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**EMBRYONIC MALFORMATIONS. BEHAVIOR AND
TREATMENT RELATED TO PRECLINICAL AND
CLINICAL STUDIES**

ABSTRACT

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CONTENT

LIST OF PUBLISHED SCIENTIFIC ARTICLES	Error! Bookmark not defined.
LIST OF ABBREVIATIONS AND SYMBOLS	Error! Bookmark not defined.
LIST OF FIGURES AND TABLES	Error! Bookmark not defined.
DEDICATION.....	Error! Bookmark not defined.
ACKNOWLEDGEMENT	Error! Bookmark not defined.
INTRODUCTION	Error! Bookmark not defined.
GENERAL PART.....	4
CHAPTER 1. EMBRYOLOGY	4
1.1. Introduction.....	4
1.2. Epidemiology of fetal malformations at birth.....	5
1.2.1. Practical challenges of the epidemiology of fetal malformations at birth	5
1.3. Prenatal diagnosis of malformations.....	6
1.4. Prenatal diagnosis of posterior fossa abnormalities.....	13
1.5. Types of cerebellar embryonic damage	14
CHAPTER 2. EXPERIMENTAL MODELS USED IN THE STUDY OF EMBRYONIC MALFORMATIONS.....	19
2.1. Introduction.....	19
2.2. Embryos of different species for the study of normal embryology	20
2.3. Biological concerns regarding the selection of animal models for teratogenic testing	22
2.3.1. Ethical and biological issues related to animal models	23
2.3.2. Specific sensitivities for teratogenic tests	25
CHAPTER 3. FACTORS WITH IMPACT ON PREGNANCY AND/OR ITS DEVELOPMENT	27
3.1. Folates	27
3.2. Ultraviolet radiation and hormones	29
SPECIAL PART.....	Error! Bookmark not defined.
CHAPTER 4. CONTRIBUTIONS RELATED TO THE SPECIFICITY OF PUBLICATIONS ASSOCIATED WITH EMBRYONIC MALFORMATIONS	31
4.1. Introductory notions.....	31
4.2. Materials and methods	34
4.2.1. Selection of databases and application criteria	34
4.3. Results and discussions.....	35
4.3.1. Neural tube defects	35
4.3.1.1. Neural tube defects, folate and primary prevention.....	43
4.3.2. Facial abnormalities	44
4.3.3. Extremity abnormalities.....	45
4.4. Conclusions.....	48
CHAPTER 5. CONTRIBUTIONS RELATED TO THE APPROACH OF EMBRYONIC MALFORMATIONS IN VITRO AND IN VIVO THROUGH FOLATES	49
5.1. Introduction.....	49
5.1.1. Transport from folate to fetus	52
5.1.2. Chemical structures of folates.....	52
5.2. Materials and methods	53
5.2.1. Reagents.....	53

5.2.2. In vitro testing	53
5.2.3. In ovo testing – CAM assay.....	54
5.2.4. Statistical data	55
5.3. Results and discussions.....	55
5.3.1. Chemical perspectives	55
5.3.2. Cellular viability	57
5.3.3. Angiogenic potential.....	61
5.4. Conclusions.....	66
CHAPTER 6. IN VIVO STUDY RELATED TO THE ASSOCIATION OF SYNTHETIC HORMONES WITH RADIATION	66
6.1. Introduction.....	66
6.2. Materials and methods	68
6.2.1. Experimental animal model	68
6.2.2. Measurement of physiological, serological and hematological parametrs	70
6.2.3. Histopathological evaluation	69
6.2.4. Statistical analysis.....	70
6.3. Results and discussions.....	70
6.3.1. Changes observed at the level of physiological parameters	70
6.3.2. Changes observed at the level of serological and hematological parameters	72
6.3.3. Changes observed at organ level	73
6.4. Conclusions.....	81
CHAPTER 7. CONTRIBUTIONS TO THE STATISTICAL EVALUATION OF THE INCIDENCE OF THE DEVELOPMENT RISK OF DOWN SYNDROME IN THE SOUTHWESTERN PART OF ROMANIA	82
7.1. Introduction.....	82
7.2. Materials and methods	84
7.3. Results and discussions.....	85
7.4. Conclusions.....	93
CONCLUSIONS AND PERSONAL CONTRIBUTIONS	94
REFERENCES	98

