Teratogenicity and Reactive Oxygen Species after transient embryonic hypoxia: Experimental and clinical evidence with focus on drugs causing failed abortion in humans

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ABSTRACT

Teratogenicity and Reactive Oxygen Species after transient embryonic hypoxia: Experimental and clinical evidence with focus on drugs with human abortive potential.

Reactive Oxygen Species (ROS) can be harmful to embryonic tissues. The adverse embryonic effects are dependent on the severity and duration of the hypoxic event and when during organogenesis hypoxia occurs. The vascular endothelium of recently formed arteries in the embryo is highly susceptible to ROS damage. Endothelial damage results in vascular disruption, hemorrhage and maldevelopment of organs, which normally should have been supplied by the artery. ROS can also induce irregular heart rhythm in the embryo resulting in alterations in blood flow and pressure from when the tubular heart starts beating. Such alterations in blood flow and pressure during cardiogenesis can result in a variety of cardiovascular defects, for example transpositions and ventricular septal defects. One aim of this article is to review and compare the pattern of malformations produced by transient embryonic hypoxia of various origins in animal studies with malformations associated with transient embryonic hypoxia in human pregnancy due to a failed abortion process. The results show that transient hypoxia and compounds with potential to cause failed abortion in humans, such as misoprostol and hormone pregnancy tests (HPTs) like Primodos, have been associated with a similar spectrum of teratogenicity. The spectrum includes limb reduction-, cardiovascular- and central nervous system defects. The hypoxia-ROS related teratogenicity of misoprostol and HPTs, is likely to be secondary to uterine contractions and compression of uterinoplacental/embryonic vessels during organogenesis.

1. Introduction

Oxygen is vital source of energy in cell metabolism across mammalian species in both adult and embryonic life [1]. Oxygen is distributed unevenly throughout the body in the adult at levels much lower than atmospheric oxygen concentrations (around 20.9% = 159 mmHg). Physiological oxygen concentrations in the adult vary depending upon the precise anatomical location, and the typical range is evidently between 1% and 14% oxygen or 7.6–110 mmHg arterial partial pressure of oxygen (pO2) (1% oxygen = 7.6 mmHg). The average oxygen concentration in the brain is 4%. However, in some parts of the brain, like thalamus and cortex, the oxygen content is lower than 1%; [2]. In the embryo the concentrations of oxygen are also low and varies between 1% and 5% (8–38 mm Hg) in various organs [1]. By definition, atmospheric air oxygen pressure can be referred to as normoxia, while partial oxygen pressure in normal physiological conditions is called “physioxia” or “physiologically relevant oxygen levels” [3,4]. Hypoxia is defined as oxygen tensions below tissue physioxia. Hypoxia is a pathologic state while physioxia is a normal state.

Furthermore, oxygen is referred to as the Janus gas, as it has both positive benefits and potentially damaging side-effects for biological systems [5,6]. The high reactivity of oxygen poses a major challenge as biological molecules are susceptible to oxidative damage as a result of excessive formation of toxic Reactive Oxygen Species (ROS) in the adult organism in many disease conditions. Examples of disease conditions in humans associated with ROS generation and reoxygenation are...
presented in Section 2.1. During normal cell metabolism at “physiologically relevant oxygen levels”, ROS is formed in small amounts and can serve as cell signaling molecules for biological processes. Slight excesses of ROS are detoxified by the cellular antioxidant reserve, including the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase [2]. Hypoxia reduces aerobic oxidative respiration and decreases electron-transport rate in the mitochondria leading to increased ROS generation, and enhanced nitric oxide (NO) synthesis. Depending on the severity and duration (e.g. acute, chronic, or intermittent) of the hypoxic state, cells may adapt, undergo injury, or die. Particularly, the massive generation of ROS in conditions when oxygen returns to oxygen-deprived (hypoxic) tissues during reoxygenation/reperfusion and overwhelms the antioxidant reserve, is known to play an important role in the induction of organ damage [7,8]. As reviewed by Chen et al. [2], the rate of the formation of ROS increases with oxygen tension, by clamping of uterine vessels or by inducing severe irregular cardiac rhythm in the embryo) with the pattern of malformations associated with transient embryonic hypoxia in human pregnancy. Focus will be paid on malformations in humans associated with the abortifacient drug misoprostol and hormone pregnancy tests (HPTs). Both misoprostol, as reviewed by Auffret et al. [11], and HPTs [12] have been reported to initiate uterine contractions and a failed abortion process in organogenesis with signs the same as those in early threatened abortion (contractions and bleeding) in some pregnant women. This can result in subsequent risks for compression of uterine and/or embryonic vessels and hypoxia/ROS related teratogenicity as discussed in this review.

2. Brief review of adverse effects of transient hypoxia and ROS in the adult

2.1. Hypoxia and ROS induced damage in the adult vascular system

Common clinical human disease or other conditions associated with reoxygenation/reperfusion damage following transient oxygen deprivation include coronary spasm, cardiac ventricular arrhythmia, cardiac arrest, surgical procedures (e.g cardiac bypass, transplantation or vascular surgery), limb injury, circulatory shock, sleep apnea and also transient hypoxia caused by vascular occlusions due to embolus/thrombosis in myocardial infarction and ischemic stroke as reviewed by Granger and Kvitets [13]. Once blood flow to a hypoxic tissue is restored, an increased supply of molecular oxygen leads to activation of pathways resulting in the increased production of ROS. As described in mechanistically oriented studies, hypoxia-related tissue injury is aggravated when blood and oxygen return (reoxygenation) after a hypoxic period; the “oxygen-paradox” [7,8]. It has also been described that hypoxia itself can generate production of ROS, however the generation during reperfusion/reoxygenation is several times higher; the longer the hypoxic period, the higher the generation of ROS during reoxygenation [14].

Primary ROS, such as superoxide and secondary highly reactive ROS such as hydroxyl, peroxynitrite, and hypochlorous are formed; there is evidence to show that ROS formed by one enzymatic pathway goes on to activate and speed up the production of more ROS by other pathways forming a vicious feedback circle, the so called ROS-induced ROS release (RIRR) [15]. The injury/damage caused by the burst of ROS after re-introduction of oxygen to hypoxic cells in various organs shares some common characteristics, namely vascular endothelial damage, resulting in impaired microvascular function, edema and cell necrosis [13]. ROS mediated endothelial cell damage is also likely to play an important role in hypoxia-related brain damage in the neonate [16]. Both endothelial cells and vascular smooth muscle cells possess the ability to generate ROS resulting in diminished barrier function, vascular damage and necrosis [2,17,18]. A large number of studies have presented evidence that ROS scavenging and/or detoxification protect against reperfusion/reoxygenation injury including damage to the human endothelium [13,19,20].

2.2. Hypoxia and ROS induced ventricular arrhythmia/cardiac arrest in the adult

In addition to tissue injury, individual organs may respond with unique functional disturbances following exposure to ROS generated via hypoxia/reoxygenation. The most striking example is the heart, which responds with rhythm disturbances including potentially fatal ventricular arrhythmia in response to hypoxia/reoxygenation and ROS generation [20–22]. Ventricular cardiac arrhythmia can also induce systemic hypoxia, and results in risk of death as well as of hypoxia-reoxygenation damage (e.g. brain damage or heart injury) if the patient survives. In animal studies, transient ligation/clamping of coronary arteries [20] or transient decrease in the arterial oxygen tension [14] followed by reoxygenation, have been used to produce ventricular arrhythmias mediated via the generation of ROS. The ROS-induced arrhythmias are linked to cellular electrophysiology changes at the level of ion channels in the heart, in particular the delayed rectified potassium (IKr) hERG channel [23–26]. Wang et al. [26] observed that intermittent hypoxia resulted in increased ROS generation, increased Ca++ ion levels as well as hERG channel protein degradation and inhibition of the Ik current; remarkably a membrane-permeable ROS scavenger prevented these effects [26]. Altogether, these mechanistic data indicate that ROS-induced arrhythmias result in hERG ion channel inactivation [26]. Furthermore, electrophysiological studies indicate that ROS-induced early after-depolarisation (EAD) preceeds reoxygenation/reoxygenation arrhythmias [27] in a similar way as QT-prolonging drugs blocking the hERG ion channel cause ventricular arrhythmia [28].

3. Hypoxia and ROS in normal and abnormal embryonic development

A delicate balance between oxygen and ROS levels is necessary for normal development of the embryo. There is evidence that a disturbed balance, particularly excessive formation of ROS, can produce a wide spectrum of malformations as will be discussed below.

