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Protective Effects of Nicotinamide on Mice Offspring Exposed to Cyclophosphamide during Pregnancy

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Abstract

Nicotinamide participates in post transcriptional activities related to the maintenance of the original structure of the DNA. The chemotherapeutic agent cyclophosphamide induces severe chromosomal abnormalities in newborns. Thus, we investigated the possible protective effects of nicotine on the toxic and teratogenic model induced by cyclophosphamide. Pregnant mice were divided into experimental groups 1 and 2 (nicotinamide 100mg/ kg and 200mg/kg respectively); Experimental Group 3 (saline); Experimental group 4 (cyclophosphamide 50 mg/kg); Associated Treaty 5 and 6 (nicotinamide 100mg/kg and 200mg/kg associated with cyclophosphamide 50 mg/kg respectively). The results indicated that the exposure to nicotinamide in two doses, did not show any parameter of toxicity. We found that associated treatments 5 and 6 have been unable to minimize the adverse effects induced by cyclophosphamide on maternal toxicity and congenital malformations. However, the group associated with nicotinamide at a dose of 200mg/kg showed a protective effect on the increase in reabsorption rate, and nicotinamide in the two tested doses showed protective effects on decreased fetal and placental weight induced by cyclophosphamide. The results of this study showed that nicotinamide in a dose-dependent manner exerted protective effect and reduced the number of abortions and that the morbidity and mortality induced by cyclophosphamide was also reduced due to the nicotinamide.

Keywords: antineoplastic agents, reproductive performance, teratogenesis

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1. Introduction

The increased incidence of cancer in recent years is due to the increase in population life expectancy and also by a number of risk factors [1]. It is estimated that in 2020 there will be about 15 million new cases, of which about 60% occur in developing countries [2].

According to Chabner [3] for the treatment of cancer in various stages are used several classes of antineoplastic agents, and alkylating agents commonly use [4]. Within this class we found cyclophosphamide, which is prescribed in combination with other drugs to treat different types of carcinomas. Its cytotoxic action is mainly due to the interweaving of DNA and RNA chain as well as the inhibition of protein synthesis [5]. Another therapeutic application of cyclophosphamide is the treatment of autoimmune diseases such as rheumatoid arthritis and graft versus host disease. It is known that this drug is often used in women of childbearing age, cancer patients, and in the event of pregnancy its use is not suspended, since pregnancy is not considered an obstruction to the application of appropriate therapeutic method. Its use during pregnancy harms the intrauterine development, being related to the occurrence of miscarriages and newborns with severe chromosomal abnormalities.

Nicotinamide is a B vitamin complex that when metabolized is converted into two coenzymes: dinucleotide adenine (NAD) and dinucleotide phosphate adenine (NADP), and among their actions we found post-translational activities of various proteins. Among them we found histones, that due to its high content of basic amino acids that serve as a skeleton, maintaining its original DNA structure. They also play a key role in oxidative and nonoxidative mechanisms of great importance for the correct maintenance and implementation of the metabolic processes of the cells and they also act to modulate the activity of sirtuin enzymes, also called silent regulatory information (SIR) that are deacetylases or ADP-ribosyl transferases, transferring ADP-ribose units to many proteins that will act in several metabolic and cellular signaling pathways that are involved in the transitional regulation, genome stability, neuronal protection and metabolic homeostasis [6]. Considering the actions of nicotinamide and its extensive empirical use [7-9] we evaluated its potential inducing effects of changes in animals in undergoing treatment with nicotinamide and its possible protective effects on toxic and teratogenic model induced by cyclophosphamide in mice during pregnancy.

2. Material and Method

All procedures described in this document are in accordance with the Ethical Principles of Animal Experimentation of the Federal Council of Veterinary Medicine and Law n. 9605 (regulated by Decree 3179, 21/12/1999)] and were approved by the Ethics Committee for Animal Experimentation of the State University of Londrina in the number 5265.2015.28.

2.1 Nicotinamide

Nicotinamide was used in the form of white crystalline powder. The experimental groups were dosed at a concentration of 100mg/kg and 200mg/kg [10, 11] diluted in sterile saline solution via gavage. The respective control groups received the equivalent volume of saline by the same way.

