THE PROTECTIVE EFFECT OF GRANULOCYTE COLONY STIMULATING FACTOR (GCSF) ON PHENOBARBITAL TOXICITY IN MOUSE FETUS

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ABSTRACT

Introduction*: GCSF is a drug That is prescribed for growing the stimulation either in some of immune system diseases or anti-concern drug users. In this study, the researchers reflected on the GCSF's effects on different organs in pregnant mice. Then, using safe doses for mother and fetus, it was applied either as a growing stimulator or a neutralizer factor for negative effects of such teratogenicity drugs as Phenobarbital. Using Phenobarbital during pregnancy can increase the possibility of abnormalities in infants like cleft palate, heart abnormalities and weight reduction in birthday. The purpose of this research is Studying the possible protective effect of GCSF against teratogenic effects of Phenobarbital in mice fetus.*

Method*: 120 mg/kg of Phenobarbital within GCSF in different doses was injected to the mice on 9th day of gestation. Their fetuses were then brought out of uterus on 15th day of gestation and were examined by microscopic and macroscopic methods.*

Findings*: Although it was observed that 1.63 µg/mL of GCSF amended the cleft palate in mice fetus (P<0.05), other doses of GCSF (3.25, 7.75, 12, 15µg/mL) yielded no difference in the cleft palate occurrence or its amendment in mice fetus.*

Results*: The results of the present study showed that GCSF may have the teratogenicity effects of Phenobarbital in cleft palate and will prevent its occurrence.*

Keywords*: GCSF, Phenobarbital, prevention, mice fetuswith language disorders.*

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Introduction

Teratogen compositions can cause deficiency in fetus and their effect is dependent on the ability to cross placenta[1, 2]. Most abnormalities occur from the 1st to 8th week of gestation[3]. Their most dangerous risks are cleft palate which may occur in blastocyst stage (6th day), during Gastrulation (14th day), early stage of organ germinate (5th week) and palate formation (7th week)[4].

The Phenobarbital drug belongs to barbiturates family by a components formula like 5-ethyl-5phenyl-barbituric acid. This drug family is often used in some epilepsy treatments.

Furthermore, Phenobarbital drugs are applied in the treatment of insomnia and stress[5].

The teratogenic effect of Phenobarbital consumption is remarkable in epileptic women. Researchers Have maintained that most of fetal abnormalities include hypoplasia or aplasia in last phalanges and nails, face palate, cleft palate and heart abnormalities[6-8].

GCSF (Granulocyte colony stimulating factor), commercially named Filgrastim, is a glycoprotein 19.8 KD made from 173 amino acids[9]. GCSF gene in human beings is encoded in chromosome 17 and total stimulating factors of colony (CSFs) - include GCSF which is known as growth factor of

blood circulation (Hematopoietic receptor). It has occasionally stimulated the stem cells of bone marrow to divide[10, 11] the monocytes and macrophages which are the basic GCSF producers[12]. GCSF production is mostly modulated by cytokines. The tumor necrosis factor (TNF) is one of the cytokines that modulates GCSF production[13]. In addition GCSF, accompanied with GM-CSF, increases the monocytes production and distinction levels (14). Tissue damage will increase the number of macrophages and expression in TNFα and GCSF. GCSF not only causes neutrophil reproduction and growth from myeloid progenitor stem cells, but it also results in the stimulation of endothelial neural progenitor cell and tissue of placenta[15].

Over many years, studies have shown that manipulation of immune system may cause positive effects on fertility[16]. According to Wegmann theory, the basic correlation between immune system and reproductive system is sharing cytokines (GM-CSF-GCSF) which has potential relation in placental growth, differentiation and embryonic defending[17, 18]. Not only does it affect the immune parameters, but has also effects on embryonic response to teratogens[19].

After studying the teratogen dosage of phenobarbital and its creative level of teratogenicity, the effects of GCSF in different organs of pregnant mice were Studied by reaching a safe dosage for mother and fetus. Finally, its usage as a growth stimulator and eliminator of harmful effect of teratogen drugs like Phenobarbital was studied.