3.1. Role of hypoxia and ROS in normal embryonic development

Embryonic development is highly conserved across vertebrate species including zebrafish, chicken, rodents, rabbits and humans, and follows the same principles, despite variations in developmental processes and differences in lengths of gestation. Oxygen is vital for normal development during organogenesis in these species. It is critical to maintain oxygen levels/tension within a low narrow range for normal development [1]. Under normal “physiologically relevant oxygen level” conditions, low levels of ROS are generated in the embryo by cell metabolism and low levels of ROS have been proposed to play a role in normal cell homeostasis and act as messengers to promote cell growth or apoptosis. In early human embryonic development highly oxygenated maternal blood is not allowed to be exchanged to the embryo until
organogenesis is almost finalised at week 8, before this the placenta vessels are “plugged” [29]. Several researchers mention that the “plugging mechanism” protects the developing embryo against ROS induced damage [29,30]. It has also been proposed that the embryo may represent a state in which antioxidant defenses (mainly catalase, glutathione peroxidase and SOD) are low and the susceptibility to injury induced by the generation of ROS is high [31]. It is essential that these defenses act in concert as an imbalance can lead to the production of highly reactive ROS. If generation of these highly reactive ROS exceeds the cellular defenses, then indiscriminate embryonic damage can occur [29].

The human embryo before week 8 – 10 is supplied with oxygen from specialized cell groups within the placenta able to gather up and store oxygen. Embryonic blood vessels gradually grow towards these cell groups, leading to the release of oxygen-laden red cells to the embryo and keeping it supplied [32]. Vasculogenesis is one of the earliest and most important events and begins in the placental villi at approximately days 18–20 post fertilization in humans [33]. Hypoxia stimulates the proliferation and migration of endothelial cells into previously avascular tissue [34]. In the rapidly developing early embryo, following vasculogenesis establishing the major vessels, angiogenesis then occurs in tissues to ensure good oxygen and nutrient supply and removal of waste products [35,36]. The formation of the vascular system is essential for normal development [35,36]. Two important vascular cell types in the embryo are vascular endothelial cells and vascular smooth muscle cells. Vascular endothelial cells initially form a monolayer throughout the entire vasculature, followed by recruitment of other cells such as podocytes and muscle cells, which stabilize and protect the vessel from potential injury and damage. There is a delay in the formation of the endothelium layer and the formation of the protective muscle layer of vessels in the early embryo [35]. Absence of the vascular smooth muscle coat presumably permit rapid changes in embryonic and organ outgrowth to occur. However, this also makes the vessels more susceptible to potential injury and damage [36–38].

In humans, by the end of week 3 postfertilization, passive oxygen diffusion becomes insufficient to support metabolism of the developing embryo, and the embryonic heart becomes vital for oxygen and nutrient distribution. The process of heart development is highly conserved between mammalian species, meaning that findings in vertebrate species may add considerably to our understanding of how the human heart develops [39,40]. Normal blood flow and blood flow dynamics play a crucial role, from when the tubular heart starts beating until the four chamber system established at around weeks 7–8 post fertilization [41]. New experimental data suggest the heart muscle tissues start to contract as soon as it forms the cardiac crescent, this period is equivalent to approximately day 16 in the human embryo [42]. The initiation of the first human heart beat via the primitive heart tube begins at around day 22–23 post fertilization, followed by active blood circulation in the embryo around day 30 [43]. Ion channels (Na+, K+ and Ca2+ channels) are critical in the embryo for all aspects of cardiac function, including rhythmicity and contractility. Major ion channels, such as the HERG (IKr channel) channel, play an important role in normal rhythm regulation and can be inactivated by excessive ROS generation in the adult heart [26]. The HERG (IKr channel) channel has been shown to be expressed and essential for rhythm regulation in the human, rat and rabbit embryonic heart from when the heart starts beating [44].

3.2. Role of hypoxia and ROS in vascular disruption induced teratogenicity

As mentioned above (see 3.1), it is well established that excessive ROS formation in the vascular endothelium causes impaired microvascular function, cell death and organ damage in the adult human when the organ is deprived from oxygen [13]. Hypoxia itself can generate ROS which is detoxified by the cellular antioxidant reserve, including the enzymes SOD, catalase and glutathione peroxidase, however, the massive generation of toxic ROS during reperfusion/reoxygenation of previously hypoxic tissues overwhelms the antioxidant reserve [14]. In a similar way, there is considerable evidence that excessive ROS generation in the vascular endothelium in the embryo plays a role in to explain the observed vascular permeability, oedema, hemorrhage and cellular injury/death preceding stage-specific malformations induced by transient periods of interrupted embryonic oxygen supply [31]. It has been proposed that the embryo is more susceptible to hypoxia-ROS induced damage due to a lower antioxidant activity compared to the adult [31]. This assumption is supported by studies showing low antioxidant activities in embryofetal tissues until just prior parturition [45,46]. In several mechanistically oriented experimental studies, Fantel and colleagues have also convincingly shown ROS generation in the vascular endothelium in the embryo to underlie teratogenicity reported after transient interrupted oxygen supply to the embryo [47–52]. For example, in one study rat embryos cultured on GD 14 were exposed in vitro to two transient 30-min episodes of hypoxia separated by normoxia. The transient exposure in vitro resulted in ROS generation (including superoxide anion radicals), oedema, vascular disruption, hemorrhage and cell death of the distal parts of the digits [47]. The pathogenesis and observed anomalies were identical to those observed after interrupted oxygen supply by clamping of uterine vessels on GD 14 or 16 in vivo in rats (Figs. 1A and 1B) [53,54] and resulted in digit reductions in the newborn after birth (Fig. 1C) [54].

A wide range of other malformations can be produced by temporary hypoxia in animals; the type of embryofetal manifestations induced is dependent on the degree and severity of the hypoxia, but also on the stage of embryofetal development when the hypoxia event occurs (see Section 4 for details). Some examples include: i) cleft lip/palate due to embryonic hypoxia induced pharmacologically by the drug almokalant on GD 11 in pregnancy in rats, the orofacial cleft is preceded by vascular disruption and hemorrhage (Figs. 2A and 2B) as discussed in a study by Webster et al. [55], and ii) severe brain damage on GD 9 in rats as demonstrated by Brent [56] (Fig. 2C) after using the clamping of uterine vessel technique which was introduced by Brent and Franklin in 1960 [57].

The developmental specificity for induction of teratogenicity by hypoxia, e.g. upper arm amputational defects on GD 9 in rats and distal digital reduction defects on GD 14 – 16 in rats (compare Figs. 1 and 2) is not known in detail. Indeed, as previously mentioned, there is a delay in the formation of the endothelium layer and formation of protective cell types (muscle cells and podocytes) in immature vessels; this permits rapid changes in growth of vessels however this also makes the vessels more susceptible to potential injury and damage [36]. These data may suggest that arteries which have recently been formed are particularly susceptible to vascular disruption and hemorrhage when exposed to toxic ROS during reperfusion/reoxygenation after a period of transient hypoxia. In support of a ROS mediated mechanism underlying the limb reduction defects, cotreatment with PBN (a potent agent with high capacity to bind reactive species like ROS), dramatically reduced both hemorrhage and limb reduction defects [52,58,59]. There is also data showing that pretreatment with PBN can prevent other hypoxia-related malformations, such as orofacial clefts, [58,59]. Furthermore, there are reports suggesting limbs and the central nervous system are particular sensitive to hypoxia/reperfusion damage due to possessing a lower antioxidant activity [52].

3.3. Hypoxia/ROS induced cardiovascular defects mediated via embryonic cardiac arrhythmia

Studies in human-relevant vertebrate experimental models, indicate that the immature embryonic heart responds with rhythm disturbances when exposed to transient hypoxia [60,61]. Indeed transient hypoxia caused bradycardia (slower heart rhythm) and the heart rate returned to normal after a period of transient hypoxia [60]. This study also showed that some embryos showed severe irregular rhythm (“heart beat became
erratic, alternating between periods of fast and slow rates) and also cardiac arrest followed by spontaneous recovery. Atrio-ventricular arrhythmias (e.g., three beats of atrium to one of the ventricle) were also occasionally observed [60]. Inactivation of ion channels, particular the (IKr) hERG channel, plays an important role for development of hypoxia/ROS related cardiac arrhythmia/arrest in the adult human heart, [23–26]. The (IKr) hERG channel is expressed and essential for rhythm regulation also in the human, rat and rabbit embryonic heart from when the heart starts beating [44]. It is thus reasonable to assume ROS related hERG (IKr) channel inactivation to be a likely mechanism that underlies cardiac arrhythmia/arrest in the embryo after a period of interrupted oxygen supply to the embryo. In support of this assumption, severe irregular rhythm induced by hypoxia/reperfusion [26], as well as drugs blocking the hERG channel in the adult and embryonic heart [62] induced the same electrophysiological changes: marked prolongation in intervals between heart beats (due to prolonged cardiac repolarisation) and occurrence of pathological early after depolarisations (EADs) resulting in severe irregular heart rhythm.

