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PHARMACOTHERAPY DURING PREGNANCY AND ITS ASSOCIATION WITH GENOME INSTABILITY IN MOTHER AND FETUS

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ABSTRACT. Pregnancy is a special physiological condition, where drug treatment presents a special concern. The use of drugs during pregnancy is increasing. Micronuclei, chromosomal aberrations, and sister chromatid exchanges are biomarkers of early biological effects which play an important role in assessing the genetic integrity of both individuals and populations. The aim of this review is to make a cross-section of previously conducted studies on the detections of genotoxic effects of drugs on human peripheral blood lymphocytes, after therapeutic exposure during the second and third trimester of pregnancy, as well as in umbilical blood lymphocytes of newborns whose mothers received the same therapy. Previous studies have shown that the cells of pregnant women and newborns are very sensitive to the effects of genotoxins, and cytogenetic biomarkers are associated with the risk of developing numerous diseases, including cancer in adults. Altogether, the administration of various doses and times of use of medications should be performed with caution.

Keywords: pregnancy, pharmacotherapy, genome instability, human peripheral blood lymphocytes, umbilical blood lymphocytes.

INTRODUCTION

Drugs have an important role in improving human health and promoting well-being. For each drug, the risk benefit ratio from preclinical studies should be carefully evaluated and it can vary for different drugs. For the pharmaceutical industry, risk benefit analysis includes

two different areas. The licensing authority analyzes health issues for the exposed population, but also for each patient, while doctors must harmonize both approaches. Preservation of the health integrity of human populations and obtaining healthy offspring is imperative in every modern society. According to the epidemiological data so far, 88% of pregnancies have a physiological course during which only basic care is needed, while in 12% of cases there is a high-risk pregnancy that requires additional help and specificity (COCO *et al*., 2014).

Pregnancy is a special physiological condition where drug treatment represents a special concern. On the other hand, pregnancy is a crucial part of life for both mother and baby, so it is very important for pregnant mothers to keep up with dos and don'ts during the gestation period. There is always a potential danger from drugs that a pregnant woman takes during pregnancy because they might contain a mixture of pharmacologically active compounds capable to interact and cause various mutagenic and teratogenic effects on the embryo/fetus, such as congenital malformations and intrauterine growth retardation. Therefore, it is very important for health care professionals and pregnant women to know which drugs can be taken and which should be avoided during this period (PEM *et al*., 2016). This review gives an insight into safe and unsafe drugs during pregnancy in order to increase awareness of their use.

Drugs used during pregnancy have to comply with very strict procedures regarding their safety, both for pregnant women and fetus, because of different possible adverse effects. The most famous is the Food and Drug Administration of the United States (FDA, USA), which has established stringent regulations regarding the labeling and use of medications during pregnancy. All drugs are classified into five categories: A, B, C, D and X based on the risks to the fetus (BRIGGS *et al*., 1998).

Table 1 shows the data on drugs commonly used during pregnancy, frequencies and the type of drug used, and the percentage of drugs prescribed in different trimesters (ANDRADE *et al*., 2004; BHAVYA *et al*., 2010; DAW *et al*., 2011; MITCHELL *et al*., 2011; ODALOVIĆ *et al*., 2012; LUPATTELLI *et al*., 2014; AYAD and CONSTANTINE, 2015; FIKADU *et al*., 2015; AYELE *et al*., 2020; PEREIRA *et al*., 2021). In addition to physicians in the health care system, pharmacists play a great role in health education by providing information not only to the health care professionals but also to the general population about the risk categories of drugs used in pregnancy (BHAVYA *et al*., 2010).

Depending on the diagnosis for pregnancy maintenance, a variety of therapeutic treatments is administered, ranging from hormone replacement to various antibiotics, tocolytics, antiarrhythmics, and vitamins. Some women have to continue their treatment for chronic diseases during pregnancy. Chronic diseases and other risk factors have been associated with maternal complications such as gestational diabetes, gestational hypertension, and pre-eclampsia during pregnancy. Chronic diseases, in particular asthma and arterial hypertension, have a great impact during pregnancy, causing both fetal and maternal complications (PINTO and MACHADO, 2017).

Among the significant gynecological problems are premature births, but also recurrent spontaneous abortions caused by cervical insufficiency. Premature births are the major cause of neonatal morbidity and mortality in developed countries. It is a multifactorial phenomenon that has significant medical, health care, and socio-economic effects and is responsible for about 75% of neonatal mortality and for half of the children's disabilities. Approximately 5-12% of newborns worldwide are born preterm (<37 weeks of gestation) (CHAWANPAIBOON *et al*., 2019).

Table 1. The data of drugs commonly used during pregnancy, frequencies and the type of drug used, and percentages of drugs prescribed in different trimesters.