2.2 Cyclophosphamide

The drug used was Genuxal® (Cyclophosphamide Monohydrate), the presentation of tablets were 50mg. The experimental group received 50 mg/Kg of the drug [12], diluted in sterile saline via intraperitoneal injection. The control group received an equivalent volume of saline in the same way.

2.3 Animals

Swiss mice (Mus musculus), male and female were used, adult, weighing about 35g, from the Central Animal Laboratory of Biological Sciences Center, State University of Londrina. During the execution of this study, the animals were kept in controlled lighting system, light / dark cycle 12 hours, at 22 ± 2 ° C, with water and feed freely. At the end of the

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experiment all the animals were euthanized by cervical dislocation.

2.4 Mating and Diagnosis of Pregnancy

The animals were put to mate late afternoon in individual cages in a proportion of female mice to a male mouse. In the following days, with a gap of 12 hours, the vagina of females was examined to verify the occurrence of the "vaginal plug", which determines the day zero of pregnancy, at which females were identified and weighed and distributed randomly in experimental groups.

2.5 Experimental Lineation

Pregnant females were divided into six experimental groups with 15 animals in each group: Experimental group 1 (G1): nicotinamide 100mg/kg via gavage the 8th to 14th day of gestation and saline intraperitoneally (ip) on day 11; Experimental group 2 (G2): nicotinamide 200mg/kg via gavage at the 8th to the 14th day of gestation and saline i.p. on the 11th day; Experimental group 3 (G0): negative control: saline via gavage the 8th to 14th day of gestation and saline i.p. on the 11th day; Experimental group 4 (G4): salt via gavage the 8th to 14th day of gestation and cyclophosphamide 50 mg/kg i.p. on the 11th day. For the evaluation of protective nicotinamide effect on the morphological and physiological changes caused by chemotherapy were assembled experimental groups of association, divided into: Associate Treatment, group 5 (G5): nicotinamide 100mg/Kg via gavage the 8th to 14th day of pregnancy plus cyclophosphamide 50 mg/kg ip on the 11th day and Associate Treatment, group 6 (G6): nicotinamide 200mg/kg via gavage the 8th to 14th day of gestation and cyclophosphamide 50 mg/kg i.p. on the 11th day.

This treatment period was chosen based on the development stages of the rodent in accordance with Manson and Kang [13], comprising the period of organogenesis phase, which occurs an intense cell proliferation and organ formation. On the 18th day of pregnancy females were euthanized by cervical dislocation and then a laparotomy and a hysterectomy were conducted to evaluate the reproductive performance, intrauterine development of the offspring and the presence of congenital malformations. For evaluation of maternal toxicity, the animals were weighed every three days during gestation and after the laparotomy the organs heart, lungs, liver and kidneys were removed and weighed. For clinical evaluation the following analysis parameters were used: piloerection, eye reddening, vaginal blood loss, changes in motor coordination, diarrhea and death.

2.6 Intrauterine Development Evaluation and Embriofetustoxicity

The uterine contents of female was analyzed by checking the number of deployments of sites according to Salewski method [14], the presence of resorptions, number of live and dead fetuses, fetal and placental weight, systematic analysis to detect defects external.

The following parameters were analyzed: fetal viability rates (number of live fetuses / number of deployments X 100), rate of post-implantation losses (number of implantations - number of live fetuses / number of deployments X 100) and embryonic resorption rates (number of resorptions / number of deployments X 100). placental index and fetal weight ratio and age of pregnancy were calculated. The evaluation of malformations in the offspring was also made through the analysis of visceral and skeletal malformations of the external fetuses. About 588 fetuses were examined under a stereoscopic microscope to check for possible external structural defects. Of these, 290 fetuses were fixed in mixing Bodian for the analysis of visceral defects, while 298 fetuses were fixed in acetone, stained with potassium hydroxide and Alizarin to perform the analysis of skeletal malformations.

Visceral analysis was conducted as follows: the fetuses were examined by combining cuts/microdissection proposed by Barrow and Taylor [15] for studying the chest and abdomen and by strategic cuts proposed by Wilson [16] to study the head. This review was performed under microscopic magnifying glass.

