AGRICULTURAL BIOLOGY,
2019, V. 54, № 2, pp. 259-268
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.2:619:578.245:618.2

doi: 10.15389/agrobiology.2019.2.259eng
doi: 10.15389/agrobiology.2019.2.259rus

INTERFERON-TAU AND FORMATION OF PREGNANCY IN COWS

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The authors declare no conflict of interests
Received July 17, 2018

Abstract

Coordinated action of steroid and peptide hormones and interferons ensure the formation of pregnancy and embryo-fetal development in cows. A special class of such interferons is interferon-tau (INFT) which is synthesized by embryo trophoblast cells. This interferon is responsible for preserving the progesterone synthesis by the ovary yellow body and embryo implantation. This paper is the first in which we report a significance of INFT under impaired embryonic development and data on evaluation of biological and clinical efficacy of bovine recombinant INFT administered to cows after artificial insemination. The aim of this work was to study the dynamics of the blood content of INFT and progesterone (P_4) during early pregnancy of Black-and-White cows (*Bos taurus taurus*) and to identify the possibility of using a bovine recombinant INFT preparation to prevent embryonic losses and to increase the effectiveness of insemination. Recombinant INFT was obtained at the Belarusian State University (V.A. Prokulevich, M.I. Potapovich). Blood content of INFT and P_4 was determined by ELISA test using Bovine Interferon-Tau Elisa Kit (USA) and Immuno-Fa-PG (Russia) 7, 14, 21 and 35 days after artificial insemination. Animals with physiological formation of embryos (n = 15) and with embryonic death (n = 3) were tested. The sensitivity of the analyses was 2.9 pg/ml for INFT and 0.4 nmol/l for P4. The phagocytic activity of leukocytes, the content of serum immunoglobulins, and the bactericidal activity of blood serum were also determined. The presence/absence of the embryo in the uterus was judged by the concentration of blood progesterone on day 21 and day 35 and by double ultrasound examination on day 35 and day 50. The evaluation of the efficacy of prescribing different doses and schemes of recombinant interferon to increase the effectiveness of insemination and to prevent delayed embryo-fetal development syndrome was carried out on 87 cows. INFT was administered parenterally once, three times, or five times in doses of 5 and 10 ml from day 12 to day 16 after insemination. Intact animals and those subjected to Progestamage administration were used as control groups. It was found that the blood concentration of INFT increased by 23.2 % from day 7 to day 14, and decreased by 30.8 % on day 35, P_4 content increased 32 times from day 7 to day 14 of embryo formation. The concentration of INFT was 7.7 % lower on day 14 and 25.2 % lower on day 35 when the embryo died. The blood P_4 level of these animals was 26.5 % lower by day 21 and 9.3 times lower by day 35. This suggests that hypointerferonemia and associated hypoprogesteronemia are among the reasons for the delay in the development and death of embryos in the early pregnancy. It was revealed that the optimal scheme of the recombinant INFT use to improve the pregnancy formation in cows is its three-fold parenteral administration in the dose of 5 ml on days 12, 14 and 16 after insemination. As compared to the intact animals, the effectiveness of insemination increased from 38.9 to 75.0 %, or by 36.1 %, and the delayed embryo-fetal development syndrome decreased from 28.6 to 16.7 %, or by 11.9 %. Metric indexes of developing embryos exceeded those of the intact animals by 32.2 % on days 28-30 of pregnancy, and by 55.3 % on days 60-65 of pregnancy, and birth weight of the calves was 14.2 % greater. This occurred along with a 33.9 % increase in INFT blood concentration, and 2.3 times increase in P_4. Direct replenishment of progesterone deficiency in animals by Progestamage administration provided 38.1 % increase in preservation of pregnancy. It is also shown that INFT

preparation has an immunomodulatory effect on the cows. The phagocytic activity of lymphocytes increased by 8.7 %, phagocytic number by 35.1 %, phagocytic index by 25.1 %, bactericidal activity of blood serum increased by 5.9 %, and immunoglobulin content by 14.3 % after the INFT triple administration. The conclusion is made about expediency of using the recombinant INFT to increase the fertility of cattle pedigree stock.