I Use of medications during pregnancy (%)			**References**
In the USA, between 1976-1978, the average number of medications used by PW was 2.6 while in 2006-2008 it was 4.2 (increased by 68%).			AYAD and CONSTANTINE, 2015
Between 1976-2008, it progressively increased by more than 60%, and 4 or more medications were used more than three times.			MITCHELL *et al.*, 2011
By 2008, approximately 50% of PW reported taking at least 1 medication. Data from the USA shows that, on average, 2.6 medications were prescribed during pregnancy. In Italy 4.6 medications were prescribed per pregnant woman.			LUPATTELLI *et al.*, 2014
II The type of drug used during pregnancy			
Prescription medicines (Rx); over the counter (OTC) medicines or such as vitamins; complementary therapies (CT) such as herbal medicine self-medication; nutrition supplements (NS)			
Rx			
↑ 90% of PW took 3-4 medicine at some stage of pregnancy.			BHAVYA *et al.*, 2010
Between 1989 and 2010: in Europe, the prevalence estimates of Rx use vary considerably across countries ranging lowest in Northern European countries (44-47%) and highest in France (93%) and Germany (85%).			DAW *et al.*, 2011
↑ 80% of PW in Europe, Australia and America used at least one medication ~90% of all PW in the USA use Rx or OTC.			LUPATTELLI *et al.*, 2014
OTC			
↑ 80% of pregnant women took prescription or OTC drugs or used social drugs (tobacco or alcohol).			MITCHELL *et al.*, 2011
NS and CT			
59% of PW used vitamins or mineral supplements, and 13% of PW took a dietary herbal supplement. Among the 297 women interviewed, 107 (36.0%) had practiced self-medication in the previous 60 days.			ANDRADE *et al.*, 2004 PEREIRA *et al.*, 2021
III The drugs prescribed in different trimesters (%)			
1st trimester:	2nd trimester	3rd trimester	
39.0 (Rx+NS)	34.4 (Rx+NS)	37.9 (Rx+NS)	ANDRADE *et al.*, 2004
41 (Rx)	39 (Rx)	20 (Rx)	BHAVYA *et al.*, 2010
11.6 (Rx)	20.3 (Rx)	/	ODALOVIĆ *et al.*, 2012

Table 1 (continued)

IV FDA risk classification system			
Category A: controlled studies show no risk or find no evidence of harm;			
Category B: animal studies show no risk, but there are no controlled studies on pregnant women;			
Category C: animal studies have shown risk to the fetus, there are no controlled studies in women, or studies in women and animals are not available;			
Category D: Evidence of fetal risk in human studies. Benefits of drug may make risk to the fetus acceptable;			
Category X: studies in animals or humans have demonstrated fetal abnormalites, or there is evidence of fetal risk. The drug is contraindicated in women who are or may become pregnant.			
V The most commonly used drugs in pregnancy (FDA category):			
A category	***B category***	***C category***	***D category***
folic acid (FA)	*Acetaminophen, fenoterol Cephalexin, amoxicilin*	*hexoprenaline verapamil*	*progesterone hydroxyprogesterone, diazepam*
VI The use drugs in pregnancy according to FDA category			***References***
During the period 1996-2000: 78% of PW were exposed to class B or C drugs; 1.1% and 3.4% were exposed to either a class X or D (almost one-half of all pregnant women received prescription drugs from categories C, D, or X);*			*ANDRADE et al., 2004 (N=152 531)*
During the period 2009-2010: 60.8% (A), 19.60% (B), 10.3% (C), and 10.9% (D) of the drugs were prescribed to PW (higher overall drug use in pregnancy than before pregnancy, particularly spotted the use of progestogens and D category drugs);			*ODALOVIĆ et al., 2012 (N=311)*
During the period 2013-2014: 26.75% (B) (approximately half of the PW utilized drugs from categories C, D, and X);			*FIKADU et al., 2015 (N=323)*
During the period 2013- 2019, a total of 9 published studies were included: 56.1% (A); 29.0% (B); 12.1 % (C); 4.1% (D), and 2.5% (X) (drugs with evidence of fetal harm were widely used).			*AYELE et al., 2020 (N=4762)*
VII The most common drugs in gynecology practice *(FDA category)*			
1st trimester	*2nd trimester*	*3rd trimester*	
Gestogens, Progesterone (D)	*Tocolytic, Ritodrine (B) Antiarrhytmic, Verapamil (C) Antibiotic, Erythromycin (B)*	*Tocolytic, Ritodrine (B) Antiarrhytmic, Verapamil (C)*	

PW – pregnant women; FDA – Food and Drug Administration of the United States; *Drugs prescribed from categories "D" and "X": doxycycline, tetracycline, co-trimoxazole, fluoroquinolones, hydroxyprogesterone, estradiol valerate, acetyl salicylic acid, diclofenac, Ibuprofen, phenobarbitone, carbamazepine, valproic acid, phenytoin, propylthiouracil, warfarin, quinine, diazepam, prednisolone, atenolol, statin and efavirenz.

More than half of premature births are spontaneous. Despite the solution problems, efforts, and medical progress, the rate of premature births in the world has increased during the last few decades. The percentage of premature births in Serbia increased from 7.23% in 2000 to 11.97% in 2014. On the other hand, the neonatal mortality rate in Serbia decreased between 1997 and 2016, excluding deaths due to short gestation and low birth weight. Data from a few studies conducted in Serbia indicated the existence of some of the premature birth risk factors, such as maternal age, smoking habits and obese or overweight mothers (LAZAREVIĆ *et al*., 2020).

Factors bringing premature births are premature uterus contraction, shortening of crannies by more than 80%, and dilatation of the cervix. Cervical insufficiency as an anatomic-physiological anomaly in pregnant women may cause preterm birth in almost a quarter of pregnancies (ACOG PRACTICE BULLETIN, 2014). Cervical insufficiency implies operative treatment (cervical cerclage) during the second trimester as well as post-operative treatment with combined therapy (ritodrine, verapamil and erythromycin).

An investigation of possible genotoxic effects of prescribed therapy is very important, especially when it comes to drugs prescribed to pregnant women, due to the potential effect on both maternal and fetal cells, given the fact that most drugs pass through the placenta. Micronuclei (MN), chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in human peripheral blood lymphocytes (PBLs) are cytogenetic biomarkers traditionally used in numerous *in vivo* and *in vitro* studies to assess the genotoxic effects of different agents (GRUJIČIĆ *et al*., 2016).