The fetuses which were submitted to skeletal analysis were evaluated for skull anomaly

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detection, sternum, vertebrae, ribs, pelvis, collarbone, phalanges, metacarpal and metatarsal according to the Taylor method [17] under microscopic magnifying glass.

2.7 Statistical Analysis

The absolute data with normal distribution were analyzed by analysis of variance (ANOVA) followed by Tukey's test and with non-normal distribution were analyzed by students' test. For the analysis of maternal parameters we used the student's test. Data for external, visceral and skeletal malformations were analyzed by Fisher's exact test. For this analysis we used the statistical program Graphpadprism 5. The results with $p < 0.05$ were considered significant, the $p < 0.01$ were considered very significant and $p < 0.001$ were considered extremely significant.

3. Results

3.1 Maternal Toxicity Assessment

The administration of nicotinamide, both in a concentration of 100mg/kg and 200mg/kg associated with cyclophosphamide (G5 and G6 groups), as well as administration of cyclophosphamide in isolation (G4 group) caused a decrease in gain and final weight of the treated females compared to G1, G2 and G0 groups. Regarding the weight average maternal organs (heart, lung and liver) females treated with nicotinamide association and cyclophosphamide, as well as females only treated with cyclophosphamide showed a significant increase when compared to the group G0. None of the experimental groups showed clinical signs of maternal toxicity, as shown in table 1.

Table 1: The effects of treatment with nicotinamide and cyclophosphamide on maternal toxicity parameters

Reproductive Parameters	Group 1 (G1)	Group 2 (G2)	Group 3 (G0)	Group 4 (G4)	Group 5 (G5)	Group 6 (G6)
Maternal weight gain	20,66±3,36	27,65±1,44	26,10±1,03	20,12±1,88 ^{c**}	14,87±2,95 ^{c*}	21,92±2,65 ^{c**}
Weight of the pregnant uterus	15,14±1,95	9,41±1,75	13,83±1,59	14,77±2,02	12,06±2,31	16,34±1,95
Final Weight	65,37±3,87	69,72±1,58	70,21±1,83	60,05±1,98 ^{c***}	56,01±3,84 ^{c**}	61,14±3,05 ^{c***}
Heart weight	0,17±0,014	0,17±0,01	0,16±0,06	0,20±0,08 ^{c**}	0,21±0,013 ^{c**}	0,22±0,01 ^{c**}
Weight of lungs	0,23±0,01	0,23±0,14	0,19±0,01	0,25±0,13 ^{c*}	0,25±0,01 ^{c*}	0,25±0,01 ^{c*}
Liver weight	2,27±0,13	2,29±0,11	2,35±0,13	2,83±0,13 ^{c*}	2,96±0,12 ^{c*}	2,98±0,09 ^{c**}
Liver Weight	0,38±0,049	0,40±0,02	0,39±0,02	0,45±0,03	0,42±0,02	0,45±0,07

Data presented as mean ± SEM. Statistically different from Group 3; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Test: One Way ANOVA followed by Tukey's test.

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Table 2: The effects of treatment with nicotinamide and cyclophosphamide on intrauterine development of mice.