Furthermore, both uterine vessel clamping and single dosing of hERG channel drugs have been shown to induce a similar pattern of stage specific cardiovascular defects, in addition to “typical” vascular disruption defects, like limb reductions [58,59]. For example, clamping of uterine vessels results in a number of rare embryonic anomalies involving the great vessels including absence of the aortic segment between the left subclavian artery and the descending aorta, absent innominate artery, right aortic arch and also three fetuses with situs inversus [63]. Drugs blocking the hERG channel blocking, in a dose dependent manner, induce cardiac ventricular arrythmia and periods of cardiac arrest in the embryo both in vivo and in vitro [62], and result in various cardiovascular defects, including ventricular septum defects and great vessel defects (e.g., absence, abnormal size/structure- and transposition of vessels) [58,59]. As discussed by Midgett and Rugony [39], numerous studies have shown that altered blood flow conditions in embryos result in a wide spectrum of cardiovascular defects. Abnormalities of the great vessels can be produced experimentally by modifying the normal blood flow pattern [64,65] or by production of episodes of embryonic bradycardia, ventricular arrhythmia and temporary cardiac arrest [66]. A wide variation of vessel defects, including the malposition of the great vessels, can be induced by selectively increasing, decreasing, or misdirecting blood flow in the developing cardiovascular system by mechanical methods or by cardioactive substances. Lack of sufficient blood flow has been related to the induction of
abnormally absent vessels [41,67]. Thus, the results suggest that the observed cardiovascular defects are the result of ROS induced embryonic arrhythmia per se.

3.4. Role of hypoxia and ROS in the development of situs inversus

Situs inversus is a congenital anomaly defined as the inverse position of thoracic or abdominal organs, including the heart. A number of recent studies indicate that motile cilia, which are hairlike organelles, on specific cells in the embryonic node (around week 2–3 postfertilisation in the human embryo) direct embryonic fluid flow in a specific direction across species (leftward). These motile cilia guide determination of the left-right axis in the embryo [68]. Failure of motile cilia function can result in abnormal postion of organs and can be of genetic origin (e.g. genetic variations resulting in defective stucture and function of the cilia), but also mediated via the external factor hypoxia [69]. A specific pathway, called TORC1 has been implicated in the formation and function of motile cilia in the embryo [69]. However, TORC1 activity needs to be tightly regulated as both hyperactivation as well as suppression can result in disturbance in cilia function. Thus TORC1 is tightly controlled and regulated to ensure proper asymmetry development (e.g heart on left side). In this context it is noteworthy that embryonic hypoxia can switch off TORC1 [69–72]. Furthermore, increased levels of a particular ROS (NO −), which can emerge by hypoxia related mechanisms in vascular beds in adults and in the embryo [48,51,52], has been linked to causing situs inversus [73].

4. Teratogenicity produced by transient hypoxia of various origins in animals

It was not until the mid1980s substantial interest was paid to ROS in the biomedical field. In this section, we discuss a range of studies inducing transient periods of embryonic hypoxia and their resulting pattern of malformations and how recent understanding of ROS induc tion can explain how embryonic hypoxia underpins such damage. Methods used to induce transient hypoxia include a decrease in maternal oxygen tension and periods of bleeding resulting in inadequate oxygen delivery (hypoxia) to the embryo in Section 4.1, by clamping of uterine vessels in Section 4.2, and by pharmacological agents in Section 4.3. All studies in Section 4.1 and most studies in Section 4.2 were conducted before 1985. This means that the concept of ROS was not generally known or mentioned in the studies conducted before 1985. However, with todays understanding, that toxic ROS generated after a period of transient hypoxia can cause damage of the endothelium in both adult arteries in various disease conditions and particular in recently formed arteries in the embryo (see Sections 2 and 3 above), we have considered it relevant to review all studies reporting an association between transient embryonic hypoxia and teratogenicity. Several of the studies conducted before 1985 (see below) showed tissue changes preceeding the malformations (e.g. vascular disruption and hemorrhage) identical to those attributed to ROS generation in more recent studies conducted after 1990 [47–52].

4.1. Decreases in maternal oxygen tension and by maternal bleeding in animals

Transient hypoxia induced embryonic death and malformations have been demonstrated since the 1950s in studies by Ingalls and colleagues [74–78]. The incidence of the teratogenic manifestations induced were dependent on the severity and the duration of the hypoxic event as well as the developmental stage of the hypoxia event [74–78]. In these studies pregnant mice were exposed to transient hypoxia (lower oxygen tensions than physiologically relevant oxygen levels) for a restricted period on different gestional days (GD) of organogenesis. The results in these studies showed that transient periods of hypoxia produced stage specific malformations in the nervous system (anencephaly, spina bifida) orofacial region (cleft palate and micrognathia), cardiovascular system (e.g. interventricular septal defects), eye but also skeletal defects (in particular hemivertebra but also fusion of ribs). A Japanese study in mice, with exposure to transient hypoxia on GD 9 and examination of embryos 4 days later, reported hydrocephalus and abnormal flexion of the spinal cord and atrophy and cell death in the spinal cord [79]. Hypoxia on GD 9 in rabbits resulted in an abnormal vertebral column in rabbits [80]. In zebrafish embryos, transient hypoxia produced limb reduction- and eye defects [77].

One of the studies mentioned previously by Ingalls [74] focused on skeletal defects; mice were exposed to periods of low oxygen on different days (GD 8–11) of pregnancy. The results showed that hypoxia-induced skeletal defects emerged in a cephalocaudal sequence corresponding to skeletal formation in the normal embryo. The study also showed that the incidence of skeletal defects in mice increased with the severity (=lower oxygen content) of the hypoxic event. The study showed that exposure to hypoxia at GD 10 in mice resulted in an increase in rib and vertebræ malformations with increasing duration of the hypoxic event; 7% of the fetuses showed rib and vertebrae malformations after 15 min exposure, 20% after 30 min, 23% at 1 h, 50% at 2 h and 79% at 5 h. In a study by Wilson [81], after repeated bleeding of rats on three successive days in pregnancy, limb defects, (mainly limb reduction malformations, but also syndactyly or polydactyly), and nervous system defects (degeneration of cerebral cortex, basal ganglia and spinal cord associated with hemorrhage in these regions) were observed (after bleeding between GD 9–11). A study in mice also showed that extensive blood sampling on GD 12, 14 or 15 in mice resulted in limb reduction defects and cleft lip [82]; the authors attributed the limb reduction defects to vascular disruption in the embryofetal tissues secondary to maternal blood loss induced hypovolemia/hypoperfusion.

4.2. Embryonic hypoxia induced by clamping of uterine vessels or uterine trauma

In this experimental model, the uterine arteries of a pregnant rat during major organogenesis are temporarily clamped (by forceps or by occlusion cuffs) for 30 min to 2 hr on one side of the bicornuate uterus with the other horn acting as the control [53,54,56,63]. The placenta and embryos on the clamped side are without any blood supply, while maternal normoxia is maintained. Implication occurs in rats on GD 6. During the period between implantation on GD 6 and when the rat embryonic heart starts beating on GD 9, many rat embryos can survive periods of severe hypoxia/anoxia of between 60 and 120 min, which is much longer than an adult organism could survive. However, surviving embryos show malformations, including microphthalmia, various cardiac -and abdominal wall defects and situs inversus. This period corresponds approximately to the first 3–4 weeks of human embryonic life (or week 5–6 if gestational length is calculated on the last menstruation). The embryo then becomes more sensitive to hypoxia with most embryos unable to survive 60 min of uterine clamping.