Experimental

Devices and tools

The devices and tools used in the present study include: Binocular optical microscope, Research Microscope (Model M3), manual rotary microtome, one-scale balance with accuracy of 0.1 g, analytical balance with accuracy of 0.0001 g, water distillation apparatus, loop binoculars, timer, surgical sets (forceps, scalpel, scissors, dissection tray, etc.), laboratory glassware (flask, graduated cylinders, desiccators, etc.), molding wax for molding glass, tissue warming plate, oven, metal cages for keeping the animals, computer and printer, slide and coverslip, filter paper, digital camera, latex gloves, cotton hydrophilic.

Compounds

Phenobarbital (Germany), GCSF (Filgrastim, Switzerland), medical alcohol (96 and100 degrees), gelatin , paraffin with a melting point of 56-60 °C, antler glue, xylene, picric acid, Bouin's fixative solution, Haupt's gelatin, glacial acetic acid, formalin, Haematoxylin, eosin powder, toluene and ether were all purchased from Sigma–Adrich Co. (Taufkrichen, Germany).

Animal

Adult mice in NMRI race (10-12 weeks) weighing 20g were purchased from Razi Institute (Tehran, Iran). Firstly, several healthy mice were selected, housed and fed with ready pallets in metal cages with floor woody chips. The necessary water was supplied by special bottles. In order to prevent pollution, the woody chips were changed at least once a week and the water bottle replaced 4 times in this period. The cages were disinfected by Formalin 6%, ethanol or liquid bleach every few days. The temperature and moisture of the room were adjusted regularly. All mice were housed in a room at temperature between 20ºC to 25ºC and relative humidity (40-50%) under the controlled light (12h light-12h dark). All experiments followed the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran. The ethical standards were based on "European Convention for the Protection of Vertebrate Animals Used for Test and other Scientific Purposes" Acts of 1986, and the "Guiding Principles in the Use of Animals in Toxicology," adopted by the Society of Toxicology in 1989 for the acceptable use of criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86 – 23, revised 1985).

The mice were randomly mated and their vaginal plugs were assessed after mating in order to ensure gestation. The pregnant mice were selected by observing the vaginal smear and plug.

In this Study, several adult female mice were selected and divided into four groups in order to consider either the teratogen effects of phenobarbital or protective effects of granulocyte colony growing factor (GCSF) on the toxicity of phenobarbital in their fetus. The following groups were accordingly examined:

1) *Experimental group 1*: this group contained four categories of mice each consisting of 4 adult female mice (10-12 week old) which had not been

gestated. The uterus of each mouse consisted of 10-12 fetuses. The mice's weights were measured by a digital balance in gram (gr). 120 mg/kg of phenobarbital was intraperitoneally injected (IP) to the mice in 9th day of gestation based on their body weights. The results were then observed in 15th day of gestation.

2) *Experimental group 2:* this group contained six categories of mice each consisting of 6 adult female mice (10-12 week old). Pregnant mice fetuses were then subcutaneously injected (S.C) with GCSF (0.8, 1.63, 3.25, 7.75, 12 and 15μg/ml).

3) *Experimental group 3*: this group contained four categories of mice each consisting of 4 adult female mice (10-12 week old) which had not been gestated. Phenobarbital (120 mg/kg) and GCSF (0.8, 1.63, 3.25, 7.75, 12 and 15μg/ml) were respectively used via intraperitoneal and subcutaneous (S.C) injection.

4) *Control group 4:* mice in this group have been in natural conditions and were not pregnant and used as baseline, compared to other experimental groups (conditions similar to other mice in other groups.)