Keywords: *Bos taurus taurus*, cows, blood, interferon-tau, progesterone, early embryogenesis, embryonic death, fertility

Modern achievements in immunoendocrine control of pregnancy in animals indicate that the intrauterine formation and development of embryos and fetuses is provided by the overlapping action of progesterone, somatotropin, placental prolactin, cortisol, and interferons produced by immunocompetent cells of the uterine and placental complex [1-3]. The output and action of the latter constitute the most important mechanism of maternal-fetal relationships [4, 5].

Interferon-tau (INFT) is a special class of interferons that is directly related to the formation of pregnancy, has a common property with all interferons and provides implantation and elongation of the embryo [6-8]. It was first discovered in 1982 in sheep [9-10] and somewhat later in cows and goats. INFT is produced by mononuclear trophoblastic cells of the embryo from the first day of pregnancy, reaches a maximum content during the implantation period, and contributes to the inclusion of all mechanisms of embryo adoption by the maternal organism into this process [11-13]. First of all, it ensures the preservation and prolongation of the progesterone-synthesizing function of the ovarian corpus luteum. The mechanism of its anti-luteolytic action is associated with blockade of oxytocin and estrogen receptors in the endometrium and inhibition of the production of luteolytic prostaglandin $F_2\alpha$ ($PGF_2\alpha$). In addition, INFT inhibits key luteolytic genes induced by $PGF_2\alpha$ in the yellow body (corpus luteum) itself [14]. Having an indirect and direct anti-luteolytic effect, it increases the life expectancy of luteocytes in early pregnancy and the active synthesis of progesterone, which provides an active endometrial secretory response and nutrition of the embryo. It has also been shown [15] that INFT stimulates the expression of interleukin IL-8 mRNA within the corpus luteum, activates the migration of neutrophils into it and thereby increases the secretion of progesterone by the luteocytes [16]. INFT has a direct effect on the synthesis of other cytokines (INF-γ, IL-2, IL-4) [17-19], has an antiviral effect and acts as one of the mediators in the induction of an anti-inflammatory reaction in the uterus of cows. This reaction to the presence of the embryo manifests itself already in the first 4 days of its life in the uterus [20, 21].

In the aggregate, the biological effect of INFT in the organism of animals is aimed at creating physiologically necessary conditions for the intrauterine development of the embryo and fetus. Its insufficient production leads to a loss of pregnancy in the early stages of gestation. It should be noted that in the domestic literature there are practically no publications on this very relevant and promising area of research.

In this paper, for the first time, the authors determined the blood levels of INFT and P_4 in cows during embryogenesis during the physiological formation of the embryo, retardation of its development and death. The pathogenetic significance of INFT in impaired embryonic development was shown. The biological and clinical efficacy of bovine recombinant INFT was evaluated for the first time when it was administered to cows after artificial insemination.

The authors' goal was to study the dynamics of blood content in cows of interferon-tau and progesterone in the early period of gestation and to identify the possibilities of using bovine recombinant INFT as a means of preventing embryonic losses and increasing the effectiveness of insemination.

Techniques. The research was carried out in 2017 in the conditions of JV Vyaznovatovka LLC (Nizhnedevitsky District, Voronezh Region) with a tie barn on Black-and-White cows (*Bos taurus taurus*) (105 cows) of 4-7 years old with an average annual milk yield of 6.5-7.6 thousand kg. The animals were fed according to the norms of the All-Russian Research Institute of Livestock.