Our previous studies have shown that progestogen therapy, administered to pregnant women during the first trimester of pregnancy for the prevention of threatening miscarriage had a genotoxic effect in PBLs of both pregnant women and umbilical blood of newborns whose mothers received the therapy (GRUJIČIĆ *et al*., 1999; MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2001). However, when it comes to the genotoxic effect of therapy prescribed in the second trimester of pregnancy, not many studies have been conducted so far. The aim of this review is to make a cross-section of previously conducted studies in humans and animals using cytogenetic biomarkers that can serve as predictive markers of the genotoxic effect of therapeutic exposure.

MATERIALS AND METHODS

The review is based on monitoring studies and analyzing MN, CAs and SCEs in PBLs used to evaluate chromosomal damage after the use of therapy during pregnancy. Our investigation consists of a literature review, based on the search of articles on Web of Science and PubMed Search engines in which the following keywords were used, separately or in combination: spontaneous miscarriage, cervical insufficiency, mid-trimester of pregnancy, pharmacotherapy treatments for maintenance of pregnancy, tocolytics, ritodrine, antiarrhythmics, verapamil, macrolide antibiotics, erythromycin, chromosomal damage, cytogenetic analysis, micronucleus assay, sister chromatid exchanges, human peripheral blood lymphocytes, umbilical blood lymphocytes.

Cytogenetic biomarkers of genomic damage in PBLs in vivo

Cytokinesis block micronucleus (CBMN) assay represents the most common method for testing genomic damage in PBLs (FENECH, 2007). This assay is a sensitive method that could be applied in monitoring cytogenetic damage in newborns, children, and adults (GRUJIČIĆ *et al*., 2008; MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2021). The MNi are small and round structures, similar to the morphology of the nucleus (Fig. 1-A). MNi can be formed from an

acentric chromosome or chromatid fragments and whole chromosomes or chromatids lagging behind in anaphase and telophase and are located outside the main nucleus in the cytoplasm of the cell (FENECH, 2007)

CBMN assay is applied on cultivated cells that divided only once which is achieved by adding cytochalasin B in the cell culture before the first mitosis. Cytochalasin B allows the division of the cell nucleus – karyokinesis but prevents cytoplasm division- cytokinesis (Fig. 2-A)). The advantage of this test is in the most objective insight into cytogenetic damage that can be obtained because thousands of cells are analyzed very quickly and easily on only one sample (FENECH, 2007).

The assay can be used for *in vivo* biomonitoring of genotoxic effects on humans, as well *in vitro* study of genotoxicity (MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2011; KIRSCH-VOLDERS *et al*., 2014). MN frequency in PBLs could be used in the prediction and assessment of different diseases such as hypertension, cardiovascular diseases, autoimmune diseases, neurodegenerative diseases, such as Alzheimer′s and Parkinson′s disease, cancers, and other diseases (FENECH *et al.*, 2020).

The CBMN cytome assay is a new and comprehensive method for measuring DNA damage, cytostasis, and cytotoxicity in different tissue types including blood lymphocytes. These events include next to an analysis of MNi, a biomarker of chromosome breakage and/or whole chromosome loss; nucleoplasmic bridges, a biomarker of DNA misrepair and/or telomere end-fusions; and nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes. A combination of CBMN assay and FISH (fluorescence *in situ* hybridisation) method is successfully used to detect the origin of MNi (Fig. 2-B)). This is how it detects the effects of agents that cause interruption of the parts of the chromosomes (clastogenic agents) as well as agents that lead to the loss of whole chromosomes (aneugenic agents) (FENECH, 2007).

Other cytogenetic biomarkers are well-known sister chromatid exchanges (SCEs) which detect exchanges of the genetic material between two sister chromatids. SCEs are induced during the G_2 phase in the cell cycle and are presumed to result from errors in replication (GRUJIČIĆ *et al*., 2016). SCEs are analyzed in the second-division metaphase using a staining method for sister chromatids differentiation by 5-bromodeoxyuridine (5-BrdU) (Fig. 1-B)).

At the cytogenetic level, SCEs are detected by differential staining of metaphase sister chromatids with BrdU-cultured cells over two cell cycles. Visibility of chromosomes by staining with Hoechst 33258 and Giemsa allows differences between sister chromatids with BrdU incorporated in one or both DNA strands. The SCE test has proven to be a powerful assay for the visual detection of physical DNA exchange between sister chromatids. SCEs are the consequences of breaking double-stranded DNA (DSBs) repaired by homologous recombination. SCEs could result from repairing DNA damage by homologous recombination during DNA replication. During two cell cycles, cells that are exposed to the thymidine analog BrdU, have different incorporation of the analog in the two sister chromatids. SCEs are clearly visualized under a microscope after the preparation of metaphase spreads (TUMINI and AGUILERA, 2021).

Therefore, SCEs are considered sensitive indicators for genome instability in cells. Most often among examinees, PBLs are used, while testing can be done *in vitro* on cell lines of fibroblasts also. The SCEs test is a relatively simple assay sensitive to most mutagenic compounds. Accordingly, the WHO included this method in the list of standard short-term screening methods. SCEs formation may indicate induced DNA damage even at low concentrations of genotoxic agents. Many authors have documented that the cells exposed to different mutagens had a significant increase in SCEs (KARELI *et al*., 2014). Within a single chromosome, the exchange of material between homologous loci of two sister chromatids is, in fact, a reciprocal exchange of its segments. This method is sensitive since it can detect

changes in the DNA molecule based on the action of many potentially genotoxic factors at lower concentrations (up to a hundred-fold) than those that produce visible chromosomal aberrations.

Test for chromosomal aberrations (CAs), which is the classical analysis of numerical and structural chromosomal aberrations in a different type of cells in the metaphase of the cell cycle, is also an important cytogenetic method. CAs as structural aberrations consisting of chromosomal, and chromatid breaks, and rearrangements are usually analyzed by light microscope (Fig. 1-C)) using the classical cytogenetic methods or the FISH technique (GRUJIČIĆ *et al*., 2016; TUMINI and AGUILERA, 2021).