Shorter periods of hypoxia (30–45 min) saw increased survival, but were associated with a range of defects during a period approximately corresponding to weeks 5–9 of embryonic development. Fetal adverse effects were observed in the nervous system (hydrocephalus, meningcele, anencephaly), eyes (e.g anophthalmia, microphthalmia), orofacial region (e.g. cleft palate, cleft lip, mandible defects, midfacial hemorrhage), but also in the urinary system (e.g. renal agenesis, fusion of kidneys) and digestive system and also in the cardiovascular system. The most susceptible period for induction of hypoxia-related defects occurred after clamping is the late organogenesis (GD 13–16 rats); during this period limb reduction defects, ranging from absent nails to loss of the entire footplate and syndactyly were observed after clamping for 30 min [53]. The limb reduction defects were preceded by edema, dilated blood vessels, hemorrhage, and blisters, with subsequent tissue degeneration [53,54]. As mentioned previously, two transient periods of normoxia (30 min), separated by 30 min hypoxia in rat embryos culered in vitro

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on GD 14, caused vascular disruption, hemorrhage and cell death of the distal parts of the digits [47] in a similar way as observed after clamping of uterine vessels on GD 14 in rats [53]. In this study by Fantel et al. [47], ROS were detected in the distal parts of the digits, suggesting ROS generation to be of importance to explain hypoxia related teratogenicity of various origins, including clamping of uterine vessels.

Interestingly, studies using uterine clamping in rats [53,54,63], show that malformations, vascular disruption and hemorrhage, not only occurred in the clamped horn of the uterus, but also in the control uterine horn (not clamped), although at a lower incidence. As described and reviewed by Webster et al. [54], a variety of procedures including clamping of the uterine wall (excluding clamping of major uterine vessels), handling of the pregnant uterus for 2 min or stretching of the uterine vessels for 2 min after midline incision in anesthetized pregnant rats GD 14 – 16 [54] or amniotic puncture [83] also produce vascular disruption and hemorrhage in the embryo. Overall these results, as discussed by Webster et al. [54], suggest that uterine wall contraction and arteriolar spasm may be the usual response during all procedures that involve disturbance of uterine horns resulting in periods of embryofetal hypoxia; the greater the disturbance the more severe the response. They also discuss that the uterine bed may vasoconstrict in response to any stimulus that evokes a generalized sympathetic discharge such as a shock or to direct local stimulation of sympathetic nerves. Webster et al. [54] conclude, that the ease which vascular disruption occurs in embryofetal tissues, particular in late organogenesis in rats (corresponding to around weeks 6–8 in human pregnancy), suggests the need for particular care in the human and attention to the outcome of pregnancies in which there has been physical or pharmacological adverse uterine adverse alterations.

### 4.3. Embryonic hypoxia induced pharmacologically with focus on drugs causing severe embryonic cardiac arrhythmia

A large number of cardiovascular active drugs have been shown to produce teratogenicity in animals attributed to transient embryonic hypoxia. Most studies have focussed on limb reduction defects and other externally visible defects as discussed in several review articles [84–86]. Examples include epinephrine, norepinephrine, nicotine, cocaine and vasopressin; which all cause a decrease in uteroplacental blood flow in animals; the underlying mechanism is most likely due to vasoconstriction of uterine vessels. Potent vasodilators like nifedipine, nitrendipine and felodipine, can also cause distal limb reduction defects and decreased utero-placental blood flow [87]. The decreased blood flow was shown to be related to diversion of blood flow from central compartments, like the pregnant uterus, to peripheral tissues in the pregnant animal at high doses [88]. The limb reduction defects, both after vasoconstrictors and vasodilators [84,89] were preceded by edema, vascular disruption in the limb buds, and subsequent hemorrhage and necrosis of embryonic tissues.

There is considerable evidence that ROS induced severe cardiac arrhythmia in both the adult and embryonic heart (across species, including the human embryo) can be induced by transient periods of hypoxia through inactivation of cardiac ion channels, in particular a specific potassium cardiac current (Ikr) channel expressed by the hERG gene (see Section 3.3). Drugs blocking the hERG channel, are classified with risk for cardiac arrhythmias and risk for sudden death in adult humans by regulatory agencies worldwide, including FDA, at therapeutic doses. The embryo tolerates much longer periods of interrupted oxygen supply than the adult until death occurs, however teratogenicity may occur. A number of studies have shown that the potent IKr blockers

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**Fig. 3.** Pharmacologically induced hypoxia in the embryo by a potent IKr (hERG channel) blocking drug (almokalant). Images taken from Danielsson et al. [91]. Images show rat embryos immunostained with antibodies against the hypoxia marker (pimonidazole) at days 11 and 13 after almokalant was previously given to the dams. Note black immunostaining, indicating generalized hypoxia in rat embryos exposed to almokalant on GD 11 (B) and GD13 (D). Control GD 11 (A) and control GD 13 (C) embryos, respectively [91]. FB, forebrain; H, heart; HB, hindbrain; Li, liver; MBA, mandibular branchial arch; Mb, mandibulum, Mx, maxilla; NL, neural lumen; T, tongue. Bars represent 2000 µm. Permission to reproduce images given by Elsevier [91].
like dofetilide, almokalant, astemizole, cisapride and dofetilide, dose dependently cause arrhythmia/cardiac arrest and periods of severe hypoxia in the embryo [62,90]. Occurrence of embryonic hypoxia after administration of these drugs was verified by using a hypoxia probe [91] after maternal dosing in rats as illustrated in Fig. 3A-D.

As discussed previously (see 3.3 above) transient embryonic hypoxia and hERG channel drugs cause very similar cardiovascular anomalies, including the great vessel anomalies. Histopathological examination of the heart in teratology studies (which was not conducted in the studies with clamping of uterine vessels) showed an increase also in ventricular septal defects [58,59]. The results suggest that the observed cardiovascular defects are the result of induced embryonic arrhythmia per se, resulting in pressure changes and misdirection of embryonic blood flow. The pattern in rats of non-cardiovascular malformations after transient embryonic hypoxia (see Sections 4.1 and 4.2) has also been compared with the pattern reported after administration of a potent IKr blocker on single days from when the embryonic heart starts beating until late periods of organogenesis corresponding to weeks 6–8 of human embryonic development (or 6–10 in gestation weeks based on last menstruation). The comparison showed that the IKr blocker almokalant induced an almost identical pattern of stage specific malformations as after clamping of uterine vessels and low oxygen tension in the atmosphere, including limb reduction defects, orofacial defects (e.g. cleft lip, small mandible), urinary defects (agenesis of kidney), anal atresia and various skeletal defects (including vertebrae, rib and amputational skeletal defects of limbs) [58,59].

The incidence of the above mentioned defects were much higher in the group treated with IKr blockers compared to concurrent vehicle treated controls and historical control data [62]. In some studies with IKr blockers, special attention was paid to cleft lip and limb reductions; the results showed that both types of defects were preceded by vascular disruption in vascular beds, and subsequent hemorrhage and necrosis of tissues in the orofacial (GD 11) and limb bud regions of the embryo (GD 13) 24–48 h after dosing [62]. The skeletal defects presented in the same way as reported by Ingalls and Curley [74] in a cephalocaudal sequence. The studies also show that 5 out of 6 defects included in the VACTRL syndrome (including Vertebræ, Anal atresia, Cardiac, Tracheo-oesophageal, Renal and Limb reduction defects) could be induced by a single hypoxic event [58,59]. Several fetuses showed three or more of above mentioned defects (=fulfills the diagnosis of VACTRL) after single dosing on GD 10, 11 or 11. Altogether the data shows that periods of cardiac irregular rhythm and cardiac arrest in the embryo are able to induce both stage specific cardiovascular defects due to hemodynamic alterations in the embryo during cardiogenesis, as well as a spectrum of hypoxia-related defects preceded by vascular disruption in other organ systems. These data also suggest that ROS induced embryonic cardiac arrhythmia plays an important role in hypoxia-related teratogenicity observed after temporary clamping of uterine vessels and after decreased oxygen content.

### 4.4. Summary of stage specific anomalies induced by transient periods of hypoxia

Table 1 summarises the wide spectrum of malformations induced by transient periods of interrupted oxygen in teratology studies by clamping of uterine vessels [53–57] or by pharmacologically induced hypoxia after administration of a teratogenic dose of a drug where embryonic hypoxia has been verified by using a hypoxia probe [55,58,59,90,91]. The table also presents information on when (stage) during embryonic development a transient period of hypoxia has been shown to induce specific type(s) malformation in rats. As mentioned previously, limb defects seem to the “easiest” defects to induce after only a relatively short period of clamping of uterine vessels during GD 13-GD 16 in rats [85]; a period of organogenesis corresponding to weeks 6–9 post-fertilisation in the human embryo. Results in studies investigating embryofetal tissues...
the interrupted oxygen supply to the embryo. As discussed in Section 4.3, there is also considerable evidence that transient periods of hypoxia followed by ROS generation can induce cardiac rhythm alterations during cardiogenesis (which occurs GD 9–13 in rats). In addition, it is known that severe cardiac arrhythmia and subsequent hemodynamic alterations in the embryo during cardiogenesis can induce a wide spectrum of cardiovascular malformations. Alterations in oxygen/ROS balance in early pregnancy in rats (GD 6–7) has also been shown to induce situs inversus (see Section 3.4). As shown in Table 1, a variety a cardiovascular anomalies as well as situs inversus, have been reported to be associated with transient periods of interrupted oxygen supply during relevant stages for induction of such stage specific anomalies.