In the first series of experiments on 18 cows, the dynamics of the content of interferon-tau and progesterone in the serum during the physiological formation of the embryo and its death were investigated. Their content was determined by enzyme immunoassay (ELISA) using the Bovine Interferon-Tau Elisa Kit test systems (Clod Clone Corp., USA) and Immuno-F-PG (Immunotech, Russia) on the 7th, 14th, 21st days and 35 days after artificial insemination of animals with cryopreserved sperm. Blood was obtained from the tailgate vein. The sensitivity of the INFT assay was less than 2.9 pg/ml, and for progesterone (P_4) 0.4 nm/l. The presence or absence of an embryo in the uterus was judged by the serum concentration of progesterone on the 21st and 35th days and on the basis of a double ultrasound on the 35th and 50th days. For ultrasound, an Easi-Scan-3 ultrasound scanner (BCF Technology, UK) with a 7.5 MHz linear probe was used.

In the second series of experiments performed on 87 cows, the authors studied the effectiveness of bovine recombinant interferon-tau for the prevention of fetal mortality and increasing the effectiveness of insemination. Interferon-tau was obtained at the Belarusian State University. The bovine interferon-tau (INFT) gene sequence optimized for expression in *Escherichia coli* cells was designed using the DNAStar program (https://www.dnastar.com/software/). The synthesis of the optimized sequence was performed by Integrated DNA Technologies (USA), the sequence was cloned as part of the pIDTSmart vector at the restriction sites Nde I and Eco RI. After that, the bovine INFT gene was recloned as part of the experimental vector pET24b(+) for the same restriction sites. For visualization of the results, the gel documentation system Fusion FX (Vilber Lourmat, France) was used. Calcium transformation, restriction analysis, and ligation were performed according to standard protocols [22]. Next, the *E. coli* strain BL21CodonPlus(DE3)-RIPL was transformed with recombinant plasmid pET24-cow INFT, in the cells of which the inducible expression of the bovine INFT gene was performed. *E. coli* BL21CodonPlus (DE3)-RIPL-pET24-cow INFT was grown in a 10-liter Biotron F15L bioreactor (Biotron, Korea) at 37 °C for 7 hours in LB medium supplemented with kanamycin and chloramphenicol. Then, induction was performed by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG), continued cultivation for another 4 hours, after which the cells were collected by centrifugation in an Avanti J30I flow centrifuge (Beckman Coulter, USA), destroyed with a Panda Plus 2000 homogenizer (Gea, Italy) under a pressure of 1000 bar, and the cell homogenate was separated by centrifugation.

The inclusion bodies containing INFT were washed and solubilized in a buffer with guanidine hydrochloride. After that, INFT was refolded and the recombinant protein was purified by chromatography (desalination, ion exchange, and size exclusion chromatography) with an NGC Scout Plus medium pressure chromatograph (Bio-Rad, United States). The purity of the protein according to HPLC (Ultimate 3000 HPLC chromatograph, Thermo Fisher, USA) and protein electrophoresis was > 99%; antiviral activity was 1.02×10^9 IU/mg. Antiviral activity was measured on the MDBK cell line with vesicular stomatitis virus, Indiana strain.

Enzymes and buffer systems from Thermo Scientific (USA) were used in the work. Plasmid DNA was isolated using a Nucleospin Plasmid reagent kit

(Macherey-Nagel, Germany), and agarose gel DNA using a Nucleospin Gel PCR Clean-up reagent kit (Macherey-Nagel, Germany) according to the attached protocols. Electrophoresis of proteins in a polyacrylamide gel was carried out according to the method described by Laemmli [23].

