the indicators at 40 min after fertilization (P<0.001)

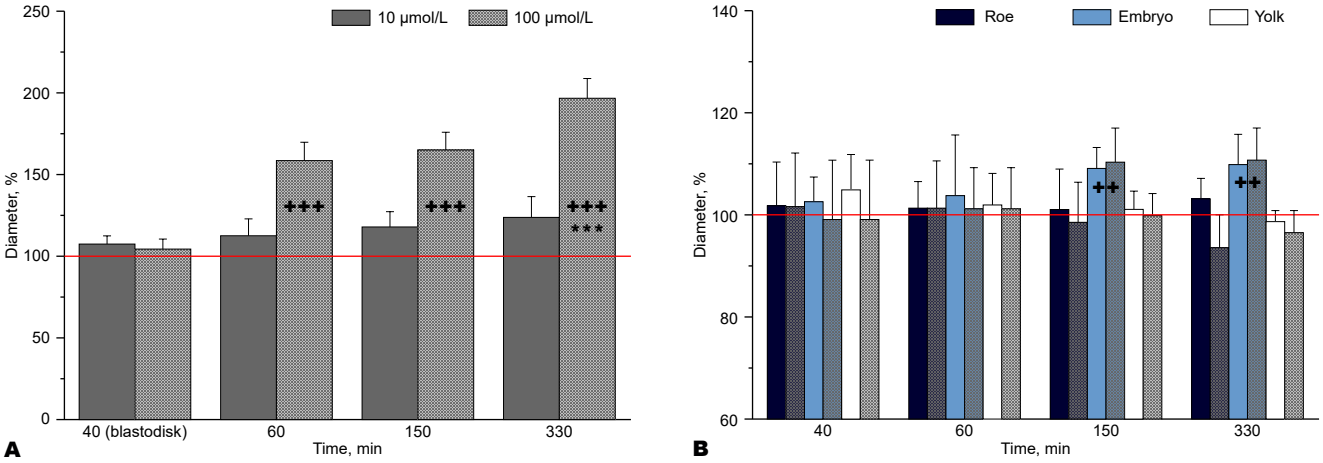

Fig. 4. Change in the blastomers (A) and embryos (B) diameter during synchronous divisions of embryonic cells under the influence of *mPEG*-carrier, 10 μmol/L and 100 μmol/L (M±CI, n=10)
Note. Designations are as in fig. 3.

It can be concluded that, in the zebrafish model, the introduction of PEG into the carbon nanomaterial system reduced the toxic effects of pure CDs on cardiac development. The obtained data demonstrated that PEGylation improves the biocompatibility of nanomaterials but does not completely eliminate the toxicity of active CDs. The significant reduction in CD toxicity could be caused by the reduction in contact between nanomaterials and cells due to the introduction of PEG.

ANOVA analysis

One suitable and adequate method for assessing the influence of exogenous factors (based on their proportional contribution to the variability of the studied indicator's values), as well as validating their role in the organism's observed metrics, is ANOVA [19]. One-way analysis of variance was analyzed for each studied parameter, obtained at specific times of embryo development: 40, 60, 150, and 330 minutes.

Additionally, four series of variance analysis were conducted under the influence of the *mPEG*-carrier at two concentrations (N_c = 2).

The *mPEG*-polymer's influence is confirmed by its substantial contribution (58.0–80.0 %) to the alterations in embryonic morphometric parameters, with the exception of variations in egg surface area and yolk during the studied period of early embryogenesis (fig. 5A). Throughout this studied period, no significant influence of the *mPEG*-carrier on changes in the area and diameter of roes and yolk was detected (Fig. 5A–B). This suggests these changes were caused by other factors, whose contributions were quite significant (18.5–98.0 %).