In 15th day of gestation, pregnant mice were anesthetized and their fetuses and placentas were brought out from uterus and ovary following laparotomy. Finally, their weights, crown- rump length (C-R) and diameters were measured after being washed by physiologic serum. The statistical analysis was conducted using SPSS program, ANOVA test and complementary Tukey (Post Hoc) test. ($P < 0.001$)

The surgery and tissue preparation

In the first stage, the mice were anesthetized by ether, fixed in supine mode and dissected. Their abdomen skin in front of vagina was cut and the uteruses which contained fetuses and placentas were vacated. The tissue sections were prepared in series and in the following, different stages of tissue preparation were summarized.

a) Tissue fixation

The tissues were fixed as quickly as possible in order to prepare the suitable sample and avoid special changes in its shape. In addition, to avoid water loss, shrinkage and evaporation in samples, due to the air exposure, they were put in liquid. The tissues and fetus were brought out and rinsed with physiologic serum and put in bouin's fixative solution for 18 hours.

b) Dehydration, Clearing, Infiltration

The water from tissues inserted to bouin's fixative solution should be removed. This process is known as dehydration in which ethyl alcohol is used. After dehydration, the toluene was applied for clearing. In the next stage, the tissues were put in paraffin bath 1&2[24] in an oven (infiltration).

c) Embedding

The tissues, completely saturated in paraffin bath 1&2, were taken out from the paraffin bath 2 and put inside glass molds containing melt paraffin.

d) Sectioning

The paraffin molds (containing samples) were then regulated (in trapezoid forms). This process is called trimming. The molds were installed on microtome set. Finally, paraffinic strips containing tissues were obtained in various thicknesses (10 μm for fetus & 6μm for placenta, uterus, and ovary) in series.

e) Sticking the paraffinic strips on slides

In this stage, the paraffinic strips, obtained by microtome set, were firstly put on covered slides by Haupt's gelatin glue in series. To avoid shrinkage in paraffinic strips, the slides' surfaces were covered by formalin 2%. They were then put on the warming plate with a temperature 10-15° C lower than paraffin melting point (40° C). Finally, after drying and spreading the sections completely, they were picked up from the warming plate.

Sequence of event	Stage	Material	Time
1	Dewaxing of paraffin	Toluene	5-6 minutes (repeated if necessary)
2	Hydration	Alcohol 100	5 min
		Alcohol 90	5 min
		Alcohol70	5 min
		Alcohol50	5 min
		Distilled water	5 min
3	Staining	Haematoxylin	3-5 min
		Washing with water	5 min
		Alcohol 50	5 min
		Alcohol 70	5 min
		Eosin	5 min
4	Dehydration	Alcohol 90	30 second
		Absolute Alcohol	30 second
5	Clearing	Toluene	5-10 minutes

Table 1: Time table of sequences of events in the staining procedure.

Staining

The nuclei and cytoplasm and connective tissues surrounding the cells were stained. The staining procedure was performed as follows (Table 1).

Mounting the cover slips

The bubble formation under the slips should be immediately avoided after the staining stage and before the sample drying; they should be stuck on the slides by antler glue.

The study of tissues

The microscopic parameters of fetuses belonging to aforementioned four experimental groups were microscopically and macroscopically examined. Then, these parameters including weight of fetuses and placenta (measured by digital balance), placenta diameter, size of fetus C-R (Crown - Rump Length) (measured microscopically by caliper) were compared.

Results

Morphologic studies

The following results were obtained from the mice fetuses exposed to phenobarbital by microscopic and macroscopic observations:

1) Cleft palate in fetus.

2) Heavier weight, compared to fetuses in control group.

3) Longer Crown-Rump (C-R), compared to fetuses in control group.

4) Bigger liver, compared to fetuses in control group.

The following results were also observed regarding the protective effect of GCSF in different doses against the teratogenicity effect of phenobarbital for adult pregnant mice in experimental groups that received phenobarbital and GCSF alone, and next time received phenobarbital and GCSF together:

1) Lighter weight of fetuses exposed to GCSF, was compared to fetuses in control group.

2) Cleft palate treatment in fetuses of pregnant mice exposed to phenobarbital (120 mg/kg) and GCSF (1.63μg/ ml).

3) In that group which received phenobarbital (120 mg/kg) and GCSF in various doses (3.25, 7.75, 12 and15μg/ml) contemporaneously, the fetus in resorption stage was absorbed to Endometrium of uterus (Figure1,2).