Reproductive parameters	Group 1 (G1)	Group 2 (G2)	Group 3 (G0)	Group 4 (G4)	Group 5 (G5)	Group 6 (G6)
[1]Implantation sites	10,70±2,055	10,00±1,75	13,30±1,20	14,80±0,78	15,40±0,84	11,40±1,34
[2]Fetal viability rate (%)	85.63 ± 13.28	90.23 ± 10.08	96.79 ± 2.13	48.13 ± 10.14[c**]	56.60 ± 11.14[c**]	50.13 ± 9.67[c**]
[2]Post-implantation loss rate (%)	6.10 ± 3.65	8.10 ± 2.77	8.00 ± 1.05	39.93 ± 10.95[c**]	43.23 ± 11.18[c**]	30.17 ± 8.08[c**]
[2]Resorption rate (%)	5.92 ± 9.7	5.43 ± 7.95	6.80± 1.32	42.58 ± 10.32[c**]	30.52 ± 11.31[c**]	19.20 ± 9.54[d**]
Fetal Development						
[1]Placental weight (g)	0,058±0,06	0,08±0,08	0,09±0,013	0,05±0,07[c**]	0,06±0,010[d*]	0,07±0,01[d*]
[1]Fetal weightl (g)	1,10±0,14	1,37±0,037	1,30±0,03	0,83±0,11[c**]	1,05±0,16[d*]	1,16±0,13[d*]
[1]Fetal length (cm)	2,21±0,25	2,63±0,03	2,63±0,04	2,07±0,23[c**]	1,66±0,33[c***]	2,15±0,23[c***]
[1]Placental índex	0,05±0,07	0,061±0,06	0,05±0,03	0,04±0,06	0,06±0,017	0,06±0,01
[1]Live fetuses	8,90±1,54	5,30±1,07	8,40±1,03	9,70±1,47	8,30±1,71	10,10±1,28
[1]Dead fetuses	0,00±0,00	0,00±0,00	0,00±0,00	0,10±0,10	0,10±0,10	0,00±0,00

Data presented as mean ± SEM.. [c]Statistically different from group 3; [d]Statistically different from group 4; * p<0,05; ** p<0,01; *** p<0,001. [1]Test: One Way ANOVA followed by Turkey's test.. [2]Test: T student's test

3.2 Evaluation of the reproductive performance and embryo-fetal development

The G6 group treated with cyclophosphamide and association with nicotinamide 200mg/kg showed a lower rate of resorption compared to the group that received cyclophosphamide (G4 group), indicating the protective effect of nicotinamide. The combination of nicotinamide and cyclophosphamide 100 and 200mg/kg (G5 and G6 groups) were not able to increase fetal viability rates and reduce post implantacional

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losses observed in the group that received only cyclophosphamide. Regarding fetal development, we observed that treatment with nicotinamide (100 to 200mg/kg) associated with cyclophosphamide was not able to promote protective effect on the reduction of fetal length caused by cyclophosphamide. The fetal weight and placental weight parameters showed statistically significant protective effect in groups with treatment associated with nicotinamide (G5 groups and G6) and cyclophosphamide compared to the G4 group that only received cyclophosphamide. Data shown in table 2.

The only external malformations were found to retroverses lower limbs presented in Table 3. The nicotinamide association in both doses 100mg/kg as a dose of 200mg/kg were not able to decrease and such defects were observed in the group exposed to cyclophosphamide.

Table 3: External alterations observed in the offspring of mice due to maternal treatment with nicotinamide and cyclophosphamide

	Group 1 (G1)	Group 2 (G2)	Group 3 (G0)	Group 4 (G4)	Group 5 (G5)	Group 6 (G6)
Analyzed parameters	%	%	%	%	%	%
Retroverses Lower Limbs	1,16	1,26	0,00	61,76^{c***}	47,57^{c**}	58,75^{c*}
Total fetuses analyses	86	79	169	68	84	102

Data presented as mean ± SEM. ^{c}Statistically different from 3; * p<0,05; ** p<0,01; *** p<0,001. Test: Fisher's Exact Test

Skeletal changes are shown in table 4. It was observed in the G4 group the presence of statistically significant malformation in the skull (incomplete ossification of the parietal, and supraoccipital exoccipital, cleft palate, incomplete ossification of the mandible and maxilla and abnormal sternebrae) compared to the G0 group. Treatment of G5 and G6 group were not able to promote protective effect.

Visceral changes shown in table 5, indicate statistically significant increase in microphthalmia in G4, G5 and G6 when compared to the group G0. Cleft palates, cerebral displacement, sided anaftalmia, difference in height and deployment of the renal pelvis, were observed but these data were not significant among any of the experimental groups.

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Table 4: Skeletal malformations observed in offspring of mice due to maternal treatment with nicotinamide and cyclophosphamide..