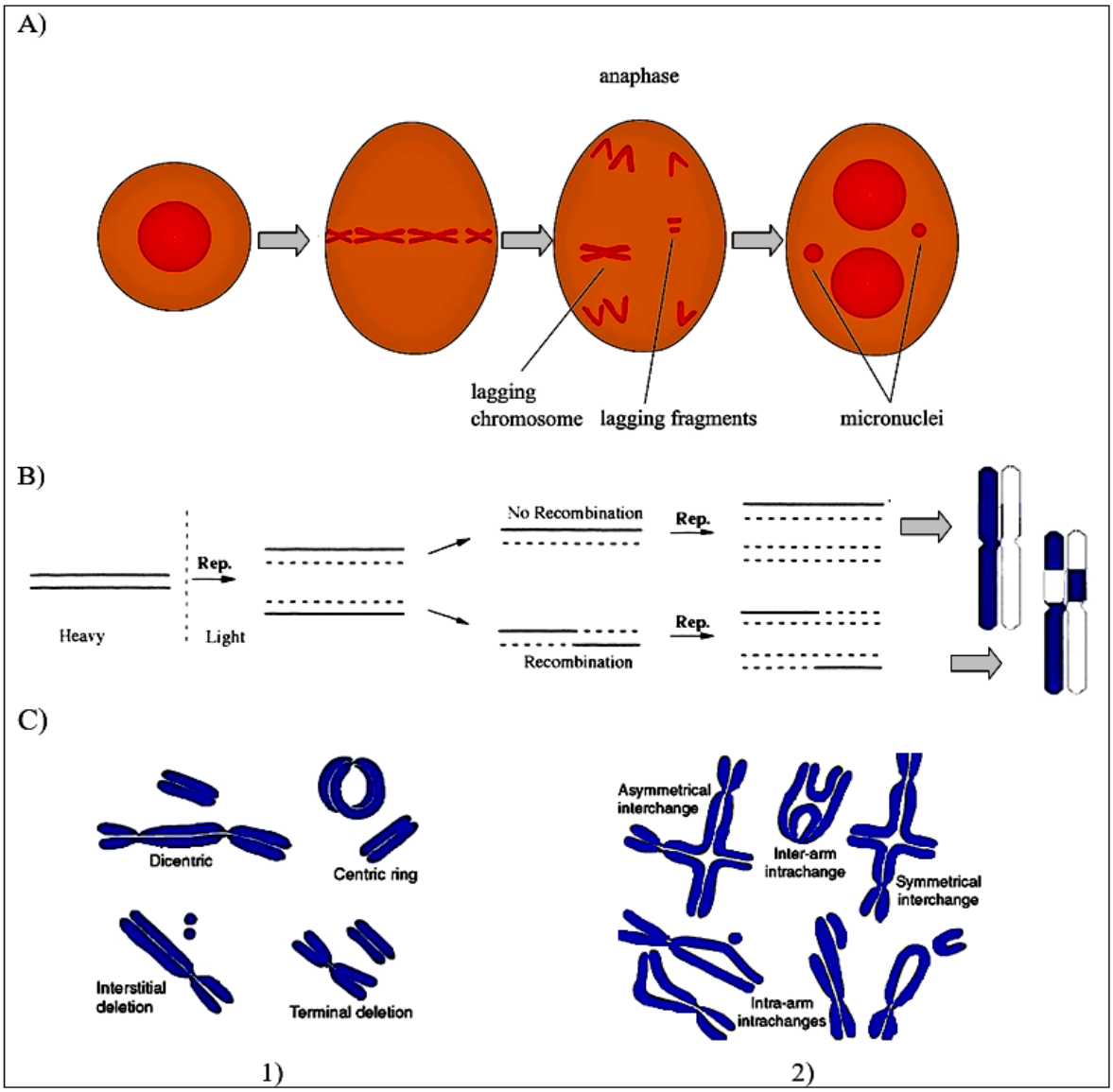

Figure 1. Mechanisms of formation A) MNi; B) SCEs C) CAs:
1) Chromosome-type and 2) Chromatid-type aberrations during cell division in PBLs.

CAs are changes in normal chromosome structure or number that can occur spontaneously or because of chemical/radiation treatment. Structural CAs in PBLs, as an important and very present biomarker of early effects of genotoxic carcinogens in the human population, has been used for over 40 years in occupational and environmental settings. Structural CAs are most commonly scored in metaphase-arrested cells that have been fixed, spread on microscope slides, and Giemsa stained. The frequency of CAs in PBLs in metaphase cells has been routinely used for decades as a tool to identify and monitor

occupational and environmental hazards of genotoxic carcinogens, confirming the importance of this biomarker. This concept of chromosomal damage is based on the evidence of an association between biomarker frequency and cancer risk (ROSSNER *et al*., 2005).

DISCUSSION

Genotoxic effects of therapy on PBLs of pregnant women

Pregnancy is a specific period in a woman's life during which a series of physiological changes take place, but also potentially pathological events that may require treatment. Many studies suggest that MN could be a significant prognostic biomarker for a successful pregnancy. TRKOVÁ *et al*. (2000) have shown that patients with unsuccessful pregnancies have an increased frequency of MNi in comparison to fertile women. Their study included couples with two or more spontaneous miscarriages, couples who were infertile, and healthy and fertile donors. FURNESS *et al*. (2010) suggest that the results of the analysis of MN frequency in PBLs in pregnant women represent an index of genomic instability in parental and/or fetal tissue. This may be due to hereditary factors that predispose to genomic instability or a consequence of environmental exposure to genotoxic agents capable to cause genetic damage. In addition, MNi may be considered as a biomarker of early genetic effects associated with cancer risk in adults (O'CALLAGHAN-GORDO *et al*., 2018).