5. Evidence for teratogenicity caused by transient hypoxia in the human embryo

The drugs misoprostol and mifepristone were introduced as abortifacient drugs in the 1990 s. As will be presented below, accumulated data from the early 1990s and onwards in a large number of clinical studies (see Section 5.1), resulted in regulatory pregnancy texts for drugs used as abortifacient today mention that human malformations can occur in the case of unsuccessful termination of pregnancy (failed abortion) [94,95]. Several investigators have proposed these abortifacient drugs act by causing uterine contractions leading to compression of uterine and/or embryofetal vessels and embryonic hypoxia during the failed abortion process to underlie the teratogenicity as reviewed by Auffret et al. [11]. This is highly interesting in view of evidence that Hormone Pregnancy Tests (HPTs) have been reported to cause similar signs such as in early threatened abortion in some pregnancies (uterine contractions and vaginal bleeding), but the pregnancy continues [12]. Exposure occurred mainly in the 1960 s and 1970 s and HPTs were used to detect pregnancy before urine tests were introduced. HPTs were supposed to initiate a menstruation bleed and expel the uterine lining (mediated via contraction of the uterus) in non-pregnant women but not affect pregnancy in pregnant women. Numerous studies published in the 1960–1980 s have showed an association between HPTs and human teratogenicity (see Section 5.2 below), resulting in regulatory concerns and withdrawal of HPTs from the market as discussed in recent publications [96–98].

An important principle for evaluation of suspected human teratogenicity is to carefully consider if the medicinal product (in this case HPTs) may have a mode of action similar to that of a known human teratogen and/or have been associated with the same type(s) of malformation(s) as an established teratogen as expressed in the EU guideline 2019 on pharmacovigilance of drugs used in pregnancy [99]. To elucidate if there is evidence that HPTs may have potential to cause teratogenicity via failed abortion in the same way as the human teratogen misoprostol, we have examined and compared the pattern of malformations associated with HPTs and misoprostol (see Sections 5.1–5.3). We also discuss the pattern of anomalies associated with use of the misoprostol and HPTs, with the pattern following transient hypoxia in animal studies. In addition, we briefly review human malformations associated with chorionic villus sampling (CVS) and various types of uterine manipulation or trauma, which have been attributed to transient interruption of oxygen to the embryo in several publications (see Sections 5.4 and 5.5).

5.1. Misoprostol

5.1.1. Indications and review of proposed mechanism for teratogenicity

Misoprostol is used as an abortifacient drug but also to prevent peptic ulcer; this was the original indication. The abortive effect is mainly mediated by its potential to cause uterine contractions. The teratogenic risk for misoprostol is today acknowledged worldwide in regulatory pregnancy labelling, which warns that if the patient chooses to continue the pregnancy despite a failed abortion; which may occur in 5–10% of the cases, the pregnancy should be followed carefully with focus on detection of limb reduction and nervous system anomalies [94]. In a study from 2016 [11], doses of 200, 400, 600 or 800 microgram of misoprostol used for therapeutic abortion as well as 200 microgram used for prevention of peptic ulcer, were all associated with teratogenicity. This and other studies also indicate that the risk of malformations after failed abortion is not related to the dose or induction; thus misoprostol can be teratogenic even at low doses and careful attention should therefore be paid to all pregnancies exposed to misoprostol [11,100, 101]. There is considerable evidence indicating that teratogenicity by misoprostol is mediated via uterine contractions and mechanical compression of the uterine artery, followed by decreased uteroplacental blood flow, generalised hypoxia and hypoxia-related vascular disruption in the embryo and subsequent hemorrhage, and tissue loss [11,101, 102]. Compression of the uterine artery may explain the wide spectrum of stage specific malformations associated with misoprostol (see 5.1.2 below) of similar types as observed after temporary clamping of uterine vessels in animals (see Table 1). Furthermore, uterine contractions may also result in compression of the subclavian artery in the human embryo and hypoxia-related vascular disruption of different branches of this artery [11,93,103]. Disruption of different branches of the subclavian artery may result in different rare human anomalies like Poland syndrome, Klippel–Feil syndrome or Moebius syndrome [103]. As will be presented below (see Section 5.1.2) particularly Moebius syndrome has been associated with misoprostol use, and is likely to be caused by hypoxia-related vascular disruption of a branch of the subclavian artery in the brainstem in the embryo [103]. The disruption results in damage of motor nuclei of cranial nerves, facial paralysis and inability to move the eyes from side to side. Most people with Moebius syndrome are born with complete facial paralysis and cannot close their eyes or form facial expressions. Limb abnormalities, like clubbed feet and/or missing fingers or toes sometimes occur with the syndrome [103].

5.1.2. Review of anomalies in human studies

Since the early 1990s, several epidemiological studies report an increased risk for human malformations after use of misoprostol. A review of four studies by Dal Pizzolo et al. [104], comprising 4899 cases of congenital anomalies compared to 5742 normal controls, evaluated the risk of fetal malformations in relation to misoprostol exposure [104]. The results showed a 3.6 times increased risk for any malformation in connection with failed abortion. In the same study, the risk for a certain type of limb reduction defects (transverse limb reductions) was estimated to be increased 25 times and the risk for cranial nerve and facial palsy (Moebius syndrome) was increased by 12 times. A few studies estimated the risk also for other subtypes of malformations. In the study by Orioli and Castilla [105], an increase in hydrocephus (4 times), urinary bladder extrophy (47 times), holoprosencephaly - a severe type of neural tube defect (18 times) were reported. Some studies have reported an overall increased risk for malformations after use of misoprostol (2.4–3.8) without estimating the risk for individual subtypes of anomalies; instead the most common malformations observed were mentioned [106,107]. In the study by Brasil et al. [106], the most common malformations in the misoprostol group (in total 77) on the organ system level consisted of neural tube defects, limb defects and cardiac defects. In the study by Barbero et al. [107], 3 out of 5 observed malformations in the misoprostol group, were neural tube defects.

Several studies were focused on characterising the pattern of anomalies associated with abortifacient drug(s), rather than to estimate risks for certain anomalies. Vargas et al., 2000 [101] in a case control study (93 cases exposed and 293 controls), showed that two types of malformations dominated in children exposed to misoprostol: Moebius syndrome (29/93) and limb reduction defects (27/93); the authors suggested a vascular disruption etiology to underly the birth defects. Both these anomalies were statistically increased; overall the results show a “very strong association between congenital defects and the
vascular disruption spectrum and first trimester use of misoprostol” [101]. Identified anomalies associated with misoprostol use in the previously mentioned study [104], in addition to Moebius syndrome and and transverse limb reduction defects, also included meningomyelocele, microcephaly, clubfoot, syndactyly, and distal limb reduction (nail hypoplasia). Auffret et al. [11], reported malformations in the nervous system (hydrocephalus, neural tube defects- including myelomeningoceles and anencephalus, corpus callosum and cerebellar agenesis and brain cysts), limb defects (lower limb reduction defect, absence of fingers and toes, oligodactyly, syndactyly, hexadactyly, club foot and club hand) and Moebius syndrome, and also urinary defects (renal agenesis and congenital mega ureter) and cardiovascular defects (transposition of large vessels). Furthermore, case reports, and case studies with a relatively large number of children [108-114], have described different types of rare major malformations (multiple malformations in several children) observed in pregnancies after use of misoprostol, including VACTERL syndrome and Poland syndrome. The subtypes of human anomalies associated with misoprostol in above reviewed studies [11, 100-114] are summarised in Table 2; the table also shows which subtypes were significantly increased. In addition, Table 3 also summarises subtypes of malformations associated with HPTs in the scientific literature as well in a regulatory document [115] in order to compare the pattern of anomalies reported for HPTs and misoprostol (see section 5.3.4 for details).

Taken together published studies show that misoprostol is a well documented cause of a spectrum of major rare human congenital malformations which are likely to be mediated via uterine contractions and hypoxia-related damage in the embryo. In this context it is relevant to stress that even prescribed low doses of misoprostol have been associated with teratogenicity [11] and that obvious uterine contractions noticed by the mother were relatively seldom; more often (but not in all women) vaginal bleed was reported.