Analyzed parameters	Group 1 (G1) %	Group 2 (G2) %	Group 3 (G0) %	Group 4 (G4) %	Group 5 (G5) %	Group 6 (G6) %
Incomplete Parietal Ossification	5,55	12,5	2,89	76,00 ^{c***}	93,18 c***	74,00 c***
Incomplete Ossification Supra and Exocipital	3,70	6,25	13,04	76,00 c***	93,18 c***	74,00 c***
Cleft Palate	0,00	6,25	1,44	85,00 c***	45,45 c***	22,00 c***
Incomplete Ossification of Jaw/Mandible	3,70	3,12	13,04	64,00 c***	29,54 c*	18,00
Abnormal Sternebio	1,85	6,25	2,89	96,0 c***	65,90 c***	40,00 c***
Reduced Numbers of Proximal Phalanges (Front paws)	1,00	1,00	0,00	4,00	4,54	2,00
Reduced Numbers of Distal Phalanges (Front paws)	0,00	1,12	0,00	4,00	4,54	6,00
Reduced Number of Proximal Phalanges (Rear paws)	0,00	0,00	0,00	4,00	4,54	0,00
Reduced Numbers of Distal Phalanges (Rear paws)	0,00	0,00	0,00	8,00	4,54	2,00
Total analyzed fetuses	54	32	69	25	44	50

Data presented as mean ± SEM. [c]Statistically different from group 3; * p<0,05; ** p<0,01; *** p<0,001. Test: Exact Fisher's test.

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Table 5: Visceral malformations observed in offspring of mice due to maternal treatment with nicotinamide and cyclophosphamide.

Analyzed Parameters	Group 1 (G1) %	Group 2 (G2) %	Group 3 (G0) %	Group 4 (G4) %	Group 5 (G5) %	Group 6 (G6) %
Microcephaly	2,12	0,00	1,92	9,30[c***]	7,5[c***]	7,69[c***]
Cleft Palate	2,12	0,00	0,00	4,65	0,00	0,00
Brain Shift	4,25	0,00	1,92	0,00	0,00	0,00
Unilateral Anophthalmia	0,00	4,25	1,92	2,32	2,5	7,69
Difference in height and deployment of the renal pelvis	0,00	0,00	1,92	0,00	2,5	0,00
Total Analyzed Fetuses	32	47	52	43	40	52

Data presented as mean ± SEM. [c]Statistically different from group 3; * $p<0,05$; ** $p<0,01$; *** $p<0,001$. Test: Fisher's Exact Test

4. Discussion

Adverse effects are studied in the toxicology of development arising from teratogens exposure during intrauterine development. Al habori et al. [18] and Birkmayer et al. [19], recommend that the toxicity of a substance can be determined from the decrease in body weight of animals. In this study it was found that nicotinamide in doses of 100mg/kg and 200mg/kg caused no significant decrease in body weight gain or the final weight of pregnant females. Also, there was no decrease in the weight of vital organs and no clinical signs of maternal toxicity. Thus we find that the nicotinamide in two tested concentration was non-toxic to animals.

According to the experimental model induced by exposure to cyclophosphamide in this study, it was found that maternal weight gain and weight gravid end in a significant decrease when compared to the control group, and in weight of the heart, lungs and liver, we observed significant increase in the weight of these organs when compared to the control group. Studies with exposure to cyclophosphamide shows impairment in maternal weight gain [20] and induces toxicity of vital organs, particularly cardiotoxicity [21], induction of pulmonary fibrosis [22] and hepatotoxicity caused by 4 - hydroxycyclophosphamide and the acrolein, cyclophosphamide metabolites [23,24]. These same results were observed in this study from the increased weight of these organs of the females

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of the G4 group. The combination of nicotinamide in two tested concentration were not able to minimize toxicity induced by cyclophosphamide.

