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EFFECT OF ORGANIC MICROELEMENTS IN LIPOSOMAL FORM ON FERTILIZING ABILITY AND THE LEVEL OF ANTIOXIDANT REACTIONS OF FEMALE RABBITS

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The purpose of this study was to investigate the effect of supplementation with organic zinc, manganese and chromium in the form of liposomal complex on the fertilizing ability and the level of antioxidant responses of female rabbits. Feeding of female rabbits with supplementation of organic forms of trace elements prior to insemination resulted in increase the numbers of corpora lutea, implantation and living fetuses compared to the control group. Moreover, there were the 4,37% and 1,74% decrease in pre- and post-implantation losses in animals receiving the organic microelements prior to insemination, respectively. The level of thiobarbituric-acid-reacting substances in ovary of experimental group was significantly higher ($P \leq 0.05$) compare to the control group, while the level of lipid hydroperoxides in experimental group was decreased. In the uterus of rabbits after addition organic compound of trace elements significantly decreasing the thiobarbituric-acid-reacting substances level was by compare to the control animals ($P \leq 0.001$). The level of the superoxide dismutase activity in uterus and ovary of female rabbits in the experimental group were significantly higher than in the control group ($P \leq 0.01$). Our studies indicated that supplementation organic microelements in liposomal form to the basal diet for 2 weeks before insemination had a beneficial effect on the metabolism intensity and maintaining antioxidant-prooxidant balance in reproductive organs that improve fertilization and embryo implantation.

Key words: organic forms of trace elements, female rabbits, antioxidant reactions.

Modern rabbit breeding is based on high-intensity using of females because the does are being inseminated every 35 or 42 days. In such a system, the females need a large of energy [1]. Reduction in fertility, may occur as a result of poor nutrition, bad feed, incorrect lighting program, suckling a large litter, health problems, moulting, etc. [2]. Deficiency in the nutrient supply during pregnancy has been highlighted as a dominant cause of developmental programming. Manal et al. found that timing of feed restriction is important for reproductive performance in rabbits [3] and also it in late pregnancy have led to increasing mortality of new-born rabbit kits [4]. In accordance with the previous researchers, Goliomytis et al. found that maternal feed restriction during gestation had an adverse effect ($P \leq 0.05$) on ratio of stillborn kits and significantly increased mortality rates [5]. Forages are the main source of trace elements for the rabbits, but its contents in forages are very variable; so when the mineral supplementation level is insufficient, reproductive problems may be occur.

Among of all trace minerals, zinc, chromium, and manganese are accumulated in the highest concentration in the conceptus, as compared to other reproductive tissues, which suggests that they play a significant role in fetal development and survival [6]. Furthermore, some research results suggest that feeding proteinated zinc, chromium and manganese to gestating females increases reproductive performance presumably because of their improved bioavailability [6]. However, the maternal-fetal transfer mechanisms of these micronutrients are not well understood. Micronutrient transport mechanisms for the conceptus are developed during the first half of pregnancy and nutrient transport is probably active rather than passive across the maternal-fetal blood barrier with specific receptors on endometrial and conceptus tissues [7].

Maternal zinc deficiency may compromise infant development and lead to poor birth outcomes. The trace elements status in general and zinc in particular of new-born depends on maternal transfer via the placenta, the colostrums or the milk [8]. Zn levels in fetuses are 1.7 to 8.7 times greater than in the endometrium and ovaries at 12 to 30 days of development [9]. Fetal storage of Mn and Cr were dependent on maternal dietary intake. Likely they play a role in fetal bone formation and may initiate estradiol secretion by the conceptus for pregnancy recognition and perhaps in progesterone.

Pregnancy is characterized by physiological changes in antioxidant systems. Oxidative stress play a role in remodeling of uterine tissues, implantation of the embryo, settlement of the villi and development of blood vessels characteristic of gestation [10]. Deficiencies of the vitamin and trace elements can lead to exaggerate oxidative stress and can induce the adverse effects on the health of the mother and on the development and viability of the new-born.

The aim of this study was to evaluate the effect of supplementation with organic form of zinc, manganese and chromium in liposomal to the basal diet on the 14th day prior to insemination on the level of antioxidant responses and reproductive function of females rabbits during the early stage of pregnancy.