ABSTRACT

Embryonic malformations are multiple, and the factors that contribute to their appearance are variable, their diagnosis and treatment being a challenge for specialists in the field. The biggest problem in the research of embryonic malformations is the lack of study material. Despite what has been described, there is still no general agreement on when, why and how the abnormal development takes place. Nowadays, the embryological assessment of many congenital anomalies in the human species is still a matter of speculation, especially due to gaps in poor knowledge of all factors involved, despite the extraordinary development of technology. As a result, various typical malformations are not yet satisfactorily explained, and medical staff in all specialties are still confused enough when faced with the background of normal and abnormal embryological development. The diagnosis of an embryonic malformation has a major impact both on the mother, the family and on the health system, which must effectively manage the delicate situation from an economic, social and medical point of view. Therefore, for a clear and rapid diagnosis and appropriate treatment it is necessary to study all aspects related primarily to the triggers, the mechanisms involved and the selected treatments.

This paper deals with a topic of great interest, both at local, regional, national and international level, namely that of embryonic malformations. Research studies on this topic are numerous and also very diverse. The material is structured according to the drafting rules in three main parts: (1) the general part, (2) the special part and (3) the own conclusions and contributions. In general part, are presented the latest aspects related to: (a) embryology - epidemiology of birth defects, prenatal diagnosis of malformations, (b) development of the cerebellum - prenatal diagnosis of posterior fossa anomalies, types of embryonic cerebellar damage, (c)) animal models used for applied embryology - embryos of different species for the study of normal embryology, surgical models, chemical models, genetic models and biological concerns regarding the selection of animal models for teratogenic testing and (d) the influence of synthetic hormones on the possible appearance and development of embryonic malformations. The special part is structured in four main chapters, one dedicated to each original research direction addressed: (a) contributions related to the specificity of publications associated with embryonic malformations in specific databases, (b) contributions related to the approach of embryonic malformations *in vitro* and *in vivo* through folate, (c) *in vivo* study related to the association of synthetic

hormones with ultraviolet radiation and (d) contributions to the statistical assessment of the incidence of the risk of developing Down syndrome in southwestern Romania.

The subject is a topical one that is in line with international and national concerns because it addresses an issue that affects millions of women around the world, from different social backgrounds and requires the approach and adaptation of diagnosis and treatment according to origin, severity and policies applied to the level of each society. Fetal malformations are the leading cause of antepartum, intrapartum or postpartum death worldwide. Their epidemiological investigation was determined by the recognition of congenital rubella syndrome and thalidomide-related focomelia. Specialists in the field require relevant data on fetal malformations at birth, as a basis for reporting their own results.

The specific objectives of the paper were represented by: (1) rigorous documentation through various scientific databases - Pubmed, Science Direct, De Gruyter, Wiley Online Library and Cochrane Library in order to identify specific current information; (2) testing of certain compounds frequently used in the treatment of embryonic abnormalities (folate) but also of those targeting the same receptors and may exert a double effect with adverse effects (anti-folate) by *in vitro* and *in vivo* methods, (3) evaluation *in vivo* by specific tests for quantification of physiological, serological parameters and histopathological analysis of the influence of synthetic hormones in the presence of radiation and (4) screening to diagnose trisomy 21 by correlating sonographic and serum markers with maternal age, over a period of four years in pregnant women from the South-West area of Romania.

The research methods adopted in this paper are currently used in various international research studies. The study in the scientific databases involved the selection of specific key terms for an advanced selection of publications in order to find the most relevant information. The *in vitro* study was performed for human keratinocytes and fibroblasts by the Alamar Blue colorimetric method (efficient, fast and economical) which involves evaluating the mitochondrial activity of cells and quantifying the number of viable cells in the presence of folic, dihydrofolic and tetrahydrofolic acids. The effects exerted on the angiogenesis process were achieved by the CAM test, a simple and easy to apply *in vivo* technique that involves the use of fertilized chicken eggs and is performed in several stages analyzing the particular reactions induced by the compounds: hemorrhage, vascular lysis, coagulation establishing an irritation score. The importance of folate