In recent scientific literature, very few studies have examined the genotoxic risks of prescribed therapy during pregnancy. One of the first *in vivo* studies has shown that drugs used during pregnancy can cause genotoxic effects (GRUJIČIĆ *et al*., 1999). This study showed that MN is one of the cytogenetic biomarkers used to detect genome instability in PBLs of 30 pregnant women in the first trimester of pregnancy with a diagnosis of threatened spontaneous abortions, after therapeutic treatment with gestagens as hormone-replacement therapy, such as progesterone and its derivates. The authors obtained a significantly higher mean MN frequency in PBLs of treated patients compared to the control sample. They concluded that gestagens had genotoxic effects on PBL of patients treated with different doses compared to values before therapy (MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2003).

However, subsequent *in vivo* studies on pregnant women in the second trimester with a diagnosis of cervical insufficiency, after cervical cerclage and combined therapy of ritodrine, verapamil and erythromycin for six days application, showed an increase of MN frequency in PBLs in comparison with their frequencies before therapy (GRUJIČIĆ *et al*., 2007; 2013).

Ritodrine is one of the most commonly used tocolytic agents and is the only drug approved by FDA in 1980 for tocolytic use. This selective β2-adrenergic agonist relaxes the smooth muscles of the uterus by stimulating the β2-adrenergic receptors, inhibiting the contractility of uterine smooth muscle which results in the arrest of premature labor (KUWABARA *et al*., 2011). To reduce the cardiovascular side effects of ritodrine, a therapy with an antiarrhythmic calcium channels blocker is used. Verapamil is a calcium channel blocker used in the treatment of different cardiac disorders (ŞAHIN *et al*., 2016). Erythromycin is a macrolide antibiotic that is largely used in pregnancy for different bacterial infections, particularly in patients allergic to penicillin (ABDELLATIF *et al*., 2019).

The results of the analysis of cytogenetic biomarkers in PBLs of pregnant women, and in umbilical blood lymphocytes (UBLs) of newborns after different therapeutic treatments during pregnancies (ritodrine, verapamil, and erythromycin), including animal studies are shown in Table 2 (FRIEDMAN *et al*., 1990; NESTEROVA *et al*., 1999; SEREDENIN *et al*., 1999; DURNEV *et al*., 2006; GRUJIČIĆ *et al*., 2007, 2008, 2013; KARELI *et al*., 2016).

GRUJIČIĆ *et al*. (2007) studied the frequency of MNi in PBLs in a sample of 19 pregnant women after the application of combined therapy. The results of this study showed that the mean MN frequency in pregnant women after the therapy was significantly higher

compared to the mean MN frequency in the same women before the therapy. In the continuation of the same research, GRUJIČIĆ *et al*. (2013) studied inter-individual differences in both baseline and induced MN frequencies in PBLs of women with cervical insufficiency. They included 32 healthy pregnant women in the mid-trimester of pregnancy and the results showed that the mean value of the MN frequency significantly increased after therapy (Tab. 2).

Subsequent research has shown that ritodrine therapy received for a month during pregnancy did not lead to a significant genotoxic, cytostatic and cytotoxic effect in PBLs in SCEs test (KARELI *et al*., 2016). On the other hand, the results of the same authors concluded that long-term use of ritodrine (1- 3 months) in combination with the mother's smoking habit can lead to a synergistic effect and, in response, there was an increase in the frequency of SCEs.

In vivo genotoxic effects of therapy on UBLs of newborns whose mothers received therapy during pregnancy

LEVARIO-CARRILLO *et al*. (2005) found a correlation between the frequency of MNi in PBLs in mothers and MNi in UBLs in their babies. Such findings indicate the validity of the use of MN frequency in maternal PBL to assess genotoxic risk in fetal tissue. Determination of MN frequency in UBLs of newborns and in PBLs of children is a precise and generally accepted method for evaluation of spontaneous and induced DNA damage (GRUJIČIĆ *et al*., 2008).

An overview of the genotoxic effect of combined therapy with one of three tocolytics (ritodrine, fenoterol or hexoprenaline) and verapamil on UBLs of neonates whose mothers received therapy during pregnancy is shown in Table 2a (GRUJIČIĆ *et al*., 2008; KARELI *et al*., 2016).

Treatment with ritodrine and verapamil is used in gynecological practice and therefore is especially important to identify adverse effects on growth, development, and the genome of the fetus. The investigation of the genotoxic effect of combined therapy with betamimetics and verapamil, which was prescribed to pregnant women in the second and third trimester of pregnancy to prevent temporary labor in a period of 7 days to 5 months, showed a significant genotoxic effect on the cells of their babies. These results indicate that administered drugs have passed the placental barrier and induced a significant increase in MN frequency in UBLs of newborns. The mean MN frequency in UBLs of neonates whose mothers received one of the tocolytics (ritodrine hydrochloride, fenoterol or hexoprenaline) and verapamil concomitantly was significantly higher compared to the baseline MN frequency in control neonates. In the same study, the authors showed that there was no significant difference in the mean MN frequencies in UBLs of newborns whose mothers smoked during pregnancy and received the therapy compared to the mean MN frequency of babies whose mothers received the therapy and were non-smokers (GRUJIČIĆ *et al*., 2008).