A two-factor analysis of variance was performed to establish the proportions of the factors' influence: investigating the effect of *mPEG*-carrier (Factor 1) concentration on changes in morphometric indicators at the previously established time points of development of loach embryos (Factor 2). It was found that for changes in the relative area

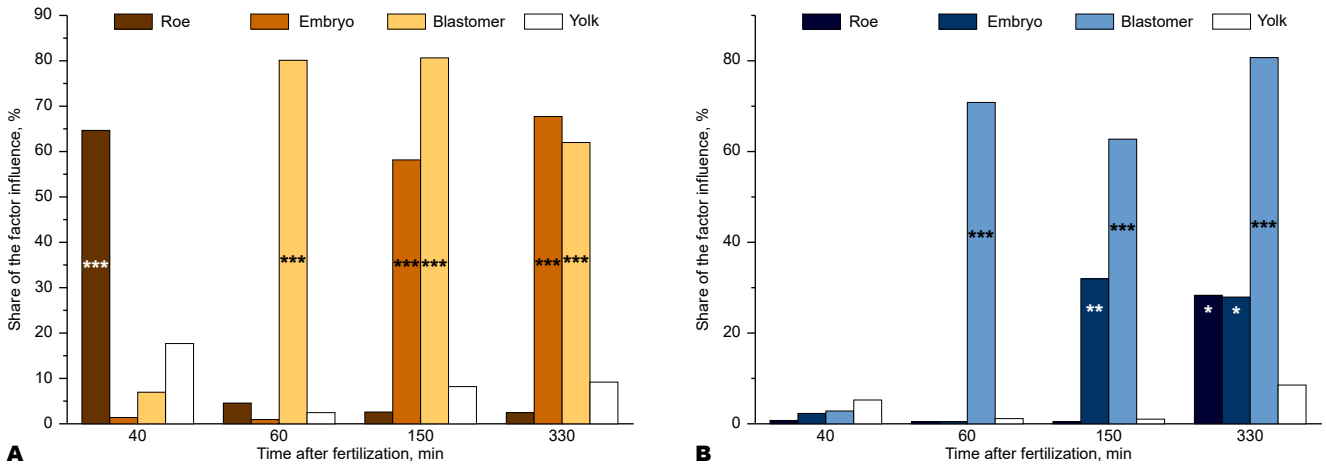

Fig. 5. One-way ANOVA data of the *mPEG*-carrier effect on the morphometric parameters of embryos (A — area, B — diameter)
Note. * — P<0.05; ** — P<0.01; *** — P<0.001.

and diameter of the roe, embryo, yolk, and blastomers, the time factor was statistically insignificant (16.2 %). Ultimately, all changes in embryonic morphometric indicators were due to the addition of polymer to the incubation medium (contributing an average of 51.3 %, P≤0.05).

This may indicate both the influence of factors not accounted for in the experiment (including female body condition, quality of reproductive products, and atmospheric pressure), and the low biocompatibility of the *mPEG*-carrier with the yolk's lipid component. However, this requires confirmation in further studies. The most notable and significant influence of the *mPEG*-carrier (P<0.001) in changes in both the area and diameter of the embryos and blastomers was found particularly at the third and sixth hours of development. As is known, an increase in the size and relative surface area of germ cells and embryos indicates cytotoxic changes, such as cell swelling and an increase in intracellular fluid [9]. Such cellular changes occur with various physical cell damages as well as with allergic reactions in the body. In these cases, the colloidal-osmotic state of the cytoplasm changes due to altered substance transport across the cell membrane. Cytotoxic swelling depletes the extracellular space of Na^+, Cl^-, and water molecules, thereby creating a new gradient for these molecules across the cytoplasmic membrane [9]. The observed increase in blastomer size provides further confirmation, but not of the embryo itself within the perivitelline membrane (*i.e.*, eggs/oocytes). Thus, cytotoxic edema is important in its own right because it signals a premorbid cellular process that almost inevitably leads to oncotic or necrotic cell death.