supplementation during pregnancy, especially for the prevention of neural tube defects in the fetus, is recognized, but there are a number of controversies related to other possible benefits of this supplementation and the different molecular forms of folate that should be administered. Folate supplementation is a common medical practice, but specialized personnel do not permanently consider the pharmacodynamic mechanisms underlying the health benefits of folate. Adequate development and growth of the fetus and the presence of disease are related to birth weight, duration of pregnancy and geographical location. A significant relationship was found between premature birth, low birth weight and poor immune system, the presence of infections, vitamin D deficiency and hormonal imbalances associated with the pre- and post-conception periods. Depending on the weather conditions, especially associated with different seasons, psychological, biological and behavioral effects can be correlated, including ovulation and pregnancy. Ultraviolet radiation plays a major role, and the amount of ultraviolet solar radiation is variable and different depending on the seasons. The *in vivo* study related to the evaluation of the action of synthetic hormones was performed on an animal model. Physiological parameters of the skin were analyzed by non-invasive methods, serological parameters by usual biochemical tests and histopathological evaluation of organs by usual methods of staining with hematoxylin / eosin and microscopic analysis. Screening for the risk of developing trisomy 21 (Down syndrome) was performed over a four-year period and nuchal translucency (NT, sonographic marker) was measured, with plasma values of pregnancy-associated plasma A (PAPP-A) and free human chorionic gonadotropin beta (β HCG) correlated with various factors such as maternal age, smoker / non-smoker status, type of pregnancy and whether or not there is diabetes.

As mentioned earlier, for the practical study the lack of material is a major problem. Currently, research is being conducted in different directions, both *in vitro*, *in silico* and *in vivo*. Any contribution made from these directions is an important one because it contributes to the achievement of a unitary whole that ultimately aims at a thorough understanding, prevention and correct approach to these types of diseases.

Following the study of specific databases to analyze the literature on the specificity of publications associated with embryonic malformations, it was concluded that the databases provide a number of important information related to the subject of embryonic malformations. The importance of using the specific key field / terms is notorious because

only through an advanced correct use can the latest information in the field be accessed. Regarding embryonic malformations, those associated with Down syndrome are the most common. Despite this, the progress in recent years is not resounding, and public awareness of the importance of proper dispensation of pregnancy, along with mass testing are the best ways to address this medical problem. The Pubmed, Science Direct and Wiley Online Library databases contain a significant number of publications, especially dedicated to a specific key term, while the Cochrane database provides much less information on a specific key term but sums up information from several articles. and clinical databases.

The group of folates, also called vitamin B9, belong to the family of water-soluble B vitamins and are composed of chemical structures derived from polyglutamates. Folates are essential micronutrients for cellular function, growth and development. During pregnancy, folate demands increase 5 to 10 times, due to their importance in processes such as vasculogenesis and angiogenesis, both key processes in placental function, fetal growth and development. Evidence accumulated over the years indicates that the risks of defective development are significantly reduced by adequate folate supplementation during both preconception and during the first trimester of pregnancy. In the present paper, the viability of healthy cells, keratinocytes and human fibroblasts was assessed in the presence of three folates (folic - AF, dihydrofolic - DHF and tetrahydrofolic acids - THF), an anti-folate (methotrexate - MTH) and combinations thereof, by the Alamar blue test. The antiangiogenic potential was also evaluated by the *in ovo* technique, the CAM test. The data revealed that MTH induced a slight decrease in keratinocytes at the highest concentrations tested, the activity was dose-dependent and none of the compounds exerted significant toxicity. Even in the case of the combination of two types of compounds, folate with anti-folate, there was no significant decrease in the number of viable cells. Human fibroblasts in the presence of folates did not show significant changes in viability, dihydrofolic acid had a more pronounced influence on viability compared to the other two folates (folic and tetrahydrofolic acids) tested. When testing the combinations between methotrexate and folic, dihydrofolic and tetrahydrofolic acids, respectively, the viability percentages varied as follows: the combination AF: MTH (1: 2) reduced the viability of fibroblasts to approximately 89%, THF: MTH (1: 2) up to about 79% and the most pronounced effect was observed in the combination of DHF: MTH (1: 2) where a decrease of up to 68% was recorded.