Materials and Methods

The study was conducted on females rabbits divided into two groups: the control group and experimental group. The control group was fed the basal diet while the experimental group was supplemented with Zn glutamate (35 µg/kg), Mn glutamate (32 µg/kg), Cr methionate (60 µg/kg), Se (20 µg/kg) with vitamins E (20 mg), A (30 000 IU), D (40 000 IU) two weeks before insemination. All group of animals were artificially inseminated with appropriate hormonal treatment. We used 40 IU PMSG (Pregnant Mare Serum Gonadotropin, Follimag, Intervet, Holland) for synchronized cycle (injected 48 h before AI) and 20 µg/doe GnRH (Gonadotropin-releasing hormone) (Fertagil, Intervet, Holland) for induction of ovulation (injected at the moment of insemination). Rabbits were fertilized intravaginally of 10×10^6 spermatozoa/ doe in 0.5 ml tris-citrate diluents.

On day 14th of gestation rabbits were euthanized with an overdose of sodium pentobarbital, and the ovaries were removed for evaluation the number of corpora lutea. Gravid and non-gravid uterine horns were weighed and the number of dead and live fetuses, number of implantations and resorptions in the uterine horns were recorded. Implantation index [(No of implants/No of corpora lutea) × 100], preimplantation losses [(No of corpora lutea — No of implants)/No of corpora lutea] × 100 and postimplantation losses [(No of implants – No of viable fetuses)/ No — of implants] × 100 were also evaluated.

The procedures and use of the animals in this experiment were approved by the Institution Ethical Committee on the policy statement for care and use of laboratory animals. The rabbits were housed in individual cages, maintained under controlled light/ dark cycles (12L:12D) and fed *ad libitum* with a commercial pelleted diet and to water via an automatic water supply system.

The tissues samples (uterus and ovaries) were collected for determining antioxidant enzyme activities and levels of lipid and protein peroxides. Each uterus and ovarian sample was homogenized in cold Tris-HCl buffer (100 mM, pH 7.4) to obtain a 10% (w/v) tissue homogenate. The homogenate was then centrifuged at 5 000 ×g for 15 min. Each supernatant was collected and stored at −20 °C until use. The protein content of each sample was determined using Bradford method (1976) and bovine serum albumin as the standard. An aliquot of the homogenate was used to determine the lipid peroxidation reactions of the sample by measuring the concentration of thiobarbituric-acid-reacting substances (TBARS), carbonyl groups as an indication of oxidative damage to proteins, as well as superoxide dismutase (SOD), catalase

(CAT), lipid hydroperoxides. All enzymatic assays were carried out at 25 ± 0.5 °C using spectrophotometer SF 46 (Carl Zeiss Jena, Germany). Each sample was analyzed in triplicate.

Lipid peroxidation level was determined by quantifying the concentration of TBARS, expressed as μmol of malondialdehyde (MDA) per mg of protein, according to Kamyshnikov [11]. The TBARS level was expressed in μmol MDA per mg protein by using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as molar extinction coefficient.

Lipid hydroperoxides (LHP) assay was developed by modifying the FOX methods described by Wolff (1994) involves the oxidation of Fe^{2-} by peroxides at low pH in the presence of both the ferric-complexing dye xylenol orange and sucrose, the amplifier of the reaction. The method proved to be a convenient, simple and efficient assay for the direct measurement of both water and lipid soluble peroxides. In fact it improves by about 60% the sensitivity of the FOX1 method for water soluble peroxides, and by 7–8 times than of the FOX2 method for lipid soluble peroxides. It allows the detection of 0.1 μM peroxide in the test solution. The method is suitable to measure the lipid hydroperoxides present in phosphatidylcholine liposomes and in human LDL. The data obtained allowed us to define a mathematical expression to calculate the lipid hydroperoxide content of liposomes knowing their oxidation index [12].

Diene conjugates (DC) were evaluated by measuring the optical density of the lipids at 232 nm on the SF-46. Lipids were read in cyclohexane (0.2–0.3 mg/ml) and the optical density (OD). OD/mg lipid was calculated. Lipid content of samples was measured by Chiang [13].

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured with the method by Kostiuk et al. [14]. SOD activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH=10.0). Absorbance at 406 nm was measured immediately and after 20 min. Activity is expressed in units of SOD per mg of tissue protein.