4) Healthy fetuses with lighter weights after the GCSF injection in 0.8 μg/ ml dose.

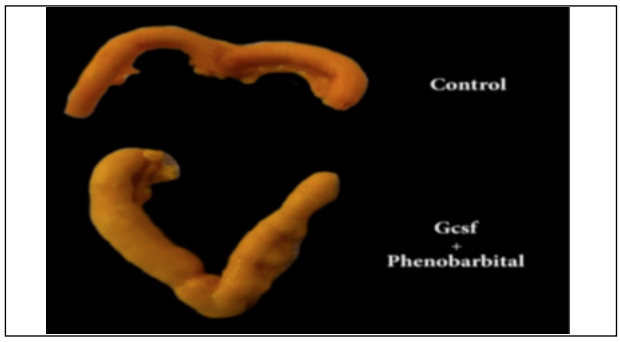


Figure 1: The comparison between the uterus in control group and the uterus exposed to phenobarbital and GCSF drugs.

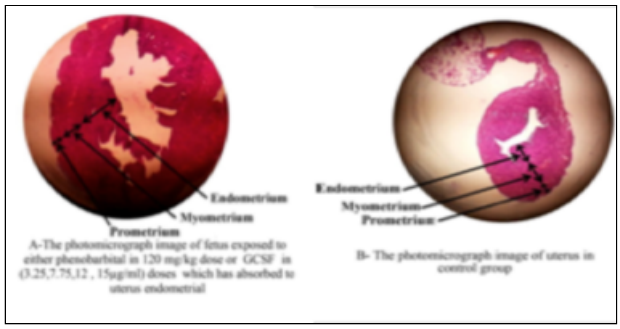
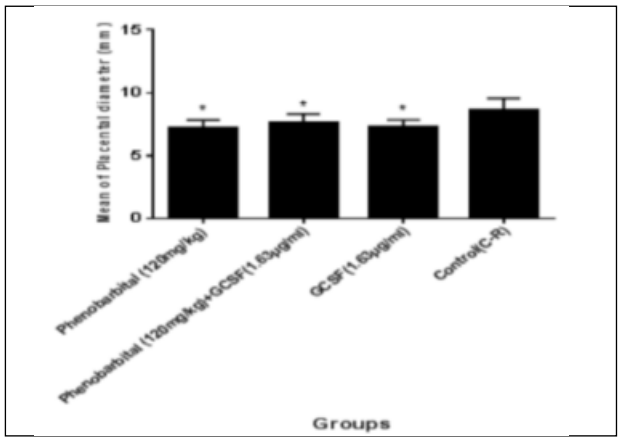


Figure 2: The comparison between uteruses in control group and groups exposed to phenobarbital and GCSF.

Alteration in the length of fetuses

As it was shown in graph 1, the average length of fetuses in 4 groups (only exposed to phenobarbital, only exposed to GCSF, exposed to phenobarbital and GCSF together, and the control group) were 9.88, 9.23, 9.79 and 10 millimeter respectively.



Graph 1: The comparison of the weight of fetuses in experimental groups and control group.

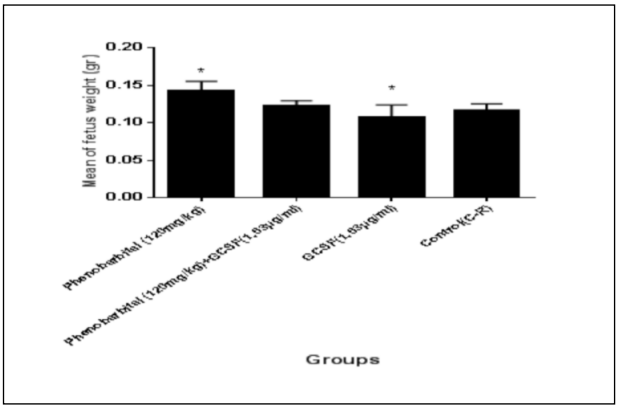
The ANOVA test showed a significant difference in the average length of fetuses among these

four groups (p=0.006).