Reactions involving folate are of major importance, especially if we consider that inhibition of transporters leads to devastating effects, such as malformations in the fetus. Despite numerous studies in recent years that have involved the administration of folate to reduce defects in the fetus and beyond, there are more and more studies that associate folate with the progression of tumor processes. Thus, it is necessary to highlight the reactions involved and to elucidate certain effects in the context of the combination of several factors that are either beneficial for the correction of certain defects or devastating for cellular replication. The studies performed require a correlation with experiments involving oxidative stress for a better understanding of the importance of the administration of compounds for prevention, but also of compounds for healing. Most often, in order to prevent embryonic malformations, it is recommended to administer folate for the future mother or for the pregnant woman at the beginning of the first trimester of pregnancy. In order to establish the optimal recommendations regarding the administration of folate, the possible negative effects produced must be taken into account, namely: masking vitamin B12 deficiency and a possible poor metabolism with increasing unmetabolized compound that can lead to aberration and deficiency grave. Both folates and antifolates can produce significant changes at the cell level that ultimately have detrimental consequences on either the fetus or the mother. *In vitro* studies provide evidence of the adverse effects of folate deficiency on placental function. In order to elucidate the mechanism exerted by the mentioned compounds and their combination, the angiogenic effect was analyzed by applying the CAM test and the obtained data indicated that folates were best tolerated, not inducing changes on the normal angiogenic process, while methotrexate showed a vessel density, slightly higher, but without inducing toxicity on vascular architecture and functionality. The process of placental angiogenesis is crucial in maintaining adequate blood flow to the development of the fetus, its modification leading to devastating effects for both mother and fetus. Folates together with fatty acids modulate specific receptors during placental angiogenesis both directly (by angiogenic factors) and indirectly (by specific modulation).

Ethinylestradiol (EES), a chemical estrogen, is the most commonly used active substance in oral hormonal formulations of contraceptives worldwide. Levonorgestrel (LNG), a synthetic progestagenic hormone, is commonly used in intrauterine systems that release LNG for its strong contraceptive effect.

The study conducted in this paper is based on the theory that many women voluntarily expose themselves to potentially harmful amounts of UVB light simultaneously with the use of hormonal contraceptives. At the same time, some intentionally use estrogen-based products, especially since most of those with topical application are in the category of over-the-counter drugs and are easy to procure. In the current experimental research, we evaluated the *in vivo* effects of: (i) EES, (ii) LNG and (iii) the association between EES and LNG on mice exposed for 21 days to UVB radiation, following the physiological parameters of the skin. evaluated by non-invasive methods, clinical and hematological parameters and histopathological evaluation. In this study, no deaths were recorded in three weeks of monitoring time in all four groups of experimental animals used. Significant changes or differences were observed in the overall appearance and weight percentage of the mice. Serum parameters of mice from all groups were analyzed to monitor liver and kidney health, and statistically significant values were recorded in the groups involving hormone administration and UVB exposure.

In the group of animals subjected to UVB radiation, the following signs were identified: in the kidneys, the capillary of the renal corpuscles showed mild hyperemia and basophilic bodies in the light of some renal tubules; in the lungs, there was moderate vascular hyperemia and few inflammatory cells were made up of small lymphocytes and a slight thickening of the alveolar septa; the spleen showed moderate red pulp hyperplasia, hemosiderin deposits in the connective tissue of the stroma, and hyperemia of the subcapsular vessels; the liver showed mild inflammatory infiltrate at the portal spaces, composed of small lymphocytes and hyperemia of the centrilobular vein and venules of the port space and the collected heart specimens showed quasi-normal histology with traces of interstitial edema. In the study group in which mice were exposed to UVB radiation and treated with EES, the samples of kidneys collected showed several changes: hyperemia of renal and peritubular capillary corpuscles, vacuolation of the apical portion of nephrocytes and basophilic bodies in the tubular system of the nephron; the lungs showed thickening of the interalveolar septa, hyperemia of the small vessels of the interalveolar septa, increased number of peribronchial mast cells and compensatory emphysema; spleen mild hyperplasia of red pulp and hemosiderin deposits, while the liver showed mild hyperemia of the venules in the centrilobular and portal spaces and also dilated sinusoidal capillaries and intracytoplasmic chromophobic vacuoles; heart samples showed interstitial edema. In mice exposed to UVB radiation and treated with LNG, the kidneys showed hyperemia of the