5.1.3. Brief review of results for human abortifacient drugs in animal teratology studies

It is important to mention that the relevant mechanism for induction of teratogenicity in humans, mediated by expulsion of an embryo via uterine contractions (abortion) is nonexistent in rodents during whole organogenesis and in rabbits in early organogenesis. Instead the conceptus undergoes gradual degradation followed by maternal resorption in the uterus. These species differences can explain why no teratogenicity was reported in conventionally designed teratology studies (dosing between GD 6 at implantation and during the whole organogenesis) in rodents or rabbits for misoprostol [116] or mifepristone [117,118], despite these human abortifacient drugs are considered to be associated with risk for human teratogenicity in the case of failed abortion [94,95]. Misoprostol, which causes abortions mainly by contracting the pregnant uterus in humans and monkeys [119]; did not affect the survival of the embryo in rodents [94,116]. Mifepristone caused a dose related increase in early resorptions in rodents as expected in view of its its anti-progesterone action, while in monkeys the anti-progesterone action was manifested by uterine contractions leading to abortions [94]. As a result of the high abortive potential of abortifacient drugs in monkeys, the number of surviving fetuses was too low to evaluate a teratogenic potential [94].

As mentioned previously, rabbits do not abort embryos in early organogenesis. Instead early resorptions occur if dosing starts at implantation (GD 6) and ends on GD18 at the end of organogenesis. This is the conventional design of teratology studies in rabbits. However, if dosing (for 1 up to 5 days) starts with an abortifacient drug at a later stage of organogenesis (on GD 11 in rabbits, corresponding to gestation week 5–6 in humans), both abortions and malformations related to failed abortions can be induced in rabbits as shown by Jost 1986 [120]. The timing and duration of dosing in the study by Jost mimicks the clinical use of abortifacient drugs in humans. In the study by Jost, very similar malformations to those associated with failed abortion after use of an abortifacient drug in humans (see Section 5.1.), were observed in rabbits secondary to failed abortion, including cranial and neural tube malformations (precedes by hemorrhage and necrosis), eye defects and limb reduction defects [120]. Indeed, Jost due to the findings in the rabbit study, already 1986 (before abortifaciant drugs were approved for use in humans), proposed that failed abortion after use of abortifacient drugs is likely to be teratogenic in humans as well [120].

5.2. Oral hormone pregnancy tests

5.2.1. Indications and review of proposed mechanism for teratogenicity

Oral hormone pregnancy tests (HPTs) with ethinylestradiol (EE) combined with a synthetic progesterone norethisterone (NET) or ethisterone (ET) were sold under different trademarks (e.g. Primodos) from 1958 to 1981 in Europe. Human clinical studies conducted during the 1970 s and 1980 s reported an association between use of HPTs and teratogenicity as will be presented below. The test principle of HPTs was that NET or ET given in high doses for two consecutive days result in a rapid spike of binding to the progesterone receptors, followed by a rapid decline. The rapid decline mimics the end of the menstrual cycle, which in non-pregnant women results in the endometrium breaking down and together with menstrual blood, is expelled through the vagina (menstruation bleed). The expulsion process is mediated via uterine contractions. It was supposed that if a pregnant woman used HPTs, she would have high levels of pregnancy-induced progesterone, which maintain pregnancy normally and do not induce a bleed or expulsion process, while use of the HPT in non-pregnant women results in a menstruation bleed [98,115,121].

There is evidence indicating that HPTs have the potential to initiate a failed abortion process, resulting in uterine contractions, bleedings and attempts to expel the uterine endometrium (with the conceptus) in a similar way as when menstruation occurs in non-pregnant women [12]. The plasma concentrations of progesterone in some pregnant women in early pregnancy (range 10–44 ng/ml) are lower than in non-pregnant women (range 2-25 ng/ml) [122]. These results imply that HPTs have the potential, and could be expected, to initiate an abortion process in some pregnant women with lower progesterone levels than in non-pregnant women. This hypothesis is supported by results in a human clinical trial with HPT (ET + EE) conducted by Rawling [12] in Australia. Five out of 66 women with 5–8 weeks amenorrhea using a HPT for two days showed neither menstruation bleed (= “not-pregnant”) nor absence of bleeding (=“pregnant”); rather they showed “spotting” (manifested as brown stainings) and signs similar to in early threatened abortion. Three of these women were subsequently proven to be pregnant; this means that three out of a total of 35 pregnant women (8.6%) using HPTs showed clinical signs such as in early threatened/failed abortion but the pregnancy continued. This failed abortion mechanism is the same as the mechanism proposed to cause teratogenicity for the established human teratogen misoprostol (see Section 5.1.).

Initiation of an abortive process has also been reported in several studies in human-relevant primate animal models after administration of HPTs. Repeated dosing resulted in a low number of fetuses examined at term (4–10 per group) due to high incidences of abortions e.g. 52–60% in Cynomolgus monkeys and 20–67% in Rhesus monkeys [123–126].

5.2.2. Review of anomalies in human studies

Several human epidemiological studies have investigated the teratogenic potential of HPTs. Most of these were conducted during the 1970 s and 1980 s [129,138,140–153]. Some were conducted more recently; 2014 [128], 2017 [127], and 2019 [121]. The main results of these studies with HPTs are summarised in Table 2.

The conducted epidemiological studies show an increase of malformations in the nervous system (neural tube defects, including spina bifida and encephalocele, and anencephalus), cardiovascular system and limbs (particular limb reduction defects of various severity), and
also an increase in orofacial clefts, atresia in the gastrointestinal system (oesophageal atresia), renal defects (unilateral renal agenesis, bladder exstrophy) and a syndrome with combined anomalies such as the VACTRL association. VACTRL is defined when at least three of the following anomalies are present: Vertebral defects, Anal atresia, Cardiovascular anomalies, Tracheoesophageal fistula, Oesophageal atresia, Renal anomalies, and Limb defects. A meta-analysis by Heneghan et al. [121], based on studies conducted in the 1970s and 1980s, showed a statistically significant increase in anomalies at these organ system levels (nervous-, cardiovascular-, musculoskeletal, renal- and gastrointestinal system) as well as combined anomalies, such as VACTRL.

Careful delineation of all observed malformations is a basic principle for evaluation of a suspected teratogen. In this respect, it is important to also study subgroups of malformations; not only an increase at the organ system level. For example, limb amputational/reduction defects of the limbs represent only around 2% all malformations, while limb defects system level. For example, limb amputational/reduction defects of the

<table>
<thead>
<tr>
<th>Congenital anomalies associated with HPTs in epidemiological studies from various countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major limb reduction malformations: 2–3-fold increases in US studies [129–132], 3-fold increase in a Swedish study [133], 30% increase in a Hungarian study [134]. In addition, three other studies (with no control group) proposed a causal association between use of HPTs and limb reduction defects [135–137], the studies were conducted in Hungary and USA.</td>
</tr>
<tr>
<td>Nervous system anomalies (all types): 3–4-fold increases [121,138,139] in UK studies</td>
</tr>
<tr>
<td>- Microcephaly: 6-fold increase [140] and 25-fold increase [141] in two French studies</td>
</tr>
<tr>
<td>- Spina bifida: 6-fold increase [142] in a UK study and 25% increase [132] in a US study</td>
</tr>
<tr>
<td>- Spina bifida and anencephaly: 5-fold increase [143] in Spain, No increase [144] in a UK study, 17% decrease [145] in a Hungarian study</td>
</tr>
<tr>
<td>- Encephalocele: 50% increase [129,130] in an US study</td>
</tr>
<tr>
<td>- Cleft lip +/- cleft palate: 40% increase in a German study [128], 60% increase in a US study [132]</td>
</tr>
<tr>
<td>- Cleft palate: 25% and 75% increase [129,130] in US studies</td>
</tr>
<tr>
<td>- Oesophageal atresia / fistula: 3–4 fold increase [129,130,132] in US studies</td>
</tr>
<tr>
<td>- Unilateral absence kidney: 2.5 fold increase [128] in a German study</td>
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<td>- Bladder exstrophy: 35-fold increase [128] in a German study</td>
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<tr>
<td>- Cardiovascular system anomalies (all types): &gt; 2 fold increase: 131, 146, 147 in US studies, 2-fold increase in a meta-analysis of studies from different countries conducted in UK [121], 3-fold increase in a Greek study [148], 1–2 fold increase [141,149,150] in studies from France, USA and Germany, 40%; decrease in a US study [138]</td>
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<tr>
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</table>

Table 2
Human malformations associated with HPTs in epidemiological studies (see text for details).