Following the ANOVA test, the complementary test of Tukey (post Hoc) also showed this significant difference between GCSF and the other groups. The average length of fetuses in GCSF group was less than the other groups. However, this significant difference is not seen (P>0.05).

Alteration in the weight of Fetuses

Considering graph 2, the average weight of fetuses in each four experimental groups (only exposed to phenobarbital, only exposed to GCSF, exposed to phenobarbital and GCSF together, and the control group), were 0.14, 0.10, 0.12 and 0.11 grams respectively. The ANOVA test showed a significant difference in the average weight of fetuses among these four groups (P<0.001). Following the ANOVA test, the complementary test of Tukey (post Hoc) showed that no significant difference was observed between the control group and that group which received Phenobarbital and GCSF together. (P=0.17).



Graph 2: The comparison of the weight of fetuses in experimental groups and control group.

On the other hand, a significant difference was observed in other groups (P<0.001). Therefore, the lowest weight of fetus was related to GCSF group and the highest weight of fetus was related to Phenobarbital group.

Alteration in the weight of placentas

As can be concluded in graph 4, the average weight of placentas in each four experimental groups (only exposed to phenobarbital, only exposed to GCSF, exposed to phenobarbital and GCSF together, and the control group), were 0.11, 0.11, 0.10 and 0.10 grams respectively. The ANOVA test showed a significant difference in the average weight of placentas among these four groups (P<0.001). Following the ANOVA test, the complementary test of Tukey (post Hoc) also showed this significant difference between GCSF and control group (P<0.001), as well as the GCSF and Phenobarbital with GCSF group (P=0.006).No significant difference was observed among the other groups (P>0.05).

Discussion

Epilepsy or convulsion is one of the most dangerous neurologic conditions in human beings. 4 to 10 in 1000 people are suffering from these conditions in different societies[20]. Over the gestation period, epileptic mothers ought to use Antiepileptic drugs. While the use of anticonvulsants may cause the risk of teratogenicity effects and malformation, their discontinuity may have also negative effects on the mother and fetus[20].

In addition to teratogen effects, other symptoms including the increase of weight, liver size and Crown-Rump length (C-R) in fetuses were also observed in the present study. Cleft palate and umbilical hernia were other effects induced in mice fetus by Phenobarbital injection. In order to consider the possible effects of GCSF on the fetus, it was only Removed injected to pregnant mice in various doses (0.8, 1.63, 3.25, 7.75, 12 and 15μg/ml). Accordingly, simultaneous injection of GCSF and Phenobarbital may effectively prevent the cleft palate occurrence in mouse fetus. Phenobarbital and GCSF respectively increase and decrease the fetus weight. Thus, the common diet of GCSF and Phenobarbital did not show meaningful differences in the fetal weight. The results show that GCSF (3.25, 7.75, 12 and 15μg/ml) could not properly decrease the phenobarbital teratogenic effects. Although this could be related to drug dose, no toxic effects were also observed in 0.8μg/ml. Nevertheless, GCSF in higher doses (3.25μg/ml) prepared conditions that led to fetal resorption and miscarriage.

No differences were observed in the fetus absorbing level of endometrial in both medical diets including 0.8 and 1.63μg/ml. Regarding the proper confidence coefficient, it is claimed that GCSF (in controlled dose) will not cause any problem to fetus. Reducing the consumption dose of GCSF (3.25 to 1.63μg/ml) will not cause any problem to fetus, too. Previous experiments approved the important role of GCSF in neutralizing the phenobarbital teratogenic effects.

Various studies indicate three common mechanisms for most of antiepileptic drugs. Some researchers believe that most antiepileptic drugs show their teratogenic effects via changing the endogenous retinoid concentration which plays an important role in growing, distinction and organogenesis of fetus[21].

In 1995, Nau-H et al. studied two groups of epileptic and healthy infants. They showed that the metabolites level of 13-cis-retinoic acid and 14-3-cis-oxo-retinoic acid obviously changed in the blood serum of patients[21]. Considering the ability of antiepileptic drugs in changing the retinol densities and its derivations, it is supposed that one of teratogen mechanisms belongs to antiepileptic drugs that will be activated by changing the retinol densities and its derivations[7].