The animals were divided into seven groups. The cows were inseminated with frozen-thawed sperm at a dose of 0.25 ml, containing 15 million spermatozoids with active forward movement. Sperm was injected through the cervical canal into the body cavity of the uterus during the manifestation of the immobility reflex. Cows from group I (n = 18) were not prescribed the drug (negative control). On the 5th and 12th day after artificial insemination, animals from group II (n = 25) were injected with the drug Progestamag − progesterone of prolonged action (Mosagrogen CJSC, Russia) at a dose of 2 ml (positive control). Group III cows (n = 8) were injected once on day 12 with bovine recombinant INFT (NPU ProBioTech, Belarus) at a dose of 5 ml, group IV (n = 8) with INFT at a dose of 10 ml, V group (n = 8) with INFT at a dose of 5 ml on day 12 and day 14, Group VI (n = 8) with INFT at a dose of 5 ml on days 12, 14 and 16, Group VII (n = 8) with INFT in a dose of 10 ml daily from day 12 to day 16. The time of interferon injection was timed to the period of nidation and implantation of the embryo (days 12-17).

Clinical evaluation of the efficacy of exogenous interferon-tau and the selection of the optimal variant of its administration was carried out according to the results of ultrasound diagnostics of pregnancy and metric indicators of developing embryos on the 28th and 30th and 60th and 65th days. Before setting up the experience and in diagnosing pregnancy or infertility in the blood of experimental cows, the content of INFT, progesterone, as well as the phagocytic activity of leukocytes, the blood serum bactericidal activity (BSBA) and the number of total immunoglobulins in it were determined [24]. At the end of pregnancy, the nature of the course of labor, the postpartum period in cows, and the body weight of newborn calves were taken into account.

The data obtained in the experiment were subjected to statistical processing using the Statistica 8.0 application program (StatSoft Inc., USA). Results were expressed as arithmetic mean (M) and standard deviation (±SD). Statistical significance was determined using the paired Wilcoxon W-test. Differences were considered statistically significant at $p < 0.05$.

Results. In the first series of experiments, the physiological formation of an embryo was recorded in 15 cows, its death in 3 animals. It was established that during embryo formation, the blood INFT concentration in cows on the 7th day was 925±35.7 pg/ml, by the 14th day it increased by 23.2% ($p < 0.05$), by the 21st day, declined by 13.7%, and by the 35th day, by 30.8% ($p < 0.001$) (Table 1). According to Kose [25], the content of interferon-tau in the blood of ruminants reaches maximum values by day 17 of gestation and then decreases by days 20-22. The P_4 concentration during this period increased 3.2 times ($p < 0.001$). Consequently, the trophoblastic interferon production peak in cows falls on the period of the embryo implantation, which ensures high progesterone-synthesizing activity of the corpus luteum, normal feeding and the formation of the embryo. When the embryo died, the content of INFT on the 14th day was 1052±36.1 pg/ml, which was lower than in healthy animals by 7.7%, and the progesterone concentration on day 21 decreased by 26.5% ($p < 0.01$) up to 22.5±1.18 nmol/l. Hypoprogesteronemia, which is formed in animals, caused a compensatory increase in INFT production by day 21, its concentration increased 1.32 times ($p < 0.01$) compared to healthy cows. However, the delayed increase in the amount of interferon did not ensure the prolongation of the function of the corpus luteum and the preservation of the emerging embryo. By the time of embryo death, the

blood INFT content decreased to 679±31.4 pg/ml, and that of progesterone to 4.0±0.21 nmol/l.

1. The concentration of interferon-tau and progesterone in serum of Black-and-White cows (*Bos taurus taurus*) during the physiological formation of the embryo and its death (*M*±SD, Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017)

The physiological state of the cows	Days after insemination			
	7	14	21	35
	Interferon-tau, pg/ml			
Pregnancy	925±35.7	1140±54.2	984±27.5	800±33.4
Embryonic death	–	1052±36.1	1297±48.9*	679±31.4
	Progesterone, nmol/l			
Pregnancy	11.8±0.21	15.8±0.98	30.6±1.16	37.3±1.67
Embryonic death	–	17.5±1.12	22.5±1.18*	4.0±0.21**

Note. A dash means that no embryonic mortality was noted during these periods.
* and ** The differences with the index at physiological pregnancy are statistically significant at $p < 0.01$ and $p < 0.001$. respectively.