5.2.3. Comparison of subtypes of human anomalies associated with use of HPTs and misoprostol

For comparative reasons, the subtypes of anomalies associated with HPTs (see Section 5.2.2. above) and misoprostol in human studies [11, 100–114] (see Section 5.1.3) are summarised and presented in the same table, see Table 3. As shown in Table 3, the pattern of subtypes of HPTs in the EUROCAT analysis is very similar to the pattern of subtypes of malformations reported for misoprostol. Furthermore, most of the malformations are known to be possible to induce by exposing the embryo to transient periods of interrupted oxygen supply to the embryo followed reperfusion/reoxygenation (see Table 1 Section 4), strongly supporting a common mechanism related to embryonic hypoxia/ROS for HPTs and misoprostol (compare Tables 1 and 3).

5.2.4. Review of results in animal teratology studies by HPTs

As mentioned previously (see 5.2.1), repeated dosing in teratology studies in human-relevant primate animal models after administration of HPTs resulted in high incidences of abortions [123–126]. One out four examined fetuses was malformed in the low dose group in a Cynomolgus study [124], showing rib and vertebral malformations of a similar type as associated with failed abortion after use of HPTs in humans. However, the low number of surviving fetuses is insufficient to evaluate a teratogenic potential. Furthermore, the human relevant mechanism for

Table 2
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<td>- Unilateral absence kidney: 2.5 fold increase [128] in a German study</td>
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</tr>
</tbody>
</table>

1. Limbs: limb reduction defects (7-fold), clubfoot, syndactyly (2-fold).
2. Nervous system: neural tube defects (2-fold), including spina bifida (3-fold) and encephalocele (3-fold), hydrocephalus, microcephaly, anencephalus,
3. Orofacial clefts: cleft palate, cleft lip with or without cleft palate (2.5-fold)
4. Ocular system: anophthalmia (18-fold), microphthalmia (6-fold)
5. Cardiovascular defects usually observed at birth: transposition great vessels (2-fold), tricuspid atresia/stenosis (5-fold), hypoplastic right heart (2-fold)
6. Cardiovascular defects usually observed after more than one week after birth: ventricular- and atrial septum defects, Tetralogy of Fallot, pulmonary valve stenosis, coarctation of the aorta
7. Gastrointestinal system: anorectal atresia/stenosis (3-fold), oesophageal atresia/fistula (2-fold)
8. Urogenital system: bladder exstrophy (3-fold), hypospadias
9. Dextrocardia (3-fold) and situs inversus (5-fold)
10. Vascular disruption defects (2 fold)

Several rare combined malformations, not included in the analysis, were observed in the study and include Moebius syndrome, Klippel-Feil syndrome (mainly characterised by vertebrae defects), and combined nervous system and limb defects: 7 out of a total of 44 (16%) showed combined nervous system and limb defects. Unilateral absence of the kidney was observed in 5/44 (10%) and VACTRL association in 5/44 (10%) in children with multiple malformations. Most of defects which were > 2-fold increased are usually detected close after birth according to EUROCAT because they are easy to detect externally (e.g. limb reductions, absence of eye, orofacial clefts or neural tube defect) or result in severe life threatening symptoms, like “blue baby” (transposition great vessels, tricuspid atresia/stenosis or hypoplastic right heart) or result in severe feeding problems (oesophageal atresia/fistula). This indicates that most of the malformations which were increased, are likely to have been detected to a relatively similar extent in pregnancies in the 1960 s and 1970 s (children born after their mothers used HPTs) as during 1980–2014 (EUROCAT register). This is also supported by the fact that the limb reductions, the types of defects which showed the highest proportional increase, have been very stable (around 2%) over the last 40 years in the EUROCAT register.

For comparative reasons, the subtypes of anomalies associated with HPTs (see Section 5.2.2. above) and misoprostol in human studies [11, 100–114] (see Section 5.1.3) are summarised and presented in the same table, see Table 3. As shown in Table 3, the pattern of subtypes of HPTs in the EUROCAT analysis is very similar to the pattern of subtypes of malformations reported for misoprostol. Furthermore, most of the malformations are known to be possible to induce by exposing the embryo to transient periods of interrupted oxygen supply to the embryo followed reperfusion/reoxygenation (see Table 1 Section 4), strongly supporting a common mechanism related to embryonic hypoxia/ROS for HPTs and misoprostol (compare Tables 1 and 3).
increase of the anomaly after use of misoprostol compared to subtypes associated with misoprostol use in humans. Comparison of pattern of subtypes of anomalies associated with HPT exposure in the EUROCAT analysis.

Malformations associated with use of misoprostol in epidemiological studies with misoprostol.

<table>
<thead>
<tr>
<th>Limb defects</th>
<th>Malformations associated with use of misoprostol in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major limb reduction malformations</td>
<td>Major limb reduction malformations in both upper and lower limbs, transverse limb reduction defects, defects affecting upper (e.g. absence of the whole or parts of arms, hands or fingers) or lower limbs (absence of the whole or parts of legs, foot or toes). Phalangeal agenesis or hypoplasia and nail hypoplasia.</td>
</tr>
<tr>
<td>Syndactyly (fusion of finger or toes)</td>
<td>Syndactyly (fusion of finger or toes), polydactyly, clinodactyly, Clubfoot (equinovarus)</td>
</tr>
<tr>
<td>Club foot (equinovarus)</td>
<td>Club foot (equinovarus)</td>
</tr>
<tr>
<td>Nervous system malformations</td>
<td>Nervous system malformations, including spina bifida and encephalocele, and anencephalus</td>
</tr>
<tr>
<td>- Hydrocephalus</td>
<td>- Hydrocephalus, Microcephaly</td>
</tr>
<tr>
<td>- Moebius syndrome - cranial nerve</td>
<td>- Moebius syndrome - cranial nerve palsy resulting in paralysis of the eye and facial muscles (mask like faces),</td>
</tr>
<tr>
<td>Facial malformations</td>
<td>- Moebius syndrome - cranial nerve palsy resulting in paralysis of the eye and facial muscles (mask like faces),</td>
</tr>
<tr>
<td>Ocular (eye) malformations</td>
<td>Ocular (eye) malformations, including Microphthalmia, Anophthalmia, Congenital cataract, and other congenital deformities of skull, face and jaw, Anomaly in jaw - cranial base relationship</td>
</tr>
<tr>
<td>Gastrointestinal system defects</td>
<td>Gastrointestinal system defects, including Obstructive atresia / fistula, Ano-rectal atresia /stenosis, and other congenital deformities of the digestive system, Anomaly in jaw - cranial base relationship</td>
</tr>
<tr>
<td>Urinogenital system defects</td>
<td>Urinogenital system defects, including Renal agenesis (absence of kidney(s)), Urinary bladder exstrophy, and other congenital deformities of the urogenital system, Anomaly in jaw - cranial base relationship</td>
</tr>
<tr>
<td>Genital defects</td>
<td>Genital defects, including Hypospadia, Undescended testes, and other congenital deformities of the genital system, Anomaly in jaw - cranial base relationship</td>
</tr>
<tr>
<td>Laterality anomalies and situs inversus</td>
<td>Laterality anomalies and situs inversus, including Dextrocardia, Situs inversus</td>
</tr>
<tr>
<td>Cardiovascular defects</td>
<td>Cardiovascular defects, including Transposition great vessels, Tricuspid atresia /stenosis Hypoplastic right heart, Ventricular septum defects, Atrial septum defects, Tetralogy of Fallot, Pulmonary valve stenosis, Coarctation aorta</td>
</tr>
<tr>
<td>Severe complex defects observed at birth:</td>
<td>Severe complex cardiac defects observed at birth: Children with severe cardiac defects were reported to not survive (see text)</td>
</tr>
<tr>
<td>Ventricular septum defects, Atrial septum defects</td>
<td>Cardiac defects observed after more than one week after birth</td>
</tr>
</tbody>
</table>

Table 3 (continued)

Malformations associated with use of misoprostol in the EUROCAT analysis

<table>
<thead>
<tr>
<th>Syndromes with combined anomalies</th>
<th>Malformations associated with use of misoprostol in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>VACTR association, Moebius syndrome, Kippel-Fel syndrome</td>
<td>Ventricular septum defects, Atrial septum defects, Pulmonary valve stenosis, Patent arterial duct</td>
</tr>
<tr>
<td>Several combined anomalies, the most prominent are combined central nervous and limb anomalies</td>
<td>VACTR association, Moebius syndrome, Kippel-Fel syndrome, Several combined anomalies, the most prominent are combined central nervous and limb anomalies</td>
</tr>
</tbody>
</table>

Table 3
Comparison of pattern of subtypes of anomalies associated with HPT exposure compared to subtypes associated with misoprostol use in humans. Italian bolded text indicate at least two times proportional increase of the anomaly compared to what could be expected in EUROCAT (HPT data set) or a significant increase of the anomaly after use of misoprostol versus a control population in epidemiological studies with misoprostol.

induction of teratogenicity in humans mediated via failed abortion is non-existing for HPTs in early organogenesis in rats, mice and rabbits since dead embryos are resorbed within uterus at an early stage of organogenesis in conventionally designed teratology studies (dosing starts at implantation on GD 6). These species differences can explain why a number of teratology studies in rodents and rabbits exposed to HPTs did not show any clear evidence of an increase in malformations but a clear evidence of an increase in resorptions (~embryonic death) at human relevant exposures of HPTs [126]. The results also indicate that conventionally designed teratology studies in mice, rats and rabbits are not relevant for detection of human teratogenicity mediated via a failed abortion process associated with use of HPTs in the same way as shown for the human abortifacient drugs like misoprostol and mifepristone (see 5.1.3. above).