Other studies on the teratogen effects of antiepileptic drugs show that epoxide metabolites, produced by antiepileptic drugs, like phenytoin, phenobarbital and Carbamazepine are the main reasons for abnormality occurrence in fetuses[7]. There has also been some research about the role of antiepileptic drugs as the reducer of serum level of folic Acid in maternal blood[22], in which an indirect teratogen mechanism is proposed. These studies have highly recommended the prescription of acid folic to epileptic mothers during gestation.

Altogether, it appears that the mechanism of teratogen effects of antiepileptic drugs, especially Phenobarbital and phenytoin, are similar. However, one should remember that the consumption of Phenobarbital for a long time during gestation may create wide teratogen effects equaled to phenytoin. Surely, the consumption of antiepileptic drugs in pregnancy will increase the risk of congenital abnormalities two times epileptic mothers (from 2 - 3 % in common population to 4 - 6 %). This risk was commonly related to old antiepileptic drugs (before 1990) like Phenobarbital, phenytoin, Primidone, Carbamazepine and etc.[23], while 1the new drugs (presented to market after 1990) like Lamotrigine, Gabapentin, Felbamate and Oxcarbazepine do not show teratogenic effects. Compared to old drugs, they produce less epoxide and free radicals[23] .

Various food and medical diets have been proposed in complement with phenobarbital in order to protect the fetus from dangerous conditions. The inhibitory effect of GCSF against Phenobarbital teratogenic effects were considered here and the experiments indicated the main effect of GCSF in 1.63 μg/ml dos in neutralizing Phenobarbital teratogenic effects.

Over the past decades, many medical and supplementary factors including folic acid, multivitamins, Acetylsalicylic Acid (ASA), vitamin E and Levamisole have been studied to verify the teratogenicity effects of antiepileptic drugs.

Finnell et al. concentrated on the role of folic acid during gestation period and congenital defect risk and concluded that a complementary of folic acid can decrease the congenital defect risk in birth time which is related to antiepileptic drugs[23]. In 2000, Sonia et al. showed that not only folic acid and multivitamin compositions decrease the cleft palate, but also can decrease other defects including cardiovascular and urinary tract defects[23, 24].

It is thus assumed that acid folic consumption decreases the congenital defects risk when exposed to antiepileptic drugs[23]. In 1998, experiments on mice and human by Fleming et al. showed that folic acid plays an important role in decreasing the urinary tract defect and more than 70% of fetal defects in urinary tract can be reduced by adding acid folic to diet[25]. Folinic Acid is a fundamental factor in metabolic process. Accordingly, the cleft palate occurrence decreased to 10.9 percent in groups which received Phenobarbital and Folinic Acid[26, 27]. In 1975, Sullivan et al. indicated that the folate deficiency can lead to drug ineffectiveness. Thus, the density level of antiepileptic drugs will increase in blood that might increase the probability of toxicity occurrence[27].

Wells et al. in 1989 resulted that phenytoin and Phenobarbital participate in oxidation by prostaglandin and produce a free reactive radical mediator which binds to protein. The teratogenicity level of phenytoin and Phenobarbital will modulated by those materials which reduce the free radical formation of phenytoin and Phenobarbital. Acetylsalicylic acid (ASA) neutralizes the prostaglandin synthesis (PGS) irreversibly. Caffeic acid in an antioxidant and alpha-phenyl-butylnitrone (PBN) is a factor for absorbing free radical. Therefore, using these compositions in pregnant mice will decrease the cleft palate occurrence in their fetus. Scientists believe that Glutathione is able to detoxify free radicals intermediates by forming an irreversible relationship. N-acetyl cysteine, which is a Glutathione precursor, is able to decrease either orofacial cleft palate or fetal weight loss in fetus[28]. It is also believed that the deficiency of vitamin k may cause cleft palate occurrence in fetus[28].