renal capillaries and peritubular capillaries; lungs a slight thickening of the interalveolar septa, hyperemia of the interalveolar septal vessels and mild inflammatory infiltrate were formed by lymphocytes; a slight hyperemia of the subcapsular vessels was also observed; the liver showed mild hyperemia of the centrilobular vein and vessels in the portal spaces and the heart samples showed no changes in normal histology. In the group in which the animals were exposed to UVB and treated with EES and LNG, most organ changes were observed. Thus, at the level of the kidneys were observed: small, atrophic renal corpuscle, with mild mesangial hyperplasia, hyperemia of the capillaries of the renal corpuscles, hyaline cylinders in some nephronic tubules and acidophilic infiltrations in arterial vessels. The lungs showed a high thickening of the interalveolar septa, with moderate hyalinization of the arteriolar walls, mild hyperemia of the small vessels of the interalveolar septum. This study is a first step in the analysis of skin physiological parameters correlated with changes in biological parameters and events that occur at the organ level with exposure to UVB radiation and the administration of active substances in the class of hormonal contraceptives. It has been shown that the association between the two hormones, EES and LNG, in the presence of UVB radiation, affects both skin parameters and biochemical parameters and has led to specific changes in organs. Estrogen being a promoter can stimulate cell proliferation with negative effects on the cell cycle with consequences of erroneous replication. Future studies are needed to investigate the mechanisms involved in the presence of UVA radiation, but also a variation in age, with a focus on specific biomarkers that correlate with the possible occurrence of malignant processes.

The disorder caused by chromosome 21 trisomy is associated with a number of physical abnormalities and intellectual disabilities. Over 40% of children diagnosed with Down syndrome have a major heart abnormality, while other major birth defects without heart damage are also common. Therefore, another major objective of this paper was to study the data recorded for a period of four years, for pregnant women in southwestern Romania in rural areas. Combinations of maternal age, measurement of fetal NT thickness, and assessment of biochemical markers were performed to assess the incidence of risk associated with trisomy 21, while emphasizing the need to implement databases to highlight cases of trisomy 21. Percentage variations related to Down syndrome incidence from country to country and from area to area are significant. In rural areas in Romania, access to medical services is more limited, and the application of screening programs is a solution to contribute to a realistic assessment of the incidence of fetal abnormalities

associated with Down syndrome. To obtain improved diagnostic performance, a combination of tests was used, including maternal age and evaluation of specific serum markers or combinations of maternal age, specific serum markers and sonographic measurements. Individual ultrasound markers or combinations thereof with one or more serum marker tests were examined in the first trimester, with or without maternal age adjustment. In the present study, 269 patients were subjected to investigations (by ultrasound and quantification of serum markers - double test), between January 2015 and December 2018.

Patients in the age group 21-29 years represented the most significant percentage (56.1%), and the lowest percentage was represented by patients over 40 years (0.7%). In the four years, correlated with age, eight minors also appeared in investigations: two 17 years old in 2015, three 17 years old in 2016, two 16 years old in 2017 and a young woman of 16 years old in 2018. Regarding the status of smokers or not, of the 269 patients included in this study for the years 2015-2018, 31 of them declared themselves smokers, while the remaining 238 stated that they are not smokers. None of the patients were reported with diabetes. Of the 269 cases studied, a percentage of 5.6% was included in the risk group ($\geq 1:250$) and a percentage of 1.5% was at the limit (in the risk range $1:251-1:300$). NT was initially implemented to assess the likelihood of a fetus with Down syndrome. Maternal age can be combined with fetal NT size and maternal serum biochemistry (free β HCG and PAPP-A) at $11 + 0$ to $13 + 6$ weeks to identify approximately 90% of affected fetuses. In the present study, no NT values ≥ 3 were recorded. A single value of 2.9 was recorded in 2015, the patient was included in a risk group after association with serum markers, age and other factors were taken into account. PAPP-A values were determined by well-known fluorescence immunochemical methods. There were only 20 MoM values ≤ 0.5 for PAPP-A that could theoretically signal some associated risks (intrauterine growth restriction, premature birth, preeclampsia, miscarriage or fetal death at ≥ 24 weeks). β HCG values were also determined by well-known fluorescence immunochemical methods. There were values of 23% ≥ 1.5 and of the 269 patients included in the risk group 60% had ≥ 1.5 values of Mo β associated with β -hCG. Screening based on maternal age, fetal NT, and the two additional serum markers resulted in a detection rate $\geq 85\%$ and a false positive rate of 3%.

The present research requires continuity in at least two directions: (a) the development of a database to contribute to a national registry that centralizes current information related to

the incidence and diagnosis of trisomy 21, not only in the southwestern part of the country. and (b) the evaluation of the mechanisms exerted by compounds of the folate class frequently used by pregnant women but also of anti-folate also frequently used in the presence of certain pathologies, taking into account the double effect they may exert - either beneficial or negative both in the presence and in the absence of various other factors such as ultraviolet radiation.