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al., 1988 [15]. The absorbance of solution was measured at 410 nm and was compared with that of the blank. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 μmol H_2O_2 per min per mg of protein.

Tests were repeated three times for every type of the samples. The results were presented as mean ± standard deviation. Differences between groups were determined by Student *t*-tests.

Results and Discussion

The different reproductive performance parameters like corpus lutea, number of implantation and fetuses, resorptions sites, fertility rates were detected in the group supplemented with organic trace elements in liposomal form for 2 weeks before insemination and in control (Table).

The data analysis showed that the numbers of corpora lutea, implantation and living fetuses in the experimental group increased in comparison with the control one. The values of resorption in the female rabbits treated with

Effect of liposome preparation on reproductive performance of female rabbits

Parameters	Control group	Experimental group
Number of corpus lutea	10.4±0.4	11.0±0.45
Number of implantation sites	9.4±0.4	10.6±0.74
Total Live Fetuses	8.6±0.4	9.8±0.49
Number of resorptions sites	0.4±0.24	0.2±0.24
Pre-implantation losses (%)	13.46	9.09
Post-implantation loss (%)	4.44	2.70
Total gestational losses	17.31	10.91

Note. The results are represented as $M \pm m$ for 5 rabbits in each group.
For all values $P \geq 0.05$ compared to control, $n = 5$.

liposomal preparation were by 2-times lower as compared to the control group. While the pre- and post-implantation losses decreased in liposomal treated group.

The decrease of gestational losses in this study is the result of supplementation with organic zinc, manganese and chromium in liposomal form before insemination which induces nutrient supply to the fetuses. In a similar study, Stanton et al. [16] observed higher pregnancy rate in cows receiving organic Cu, Zn, and Mn vs. the inorganic forms.

The correlation between nutrition and reproductive performance has been investigated in different animals. Phiri E. C. J. H. et al. [17] found that ewes receiving zinc supplementation had a higher fertility rate and were more prolific (89% vs. 40%). Also, Tang Xiao-lin et al. [18] compared the effects of chitooligosaccharide-zinc (COS-Zn) as a new organic zinc source with zinc sulfate on the growth and reproductive performance of female mice and found that the utero-ovarian index and the number of embryos were significantly increased in COS-Zn group ($P \leq 0.05$).

In some studies it has been indicated that changes in mineral metabolism could have a harmful effect on fetal growth [19, 20]. For example, zinc element of mothers affects fetal growth, levels of serum Insulin-like Growth Factor I and leptin [21]. In addition, another systematic review showed that zinc supplementation during pregnancy is associated with a 14% reduction in premature deliveries [22].

In Fig. 1, the changes in the intensity of peroxidation processes after supplements of organic forms of trace elements in the reproductive organs of rabbits were determined.

The level of TBARs in ovary of experimental group was significantly higher ($P \leq 0.05$) as compared to the control group, while the lipid hydroperoxides level was decreased in experimental group (Fig. 1).

In the uterus of rabbits after addition of organic compound of trace elements significantly decreasing of TBARs level was compared to that parameter in the control animals ($P \leq 0{,}001$) (Fig. 2). Content of diene conjugates also was lower in the experimental group. While the level of lipid hydroperoxides in experimental group was significantly increased after adding of preparation, as compared to the control group.

The improvement of reproductive performance in our current results for the group supplemented with organic forms of trace elements as compared to the control were accompanied by using liposomal emulsion with trace elements in organic form during the fertilization period of rabbits. So that, for instance, Mn^{2+} inhibits the free radical chain which follows the formation of hydroperoxides and that lead to the formation of MDA. It has been reported that Mn^{2+} is able to reduse the lipid free radicals (RO and ROO) making them unable to carry on the process of LPO [23].

During the embryo implantation, the increased fluidity in the membranes of endometrial cells is caused by a slight increase in lipid peroxidation, aids the fusion of the trophectoderm with the endometrial cells [24]. It has been suggested that Mn^{2+} supplementation inhibits LPO, thus increasing the membrane integrity and viability, which are required for storage of lipids and phospholipids [25]. It has been reported that Mn^{2+} protects placental membrane from peroxidative damage produced by the superoxide radicals (O^{2-}) [26].