Direct replenishment of progesterone deficiency through parenteral administration of progesterone-containing drugs increased the safety of pregnancy in such animals by 33.1% compared with intact cows (Table 2). The efficiency of interferon-tau administration depended on the dose and frequency of administration. The optimal was 3-fold administration at 48-hour intervals at a dose of 5 ml (on days 12, 14, and 16 after insemination). At the same time, the rates of pregnancy preservation exceeded those of animals from the intact group by 36.1%, and the incidence of embryo development retardation syndrome decreased by 11.9%. Increasing the dose and frequency of administration of interferon-tau did not provide an improvement in the clinical effect.

2. Results of the use of interferon-tau for the prevention of embryonic mortality and increasing the effectiveness of insemination in of Black-and-White cows (*Bos taurus taurus*) (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017)

Group	Inseminated cows	Diagnosed pregnancy		Fetal developmental delay syndrome, number/%
		number	%	
I (negative control)	18	7	38.9	2/28.6
II (positive control)	25	18	72.0	4/22.2
III	8	3	37.5	1/33.3
IV	8	5	62.5	1/20.0
V	8	6	75.0	1/16.7
VI	8	4	50.0	1/25.0
VII	8	5	62.5	1/20.0

Note. For a description of the groups, see theTechniques" section.

In cows of group V, the blood INFT concentration increased from 818±42.0 to 1079±46.8 pg/ml, or by 31.9% ($p < 0.01$) from 12 to 20 days after insemination, and decreased to 694 ± 24.1 pg/ml ($p < 0.01$) by day 35. At the same time, the blood P_4 content within the same period increased 2.7 times from 17.1±1.16 to 46.9±2.41 nmol/l ($p < 0.001$) and exceeded that in the intact animals with physiological pregnancy by 25.7% ($p < 0.01$). In group II, the blood INFT increased from 858±27.0 to 919±38.5 pg/ml, or by 7.1%, while the content of progesterone increased from 15.8±0.87 to 41.8±2.64 nmol/l, or 2.6 times ($p < 0.001$). Therefore, exogenous progesterone reduces the interferon-producing function of the embryo trophoblast via negative feedback.

The positive effect of bovine recombinant INFT on the morphofunctional state of the ovarian corpus luteum and the emerging embryo and fetus confirmed their metric indices during ultrasound scanning (Figs. 1, 2).

The size of the ovarian corpus luteum in cows in group V compared to the intact animals was 25.2% ($p < 0.01$) higher at days 28-30 after insemination

and 32.1% ($p < 0.01$) higher at days 60-65. The metric indices of developing embryos under INFT administration were 32.2% ($p < 0.01$) higher than those of intact cows on the 28th-30th day of pregnancy, and 55.3% ($p < 0.01$) higher on the 60th-65th days.