Increasing the antioxidant effects can protect the fetus from teratogens like antiepileptic drugs (phenytoin and Phenobarbital)[29].

Shoshona et al. considered the possible role of Granulocyte macrophage colony stimulate (GCSF) on teratogenic effects in 2001. The manipulation of maternal immune system by leukocytes as a mediator affects the responsiveness capability to various cytokines like granulocyte macrophage colony stimulating factor (GCSF-GM-CSF)[30]. They tried to evaluate the ability of GCSF-GM-CSF in modulating the teratogenic activity along with possible changes in maternal immune system responses. They found that GCSF-GM-CSF have an important effect on this process in experimental population and compared to leukocyte operation results, they found that therapy by these drugs increases IL2 , IL3 production impressively[30].

In 1990, Wegmann found that the basic interaction of immune and reproductive system is their common cytokines of GCSF-GM-CSF which are potential modulators of placental growth, differentiation and fetal protection[17, 18].

It has been shown that the cytokines of GCSF-GM-CSF in experimental mice with lost pregnancy cause the fetal survival by decreasing the resorption level and supporting placental growth and function. Thus, manipulation of maternal immune system may influence not only the immune parameters but also fetal response to teratogens[17-19].

Torchinsky in 1994 observed that embryonic sensitiveness to teratogen defects is individual and depends on the genetic background and mother's life environment factors including nutrition, temperature and stress. Cyclophosphamide (CP) is another teratogen composition that is important in manipulation of maternal immune system[31].

In embryonic response, various cytokines will expression to teratogen factors , so, the expression of CSF, GCSF, TGFβ and growth factor will decrease after teratogen composition functioning like cyclophosphamide(CP)[32, 33].

The obtained data from various teratogens show that these mechanisms delay the growth and the formation of structural abnormalities is different in them. Thus, they might be influenced by various cytokines. The present findings are in agreement with many Studies that suggest various cytokines belong to GCSF_GM-CSF_CSF family which have protective and therapy features. For instance, GCSF_GM-CSF can protect mice from the lethal effects of ionizing radiation[34, 35].

Savion et al. found that manipulation of maternal immune system may decrease the morphologic defects resulted from chemical and medical factors by an indirect effect via regulation of cytokine production of activated immune cells[30]. The injection of GM_CSF and GCSF to mice prior to breeding increases this type of Cytokine treatment and decreases the interleukin (IL2_IL3) production. This change in produced regulative molecules by activated immune cells has an important role in protecting the fetus against defects[30].

The effect of manipulating maternal immune system in reducing the morphologic defects resulting from teratogen in mice has been proved. Although the mechanism of this process has been unknown up to the present time, it is assumed that one of the proteins belonging to maternal immune system affects the fetal gene expression. It should be noted that the effect of modulating activity of proteins belonging to congenital immune system on the normal growth of fetus is unknown.

The most important point is that achieving optimal health for the mother's immune system might be very important for avoiding congenital abnormalities. Considering that most teratogens are immunotoxic factors, many questions about the possible role of stimulation of maternal immune system in fetal morphologic defects are aroused which need to be widely explored.

Conclusions

In the present study, the teratogenicity effects of phenobarbital were Studied. The data shows that phenobarbital will probably influence the immune system and the teratogenicity effects cause cleft palate. As it is known, phenobarbital effects induce oxidative stress and indirectly influence the immune system and finally, disable it.

GCSF with dose of 1.63μg/ml is healthy and like the folic acid and vitamin E, decreases the teratogen effects of phenobarbital (cleft palate). Considering the positive results obtained in the animal level, the researchers wish to apply various doses in different pregnancy period and introduce GCSF in emergency moments as an anti-teratogenic drug.

The inhibitory mechanism of GCSF drug against phenobarbital teratogenic effect has been one of the main issues in recent years. The role of cellular and molecular mechanisms in this process is also a matter of question that can be answered by

complementary studies in the field. These questions produce a better comprehension of cellular processes in drugs or other compositions which can prevent phenobarbital teratogenic effects as well as other antiepileptic drugs with similar mechanism and fewer side effects.

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