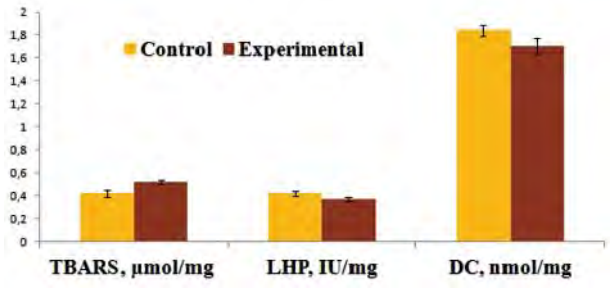

Fig. 1. **Effect of organic microelements in liposomal form on the level of oxidative stress biomarkers in the ovarian of rabbits**
* — $P \leq 0{,}05$ compared to control, $n = 5$.

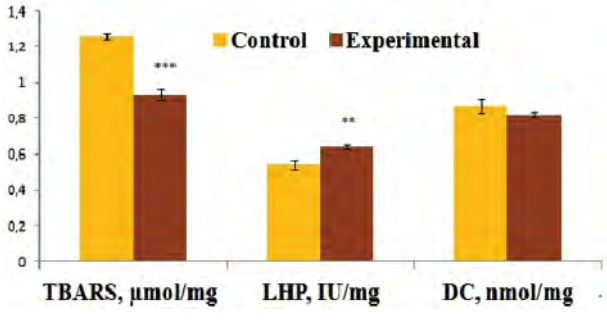

Fig. 2. **Effect of organic microelements in liposomal form in the TBARS, LHP and DC in the uterus of rabbits**
Here and after: *** — $P \leq 0.01$, ** — $P \leq 0.001$ compared to control, $n = 5$.

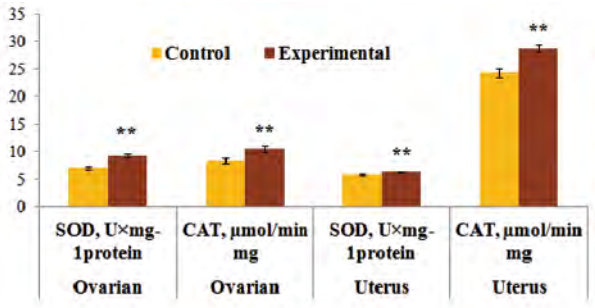

Fig. 3. **Effect of organic microelements in liposomal form on SOD and CAT activity in the ovaries and uterus of rabbits**

Several studies have indicated that antioxidative defense is modified during normal pregnancy [27]. It is worthwhile to mention that the decrease of antioxidant enzymes level during pregnancy is dependent on trace elements profile. Accordingly, addition of organic trace elements in liposomal form to the basal diets caused activating effects on antioxidant enzymes superoxide dismutase and catalase.

The level of the SOD activity ($P \leq 0.01$) in uterus and ovary of female rabbits in the experimental group were significantly higher than in the control group (Fig. 3). The activity of catalase in those tissues of experimental groups were also significantly increased, as compared with the control group ($P \leq 0.01$).

Oxidative stress is generated during normal placental development; however, when the supply of antioxidant micronutrients is limited, exaggerated oxidative stress within both the placenta and maternal circulation occurs, resulting in adverse pregnancy outcomes [28]. Manganese and zinc are essential metal acts as a cofactor of various enzymatic systems such as SOD and CAT, and participate in the structure of ceruloplasmin [29] and, therefore, play important biological functions. The deficiency of these essential trace elements could have devastating effects on the health of the mother, fetus and newborn. Use of organically complexed trace minerals can help prevent these losses, due to their increased stability in the upper gastrointestinal tract of the animal. In current investigation we used liposomal emulsion as a method of direct delivery of trace elements in organic form to the target organs, where the active compounds of our formulation expressed their biological action. Our results have been supported by Tian X. and Diaz F.J. studies [30]. They show that feeding a zinc deficient diet for 3–5 days before ovulation (preconception) dramatically decreases oocyte quality and developmental potential including a decrease in DNA and histone methylation and associated increase in expression of repetitive elements. These epigenetic defects along with previously shown meiotic defects [31] severely compromise fertilization and preimplantation embryonic development.