KARELI *et al*. (2016) showed that the use of ritodrine for up to one month revealed a significant decrease in SCE/cell in newborns, while administration of ritodrine from 1 to 3 months induced an increase in SCE in neonates compared to the control group. That means the administration of ritodrine as a tocolitic agent should not last longer than a month. The same authors consider that accumulation of mutations and unrepaired DNA damages during neonates embryonic life increases the fragility of their genome and could act as an initiative fact for possible future carcinogenesis and/or other genetic-related diseases.

Table 2a. Cytogenetic biomarkers in PBLs of pregnant women, and in UBLs, FBLs of newborns after different therapeutic treatment during pregnancy.

Treatment	N	Diagnosis	WG	TDP	TC	CB	Effect	Ref.
In vivo cell/type								
Pregnant women/PBLs	19	*Cervical insufficiency*	13-24	ritodrine, verapamil erythromycin	2x50 mg/day 2x40 mg/day 4x500 mg/day	MN	sig. ↑ MN compared to control (after admin. of 1 week)	GRUJIČIĆ *et al*., 2007.
	32	*Cervical insufficiency*	13-24	ritodrine, verapamil erythromycin	2x50 mg/day 2x40 mg/day 2x500 mg/day	MN	sig. ↑ MN compared to control (after admin. of 1 week)	GRUJIČIĆ *et al*., 2013.
	10	*Premature labor*	37-38	ritodrine	4x10 mg/day	SCEs	sig. ↑ SCEs compared to control, after long admin. (1-3 months)	KARELI *et al*., 2016.
Newborns/UBLs	6	*Premature labor*	13-36	ritodrine, verapamil	3x40 mg/day 2x40 mg/day	MN	sig. ↑ MN compared to control (after administration of 7 days to 5 months)	GRUJIČIĆ *et al*., 2008.
	10		13-36	fenoterol, verapamil	6x5 mg/day 2x40 mg/day			
	7		13-36	hexoprenaline, verapamil	6x0.5 mg/day 2x40 mg/day			
	10	*Premature labor*	37-38	ritodrine	4x10 mg/day	SCEs	sig. ↑ SCEs compared to control (after admin. of 1 to 3 months)	KARELI *et al*., 2016.
Newborns/FBLs	10	*Premature labor*	37-38	ritodrine	4x10 mg/day			
Other patients/PBLs	5	*Supraventricular tachicardia*	/	verapamil	5-10 mg/day i.v.+ 80 mg (3x p.o.)	CAs	sig. ↑ Cas compared to control	FRIEDMAN *et al*., 1990.

WG – Week of gravidity; TDP – Therapy during pregnancy; TC – Treatment concentrations (mothers); CB- Cytogenetic biomarker; admin. – administration; Ref. – Reference; PBLs- human peripheral blood lymphocytes; UBLs – umbilical blood cells; FBLs – fibroblasts from the fetal surface of placenta; i.p. – intraperitoneally; MN – micronuclei; SCEs – sister chromatid exchange; CAs – chromosomal aberrations.

Table 2b. Cytogenetic biomarkers (CAs) in detection mutagenic effects in animal studies *in vivo.*

Treatment	Therapy	TC	CB	Effect	Ref.
Tests in animal model/ animal cells					
BALB/C mice/ BMCs	verapamil	0.2- 2.5 mg/kg	CAs	non-clastogenic *per se*.	NESTEROVA et al., 1999.
C57BL/6 mice/ BMCs	verapamil+acrilamide, cyclophospamide, and dioxidine	2.5 mg/kg+50 mg/kg i.p. 10 mg/kg i.p. 100 mg/kg i.p.		co-mutagenic effect potentiates the clastogenic effect of known mutagen	
BALB/C mice/ BMCs C57BL/6 mice/ BMCs	verapamil + cyclophospamide	0.1- 0.4 mg/kg i.p. + 10 mg/kg i.p.	CAs	co-mutagenic effect potentiates the clastogenic effect of known mutagen	SEREDENIN *et al*., 1999.
C57BL/6 mice/ BMCs	verapamil + antiviral drug ribavirin	0.25–10 mg/kg + 10-400 mg/kg	CAs	co-mutagenic effect co-clastogenic activity	DURNEV *et al*., 2006.

BMCs-bone marrow cells; i.p. – intraperitoneally; CAs- chromosomal aberrations; TC - treatment concentrations (mothers); CB- cytogenetic biomarker.

Table 3. Cytogenetic biomarkers (MN, CAs, SCEs) in human cells (PBLs, UBCs, FBS) and non-human model (CHO) after single treatment with different concentrations of tocolytic, antiarrhythmic, macrolide antibiotics and combined treatment *in vitro.*

Treatment		cell type	Donors (repetition)	C	CB	Effect	Ref.
Tests in human models							
Tocolytic	ritodrine	PBLs	3	$8.4x10^{-6}$ — $12.4\ x10^{-4}$M ($8.4\ x\ 10^{-5}$M*)	MN	sig. ↑ MN	MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2011.
Antiarrhythmic	verapamil	PBLs	3	$0.56- 11\ x\ 10^{-4}$M ($1.1\ x\ 10^{-5}$M*)	MN	sig. ↑ MN	FRIEDMAN *et al*., 1990.
	verapamil	PBLs	5	$0.1-6\ x\ 10^{-5}$M ($3\ x\ 10^{-5}$M*)	CAs	sig. ↑ CAs	