Drawing from literature data and our results, it can be concluded that while PEG-modification often improves nanomaterial biocompatibility, it doesn't always completely eliminate their toxicity. It's well known that neither the thiazole derivative, *mPEG*-nanomicelle, nor their complex altered antioxidant enzyme activity in hepatocytes from mice with Nk/Ly lymphoma [32]. Our findings are partially consistent with data on poly(PEGMA) cytotoxicity against tumor cell lines [12–13]. Another PEG-nanomaterial, the polymer *mPEG*-b-PGC, is non-cytotoxic, but paclitaxel (PTX)-loaded nanoparticles are cytotoxic to lung, breast, and ovarian cancer cell lines [14]. Optimal *mPEG*/PLGA ratios, which influence surface PEG density and nanoparticle size, can ensure prolonged PLGA-*mPEG* nanoparticle circulation in the bloodstream [2]. It's also important to note that the sarcoplasmic reticulum's recovery rate significantly depends on both PEG's molecular weight and pre-incubation time, with PEG200 incubation for 2–6 hours notably increasing this recovery rate [35].

We should approach using *mPEG*-carrier with great caution, and they require further thorough research and optimization of loading conditions. Their low toxicity at small doses and ability to improve biocompatibility make them promising [24]. However, their clear dose-dependent toxicity, potential immune responses [22], and biodegradability issues necessitate strict control over concentration, structure, and administration regimen to ensure safety and effectiveness in clinical settings.

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Морфометричний та дисперсійний аналіз параметрів ембріона в'юна за впливу ПЕГ-носія

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У біомедицині ПЕГ-матеріали демонструють значний позитивний перспективний потенціал як системи доставки ліків, у загоєнні ран і тканинній інженерії. Відомо, що комплекс поліетиленгліколю та похідної тіазолу продемонстрував високу цитотоксичність проти різних ліній пухлинних клітин, як-от меланома, гліобластома, гепатокарцинома та лейкемія. Однак також заслуговують уваги дослідження впливу наноносіїв на такі чутливі тест-системи, як зародки холоднокровних. Наші дослідження на ембріонах в'юна встановили: 10 мкмоль/л ПЕГ-носія ініціювало позитивні достовірні зміни виживання зародків та передличинок в'юна, тоді як 100 мкмоль/л призводить до набряку ембріонів. Метою роботи було ідентифікувати морфометричні параметри ембріонів холоднокровних на ранніх стадіях розвитку та аналіз їх змін під дією ПЕГ-носія за допомогою дисперсійного аналізу (ANOVA). Найінформативнішими параметрами були відносна площа та діаметр ікри, бластомерів, зародків і жовтка. ПЕГ-носій у концентрації 10 мкмоль/л не виявляє ембріотоксичних властивостей і навіть позитивно впливає на виживання зародків. Достовірне збільшення відносної площі та діаметру бластомерів та ембріонів (ікри, зародка, жовтка) спостерігали лише за впливу 100 мкмоль/л носія. Частка впливу *mPEG*-полімеру на морфометричні зміни ембріонів була значною (58.0–80.0 %) на 3-й та 6-й годинах розвитку, за винятком площі ікри/жовтка. Двофакторний аналіз підтвердив, що зміни відносної площі та діаметру ікри, зародка, жовтка та бластомерів в'юна достовірно обумовлені додаванням в середовище *mPEG*-полімеру (51.3 %, P≤0.05), і не залежать від чинника часу розвитку. Як відомо, збільшення розмірів зародкових клітин та ембріонів свідчить про ембріотоксичні властивості речовин, що потенційно веде до загибелі. Отже, ПЕГ-модифікація покращує біосумісність наноматеріалів та може значно зменшити токсичність активних компонентів. Однак застосування *mPEG*-полімеру вимагає суворого контролю концентрації, структури та режиму застосування через чітку дозозалежну токсичність, потенційні імунні відповіді та проблеми з біодеградацією.

Ключові слова: морфометричні параметри, в'юн, зародок, поліетиленгліколь, полімерний носій, дисперсійний аналіз

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