The result of our studies indicate that supplementation organic microelements in liposomal form to the basal diet on the 14th day prior to insemination provided an increase of reproductive ability — growth and development of embryos and their implantation and improvement in the antioxidant activity, and decrease the oxidative stress in female rabbits during early state of gestation.

In conclusion, supplementation of rabbit does to the basal diet prior to insemination mineral biocomplex improved fertilizing ability in particular it at 4,37% and 1,74% decreased pre- and post-implantation losses. This suggests that organic trace elements in liposomal form able to compensate microelements losses that the usual may occur during gestation.

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ВПЛИВ ОРГАНІЧНОЇ ЛІПОСОМАЛЬНОЇ ФОРМИ МІКРОЕЛЕМЕНТІВ НА ЗАПЛІДНЮВАЛЬНУ ЗДАТНІСТЬ ТА РІВЕНЬ АНТИОКСИДАНТНИХ РЕАКЦІЙ САМИЦЬ КРОЛІВ

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Метою роботи було вивчення впливу органічних сполук цинку, мангану та хрому у формі ліпосомального комплексу на запліднювальну здатність та рівень антиоксидантних реакцій самиць кролів. Згодовування кролицям органічних сполук мікроелементів підвищує кількість жовтих тіл вагітності, імплантацій та живих ембріонів порівняно з контрольною групою. Окрім того, у тварин, які отримували органічні мікроелементи перед осіменінням, відзначено зниження на 4,4% та 1,7% передімплантаційних і постімплантаційних втрат. Рівень ТБК-активних продуктів у яєчниках самиць дослідної групи був вірогідно вищий ($P \leq 0,05$), ніж у контрольній групі, тоді як вміст гідропероксидів ліпідів знижувався. У матці кролиць після додавання органічних сполук мікроелементів істотно знижувався вміст ТБК-активних продуктів порівняно з аналогічним показником у контрольній групі ($P \leq 0,001$). Активність супероксиддисмутази у матці та яєчниках тварин дослідної групи була вірогідно вищою порівняно з контролем ($P \leq 0,01$). Наші дослідження показали, що додавання органічних сполук мікроелементів у ліпосомальній формі до основної дієти впродовж двох тижнів до осіменіння позитивно впливає на інтенсивність обмінних процесів у репродуктивних органах та зберігання антиоксидантно-прооксидантної рівноваги, що покращує запліднюваність та імплантацію ембріонів.

Ключові слова: органічні форми мікроелементів, самиці кролів, антиоксидантні реакції.

ВЛИЯНИЕ ОРГАНИЧЕСКОЙ ЛИПОСОМАЛЬНОЙ ФОРМЫ МИКРОЭЛЕМЕНТОВ НА ОПЛОДОТВОРЯЮЩУЮ СПОСОБНОСТЬ И УРОВЕНЬ АНТИОКСИДАНТНЫХ РЕАКЦИЙ САМОК КРОЛИКОВ

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Целью работы было изучение влияния органических соединений цинка, марганца и хрома в форме липосомального комплекса на оплодотворяющую способность и уровень антиоксидантных реакций самок кроликов. Скармливание крольчихам органических соединений микроэлементов повышает количество желтых тел беременности, имплантаций и живых эмбрионов по сравнению с контрольной группой. Кроме того, у животных, получавших органические формы микроэлементов перед осеменением, отмечено снижение на 4,4% и 1,7% предимплантационных и постимплантационных потерь. Уровень ТБК-активных продуктов в яичниках животных опытной группы был достоверно выше ($P \leq 0,05$) по сравнению с контролем, в то время как содержание гидропероксидов липидов снижалось. В матке крольчих после добавления органических соединений микроэлементов существенно снижалось содержание ТБК-активных продуктов по сравнению с аналогичным показателем контрольных животных ($P \leq 0,001$). Активность супероксиддисмутазы в матке и яичниках крольчих опытной группы была достоверно выше по сравнению с контролем ($P \leq 0,01$). Наши исследования показали, что добавление органических соединений микроэлементов в липосомальной форме к основной диете в течение двух недель до осеменения положительно влияет на интенсивность обменных процессов и сохранение антиоксидантно-прооксидантного равновесия в репродуктивных органах, что улучшает оплодотворение и имплантацию эмбрионов.

Ключевые слова: органические формы микроэлементов, самки кроликов, антиоксидантные реакции.