Table 3 (continued)							
Macrolide antibiotic	erythromycin	PBLs	3	0.68-5.45 x 10^{-4}M (1.36×10^{-4}M*)	MN	did not have genotoxic effect	GRUJIČIĆ *et al*., 2009.
	dirithromycin	PBLs	4	37.75- 250 µg/ml	MN, SCEs	sig. ↑ MN, and SCEs	KAYRALDIZ *et al*., 2015.
	natamycin	PBLs	4	13- 28 µg/mL	MN, SCEs, CAs	sig. ↑ MN and CAs except the lowest conc. sig. ↑ SCEs at the highest conc. for 48 h only	RENCÜZOĞULLARI *et al*., 2009.
Combined treatment	ritodrine + verapamil	PBLs	3	8.4 x 10^{-5}M* 1.1 x 10^{-5}M*	MN	sig. ↑ MN	MILOŠEVIĆ-DJORDJEVIĆ *et al*., 2011.
	ritodrine + verapamil + erythromycin	PBLs	3	8.4 x 10^{-5}M* 1.1 x 10^{-5}M* 1.36 x 10^{-4}M*	MN	sig. ↑ MN in higher conc.	
	ritodrine + CPT-11	PBLs UBLs FBS	6 6 6	50 ng/ml	SCEs	sig. ↑ SCEs	KARELI *et al*., 2016.
Tests in non-human model							
Antiarrhythmic	verapamil + bleomicin + MMC	CHO		n.d.	CAs	co-mutagenic potentiates the mutagenicity of cancer drugs	SCHEID and TRAUT, 1993
	verapamil + arsenite	CHO		100 µM 40 µM	MN	co-mutagenic potentiate arsenite-induced MN	LIU and HUANG, 1997

C – concentrations; CB – cytogenetic biomarker; PBLs – human peripheral blood lymphocytes; conc. – concentration; *DTD – equivalent day therapeutic doses. sig. ↑ MN/CA compared to control cells; CHO – Chinese hamster ovary cells; CPT-11 – irinotecan; FBLs – fibroblasts from the fetal surface of placenta; MMC- mitomycin.

Verapamil, as well as all betamimetics, passes through the placenta which does not represent absolute protection of the fetus against external impacts (VÄHÄKANGAS *et al*., 2006). There are studies suggesting that verapamil-induced CAs in bone marrow cells (BMCs) in various genotypes of mice. It has been demonstrated on BMCs of C57BL/6 and BALB/C mice that verapamil displays a co-mutagenic effect in a combination with cyclophosphamide or the antiviral drug ribavirin (NESTEROVA *et al*., 1999; SEREDENIN *et al*., 1999; DURNEV *et al*., 2006) (Tab. 2b). These results confirm the possibility that a fetus responds to the changing conditions of intrauterine development at the cellular level, by increasing the MN frequency, which can be detected in UBLs.

In vitro genotoxic effects of ritodrine, verapamil, and erythromycin on PBLs

Our results of the analyses of different cytogenetic biomarkers (MN, CAs, SCEs) in PBLs after treatment with different concentrations of ritodrine, verapamil, and erythromycin *in vitro* are presented in Table 3.

MILOŠEVIĆ-DJORDJEVIĆ *et al*. (2011) analysed MN frequency to determine the effects of ritodrine and verapamil in cultured PBLs of healthy donors. The results showed a concentration-dependent increase in MN frequency in both separate and combined treatment with tested drugs until the plateau was reached and the maximum genotoxic effect was manifested (Fig. 2).

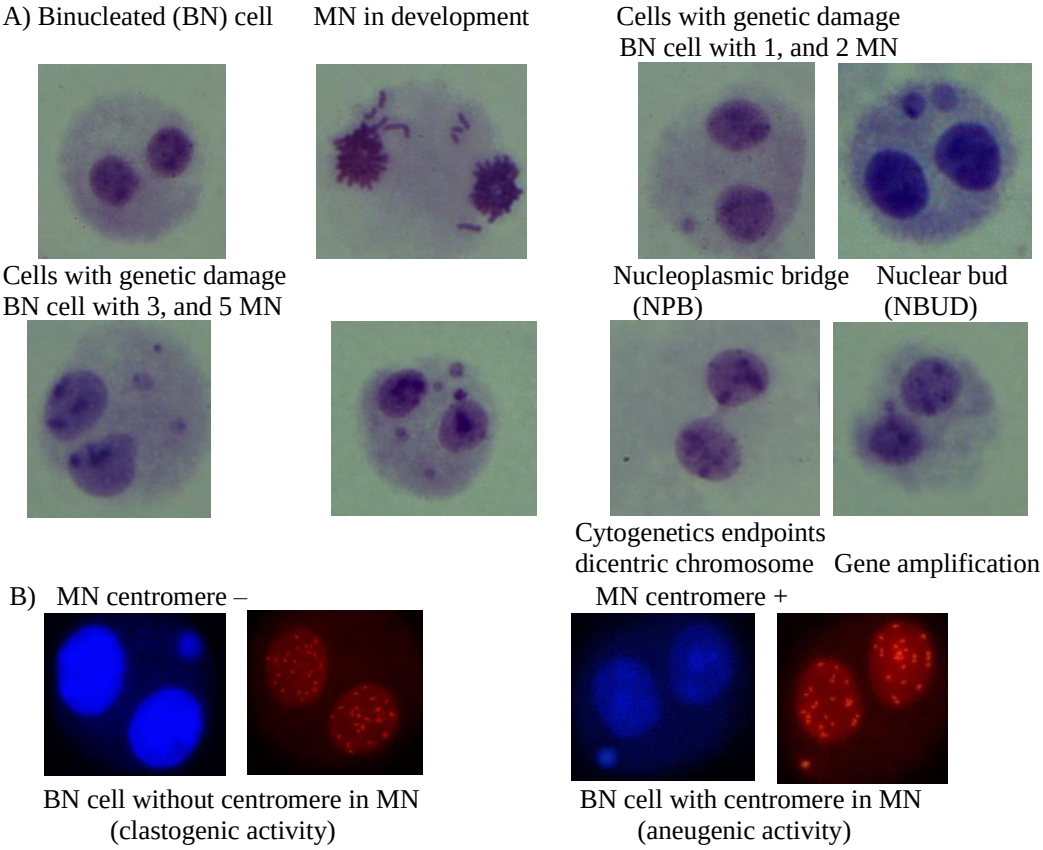

Figure 2. Cytogenetic endpoints in human PBLs by application of CBMN test
A) Cytognetic endpoints B) CBMN + FISH method (determination of origin MN).