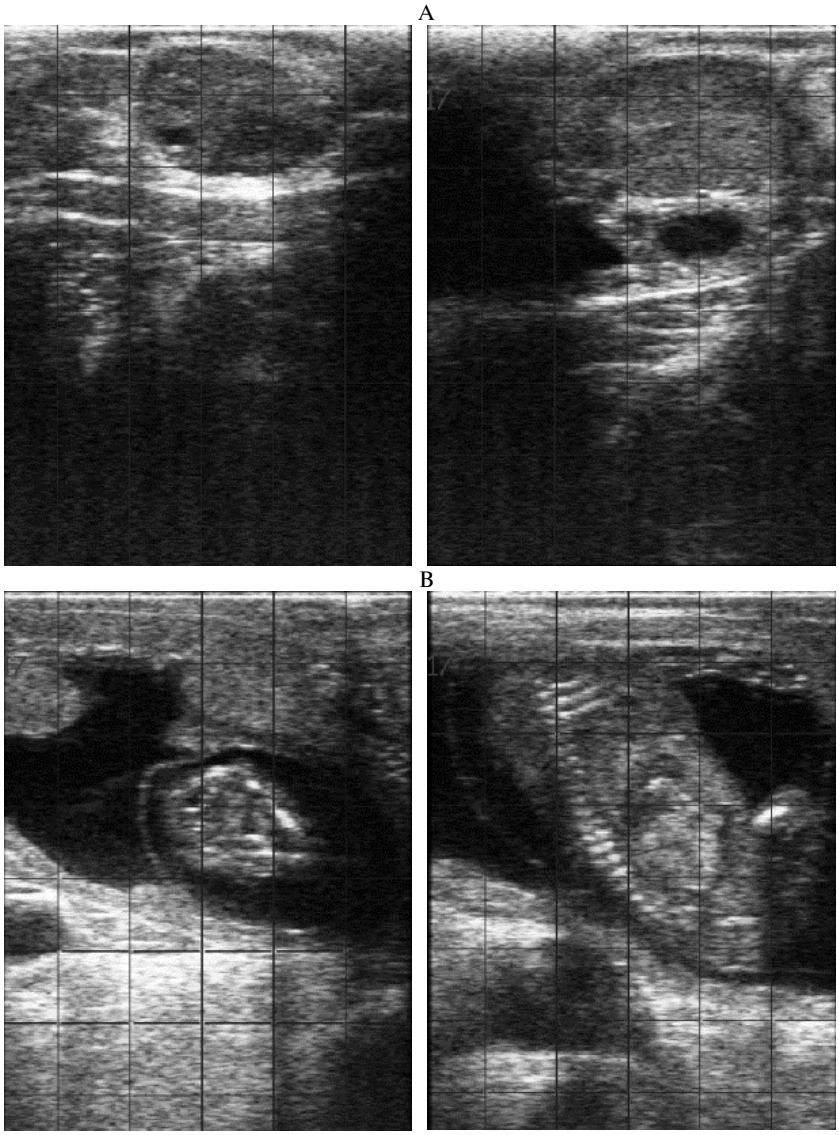

Fig. 1. The ovarian corpus luteum (A) **and the embryo** (B) **in intact** (left) **and treated with bovine recombinant interferon-tau** (right) **Black-and-White cows (*Bos taurus taurus*) on day 60 of pregnancy** (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017).

Evaluation of the immune status of cows before and after the recombinant INFT administration showed that this interferon also has an immunomodulatory effect. This was evidenced by changes in the phagocytic activity of leukocytes (PAL), the bactericidal activity of blood serum and the content of serum immunoglobulins (Table 3). Two weeks after 3-fold injections of interferon, the PAL increased by 8.7%, phagocytic number (PN) by 35.9%, the phagocytic index (PI) by 25.4%. The blood bactericidal activity increased by 5.9% ($p < 0.05$), the content of immunoglobulins by 14.3% ($p < 0.01$). In intact animals, no such changes were observed. Differences between animals of groups I

and V were for PAL 9.3% ($p < 0.001$), for PN 15.2%, for PI 6.2%, for blood bactericidal activity 10.8% ($p < 0.001$), and for immunoglobulins 18.9%.

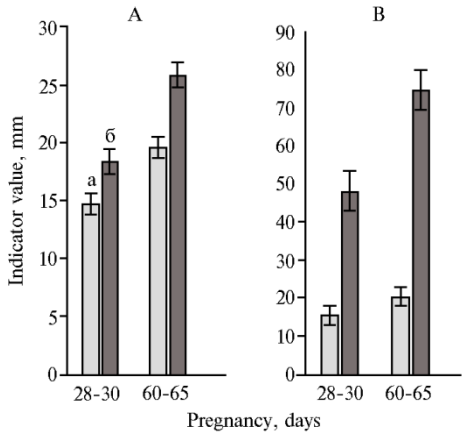

Fig. 2. The diameter of the ovarian corpus luteum (A) **and the coccygeal-parietal size of the embryo and fetus** (B) **in intact** (a) **and treated with bovine recombinant interferon-tau** (b) **Black-and-White cows (*Bos taurus taurus*) during pregnancy** (Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Province, 2017).