It has been shown by Oliveira et al. [25] that cyclophosphamide when administrated in pregnant females caused significant decrease in reproductive parameters, according to the data that corroborate the experimental model induced with cyclophosphamide in this study. During the early stage of the mice, between the 6th and the 8th day of pregnancy, the trophoblast cells invade the endometrial tissue in order to establish a maternal-fetal contact that provides one exchange surface [26]. At this stage the process of differentiation of trophoblast cells that will form the placenta is not yet finalized [27]. Thus, the presence of cyclophosphamide in maternal circulation during this period associated with immaturity of the placenta, contributed to greater drug access to primary embryonic tissue, allowing the occurrence of lethal events to the newly implanted embryo. Only the group nicotinamide 200mg/kg when administrated concurrently with cyclophosphamide, G6 experimental group, showed protective effect on resorption rates compared to the G4 group. The imbalance between the production of oxidants and antioxidants agents has been touted as one of the causes of preeclampsia, intrauterine restrictions on development and the occurrence of spontaneous abortions [28]. The supplementation with antioxidants improves reproductive performance on mice with advanced age [29]. According OH, et al. [30], the antioxidant properties of nicotinamide are related to the prevention of nephrotoxic effects induced by cisplatin chemotherapy. Considering the previous findings, we can suggest that the decrease in abortion rates may have been promoted by antioxidants reactions produced by nicotinamide in cellular energy metabolism and DNA repair damaged by cyclophosphamide.

The treatment with cyclophosphamide caused a decrease of the weight and length fetal and placental weight. G5 and G6 treatment were able to promote protective effects parameters in fetal weight and placental weight. The presence of nicotinamide in maternal circulation during this time was able to prevent the occurrence of lethal events to the newly implanted blastocyst and damage to the placenta formation. Our studies are in agreement with findings of Lapas & Permezel [31], which demonstrated the anti-inflammatory action and nicotinamide antioxidant on the placenta affected by damage caused by exposure of cyclophosphamide. It is known that the placenta ability to transfer nutrients and oxygen is proportional to its size. This explains the correlation between placental weight and fetal weight [32].

The literature provides some studies to enhance the therapeutic effect of nicotinamide, such as the study of O'Brien et al. [33] It shows that the protective effect of nicotinamide on the beta cell apoptosis number in experimental models of diabetes induced by cyclophosphamide. Another study is to JONH et al. [34] having antioxidant effects of nicotinamide on gestational diabetes. In addition, the effectiveness of nicotinamide has also been demonstrated by Ieraci & Herrera [35] on prevention of fetal alcohol syndrome.

In this study the only detected external malformation was the retroverses lower limbs, observed in the G4 group. This fact was also observed by Leyder et al. [36], who reported the case of a newborn who was exposed to cyclophosphamide during the first trimester of pregnancy and had defects in the hands and feet. Leyder et al. [36] reported the case of a newborn who was exposed to the chemotherapy regimen of cyclophosphamide and was found micrognatia and bilateral malformations of the hands and feet. Thus, external malformations found in this study are in agreement with the induced model.

According to Moore and Persaud [37] during the first days of development, teratogenic agents usually cause embryotoxicity leading to death of the embryo. During the period of organogenesis, these agents disrupt development and can cause major congenital anomalies and during the fetal period can produce morphological and functional abnormalities. Thus, the most severe malformations tend to be aborted in early development.

In the skeletal malformations, the occurrence in the group treated with cyclophosphamide was

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significant compared with the control group. These data are in agreement with the bone changes reported by LEITE et al. [38] in their studies using cyclophosphamide. Nicotinamide in the two doses tested was not able to reduce these defects.

Paladini et. Al [39] reported that cyclophosphamide was involved in the emergence of abnormalities in phalanx formation in embryos exposed to cyclophosphamide in the first trimester of pregnancy. This study showed the presence of reduced numbers of distal and proximal phalanges of both front and back paws of the fetus of G4, G5 and G6. But these changes were not statistically significant when compared to the control group.

The analysis showed visceral malformations: microphthalmia, cleft palate, brain shift, unilateral anophthalmia, difference in height and deployment of the renal pelvis, but only microphthalmia was significant in the G4 group compared to the group G0. Anomalies in the formation of the eyeball have been described in studies where there was exposure to cyclophosphamide during the first trimester of pregnancy [36]. We found that nicotinamide association with both the dose of 100mg/kg as in 200mg/kg were not able to produce a protective effect.

5. Conclusion

Nicotinamide inhibited DNA damage induced by cyclophosphamide in a dose-dependent manner exerting protective effect, reducing the number of abortions and reducing the morbidity and mortality associated with intrauterine growth retardation. The results of this study are promising. Nicotinamide is safe to use, clinically established and has proven protective effect. These results can be used as a basis for future research considering the use of higher concentrations of nicotinamide.

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