A further increase of tested concentrations of both ritodrine and verapamil caused the decline in MN frequency in analyzed binucleated (BN) cells, while the highest tested

concentrations manifested the cytotoxic effect. The authors also concluded that combined treatment of ritodrine and verapamil in concentrations equivalent to daily therapy doses (DTD) expressed a co-mutagenic effect and that the MN frequency was increased three times compared to the control untreated PBLs. Verapamil showed a cytotoxic effect at the highest tested concentration which resulted in the complete absence of BN cells. To determine the origin of MN, the FISH method with centromeric probes was used. The results showed that the majority of the analyzed MNs were with centromeric signals suggesting that the treatment with verapamil alone or in combination with ritodrine induced chromosome losses indicating aneugenic activity (Fig. 2).

In comparison to non-pregnant women, pregnant women and their fetuses are more susceptible to bacterial infection. Macrolides are widely used in the treatment of pregnant women, so the interest of researchers to determine the genotoxicity of macrolide antibiotics seems fairly understandable. GRUJIČIĆ *et al*. (2009) showed that all tested concentrations of erythromycin did not increase MN frequency in PBLs in comparison to untreated cultured cells. In contrast, *in vitro* studies have shown that macrolide antibiotics, such as dirithromycin and natamycin, can induce genotoxic effects in PBLs when compared to controls (KAYRALDIZ *et al*., 2015; RENCÜZOĞULLARI *et al*., 2009).

Recommendations

All therapeutic treatments aim to maintain pregnancy and give birth to healthy offspring. Numerous studies have shown that fetuses and children are more sensitive than adults to many drugs prescribed during pregnancy when compared to their mothers (Fig. 3). One of the reasons is that the placenta does not provide absolute protection to the fetus from external influences. In recent years, many authors have pointed out that damage of hereditary material in newborns affects the disease onset in later life (GRUJIČIĆ *et al*., 2009).

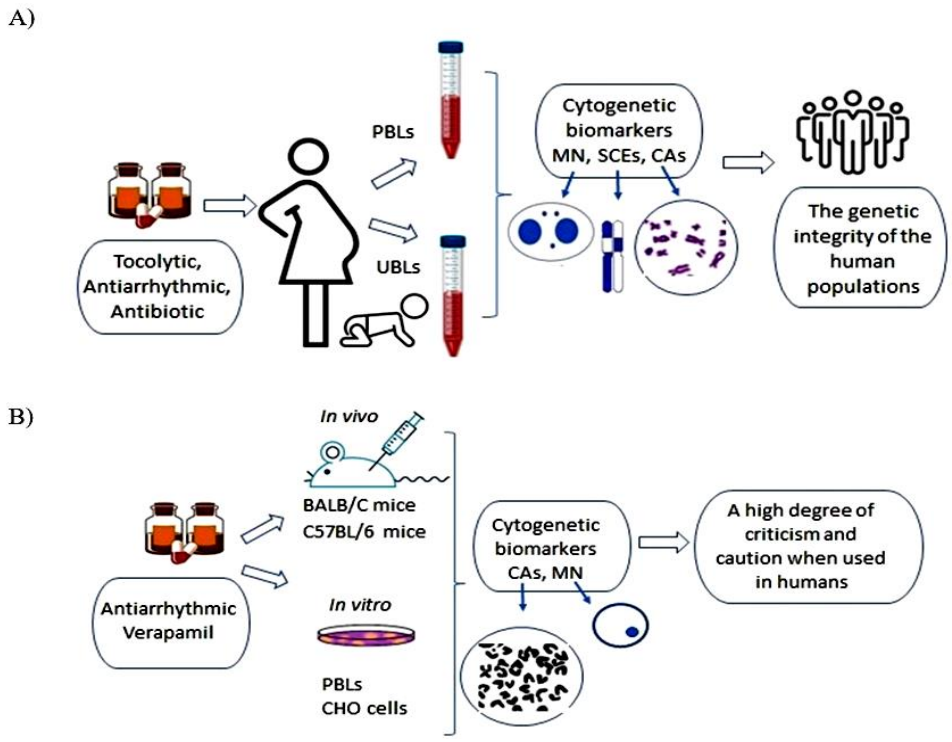

Figure 3. Schematic presentation of cytogenetic biomarkers in human studies (A) and in non-human studies (B) and its endpoint (PBLs- human peripheral blood lymphocytes; UBLs- umbilical blood lymphocytes CHO- Chinese hamster ovary cells).

The importance of using cytogenetic biomarkers as predictive factors of early biological effects in disease risk assessment in both children and adults is crucial for the detection of sensitive phenotypes and obtaining genetically healthy offspring. Based on the presented results in this review, we can conclude that therapy administered during pregnancy affects genetic damage in PBLs in both mothers and their babies. Animal studies have confirmed such findings. Altogether, extremely constructive criticism and utmost caution are suggested when using them in humans, including administered doses and the time of therapeutic use (Fig. 3).

CONCLUSIONS

The therapy treatments are very important because they enable couples and pregnant women with a physiological or genetic imbalance to have healthy offspring. A constant balance between risks and benefits when it comes to therapy is necessary for the daily work of physicians and pharmacists, especially in the field of reproductive health. Altogether, caution is suggested in various doses and times of therapeutic regimes.

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