At birth, the body weight of calves from cows treated with INFT exceeded the corresponding figure in intact animals by 14.2% (respectively, 33.7±1.9 kg and 29.5±1.5 kg). In mother-cows, when interferon was used, the number of postpartum complications decreased 1.5 times.

3. Indicators of the immune status of intact and interferon-treated Black-and-White cows (*Bos taurus taurus*) (*M* ± SD, Vyaznovatovka JV, Nizhnedevitsky District, Voronezh Region, 2017)

Indicator	Group			
	I (negative control)		V	
	before the administration	2 weeks after the administration	before the administration	2 weeks after the administration
PAL, %	69.3±1.2	70.9±0.8	71.3±1.5	77.5±1.0**
PN, microbial cells per phagocyte	4.5±0.28	4.6±0.19	3.9±0.22	5.3±0.31**
PI, microbial cells per active phagocyte	6.5±0.39	6.5±0.18	5.5±0.21	6.9±0.42*
General immunoglobulins, g/l	24.9±1.5	24.9±1.4	25.9±1.1	29.6±1.5**
BSBA, %	58.6±1.2	58.4±0.7	61.1±0.8	64.7±0.8*

N o t e. PAL is phagocytic activity of leukocytes, PN is phagocytic number, PI is phagocytic index, BSBA is blood serum bactericidal activity. For a description of the groups, see the Techniques section.
* and ** Differences with background values are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

The pleiotropic effects of IFN-tau provide for the physiological formation of pregnancy in cows [16, 26]. It has been shown that INFT not only prolongs the progesterone-synthesizing function of the corpus luteum but also, together with progesterone, provides for the synthesis and secretion of endometrial histotroph, including amino acids, enzymes, glucose, cytokines, and growth factors critical for the embryo's nutrition, implantation, and placentation [27]. However, the threshold value of the concentrations of INFT in the blood of animals, necessary to maintain pregnancy, has not yet been established. In the authors' experiments during the nidation period of the embryo, they were within 1090-1200 pg/ml. The death of the embryo was recorded at a concentration of 1000-1080 pg/ml. Continuing research to assess the interferon status of animals in the early stages of gestation, in the authors' opinion, will allow proposing a standard indicator of the INFT content and using it as an indicator of the emerging pregnancy state. In experiments performed in mice [28], it was also shown that exogenous INFT prevents lipopolysaccharide-induced implantation failure and increases the number of implanted embryos in such animals by suppressing the production of proinflammatory cytokines (IL-1, TNF).

Thus, the obtained results showed that the concentration of INFT in the serum of cows from the 7th to the 14th day of embryo formation increased by 23.2%, and by the 35th day it decreased by 30.8%. With the death of the embryo, the concentration of INFT on the 14th day was lower by 7.7%, on the

35th day – by 25.2%. Normal embryo formation, the course and preservation of pregnancy in cows in early gestation are largely determined by the production of ovarian progesterone and trophoblastic interferon-tau as one of the autocrine regulators of early embryogenesis and implantation. The interferon preparation used by the authors meets the stated requirements for biological and therapeutic qualities. Its parenteral injections to animals during the implantation of the embryo provide prolongation and activation of the progesterone-synthesizing function of the ovarian corpus luteum by 25.7%, insemination efficiency – by 36.1%, reduction of the manifestation of intrauterine growth retardation syndrome – by 11.9%, as well as an increase in the natural resistance of the organism of mother-cows by increasing the intensity of phagocytosis, the synthesis of serum immunoglobulins and the bactericidal activity of the blood serum. The pharmacological control of interferon, progesterone and cytokine statuses of inseminated animals during the period of blastogenesis and implantation can be the basis for preventing embryonic losses and increasing the fertility of cows in highly productive dairy herds.

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