5.3. Uterine manipulation due to chorionic villus sampling (CVS) in humans

CVS is an established risk factor for malformations in early pregnancy, and due to this risk, FDA in US recommends that CVS should be delayed until the 10th week of pregnancy. CVS comprises taking a sample of chorionic villus cells from the placenta by a needle at the point where it attaches to the uterine wall. The pattern of the prominent malformations associated with CVS in the literature [156–158] is very similar to the pattern of the most prominent malformations associated with HPTs and misoprostol and consist of:

1. Major limb reduction defects, ranging from hypoplasia /aplasia of distal phalanx or nails, partial (brachydactyly) or complete (acatly) absence of fingers or toes, severe limb reduction defects, and missing the whole limb (amelia) or distal parts of defect limbs and transverse terminal limb reduction defects but also syndactyly, clubfoot and arthrogryposis.
2. Major nervous system defects; Neural tube defects, including spina bifida encephalocele, hydrocephalus, microcephaly, corpus callosum agenesis.
3. Major craniofacial malformations: Moebius syndrome (cranial nerve palsy resulting in paralysis of eye and facial muscles), Cleft lip alone or in combination with palate, cleft palate, oromandibular hypoplasia, including underdevelopment of jaw (micrognathia) and other effects in the urinary- (renal agenesis, double monolateral ureter), the cardiovascular- (cardiac septal defect) and digestive systems (omphalocoele) were reported in these studies.

The sequence of events after the CVS procedure resulting in malformations has been proposed to be related to uterine cramping and bleeding in the placenta, followed by decreased utero-placental blood flow, resulting in generalized embryonic hypoxia and vascular disruption in the embryonic tissues with subsequent hemorrhage, necrosis and tissue loss [157,159]. The type of limb reductions associated with CVS have been studied in relation to timing; and Firth et al. [160,161],
showed that proximal defects (limbs ending at the upper arm or thigh level) were found to be associated with CVS performed before 60 days of pregnancy (= days 44 of human embryonic development), whereas toe and finger defects or nail defects (distal defects) were associated with CVS around 70 days of pregnancy (= day 54 of human embryonic development). The risk is considered to be highest in early pregnancy (7–8 weeks =5–6 weeks of embryofetal development), but malformations have been observed as early as the 4th week [160].

5.4. Uterine manipulation or trauma in human pregnancy

The procedure dilation and curettage at weeks 6–10 of gestation has been associated with the occurrence of extensive malformations, including limb reduction defects, orofacial clefts, brain malformations and heart defects [162] and club foot [163] Incomplete uterine curetage at 7 weeks gestation resulted in induction of oro-mandibular limb hypogenesis at birth [164]. It was proposed by Holmes et al., 2018, that the circulation in the exposed embryo was compromised by the unsuccessful curettage procedure causing periods of embryonic hypoxia, but the pregnancy continued [165]. Severe limb reductions (amputation of legs and one arm occurred) after the mother had uterine lavage performed at week 7–8 of gestation to obtain cells for sex determination of the embryo [85]. Abdominal trauma (e.g. car accident) in pregnancy has been associated with hypoxia-related teratogenicity in human pregnancy, particularly limb reductions [165], Lipson et al. [93] reported that 8 out of 15 cases of Moebius syndrome in Australia were associated with potential trauma during pregnancy, and limb defects of varying severity were present in 13 of 15 of the cases. The authors concluded that any event that interferes with uterine/fetal circulation could cause teratogenicity.

6. Concluding remarks

Altogether, as shown in Fig. 4, which summarises the results in the current review article, transient interruption of oxygen supply to the embryo of varying origin is teratogenic across species (including the human embryo). As also shown in Fig. 4, there is strong evidence teratogenicity is highly likely to be directly related to ROS formed when oxygen returns (reoxygenation) to hypoxic tissues in the embryo.

Hypoxia itself can generate ROS, but particularly when oxygen return to severely hypoxic tissues during reperfusion/reoxygenation after a period of interrupted oxygen supply, it results in a massive burst of ROS which overwhelms the antioxidant defenses (mainly catalase, glutathione peroxidase and SOD). This results in indiscriminate damage; the longer the hypoxic period, the higher the generation of ROS during reoxygenation. There is also evidence that the embryo may represent a state of which antioxidant defenses and the susceptibility to injury induced by the generation of ROS is high as proposed by Fantel and colleagues in several studies [31,47–51]. The results also suggest that the developing cardiovascular system is the primary target for generated ROS. ROS generation in the vascular endothelium of recently formed arteries, resulting in vascular disruption/hemorrhage is likely to underlie a wide variety of stage specific malformations in embryonic tissue/s/ organs, which normally should have been supplied by the artery. Also, the embryonic heart, from when the tubular heart starts beating until the four chamber heart has been formed, is susceptible to ROS. ROS is likely to induce periods of severe irregular rhythm and cardiac arrest as well as severe alterations in blood flow and blood pressure in the embryo; these alterations are likely to underlie the wide pattern of cardiovascular defects associated with transient hypoxia of varying origin in both human and animals. Furthermore, there is evidence that the ROS induced severe irregular rhythm in embryos further aggravates the embryonic hypoxia (see Fig. 4).

Finally, even if transient hypoxia has been known to be teratogenic in animals since the 1950 s (by decreasing the oxygen content in the atmosphere) and the 1960 s (by clamping of uterine vessels), it is not until more recently, when mechanistic information became available on the teratogenic effects of excessive ROS generation in embryo, that it became apparent that a delicate balance between oxygen tension and ROS is necessary for normal left-right axis of organs. Furthermore, it is not until the last 10 years that transient hypoxia has been considered likely to be teratogenic in humans; this is mainly based on scientific data (including pattern of malformations) in newborn babies surviving a failed abortion process when the mother used misoprostol in organogenesis. The data in our review clearly show the similarities in subtypes of specific malformations between failed abortion in humans after use of misoprostol and hypoxia of various origins in animal studies. The almost identical pattern of anomalies associated with Hormone Pregnancy Tests

Fig. 4. Schematic drawing (based on the results in this review) on a proposed common ROS mediated mechanism for how a period of transient hypoxia of various origins in the embryo has the potential to cause specific subtypes of anomalies across species, including humans.
(HPTs) and misoprostol, strongly indicate that HPTs also should be considered teratogenic in humans by a similar pathogenesis as misoprostol in some pregnant women: after initiation of an abortion process with uterine contractions but the embryo survives (failed abortion) – compression of uteroplacental/embryofetal vessels results in periods of interrupted oxygen supply to the embryo and generation of toxic ROS during reoxygenation. In this respect, it is important to note that HPTs were used in the 1960 s and 1970 s, a long time before misoprostol was registered as a medicinal product and known to cause teratogenicity via a failed abortion mechanism. Finally, the similar types of prominent human malformations observed after Chorionic Villus Sampling and uterine manipulation (including failed abortion after curettage and abdominal trauma) as described for HPTs and misoprostol further support that transient embryonic hypoxia of various origins can cause teratogenicity across species, including humans.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bengt Danielsson reports a relationship with Bayer AG that includes: consulting or advisory. Bengt Danielsson reports a relationship with Canadian Govt that includes: consulting or advisory. Neil Vargesson is a member of the editorial board of Reproductive Toxicology. Neil Vargesson reports a relationship with Canadian Govt that includes: consulting or advisory. Neil Vargesson is a member of the editorial board of Reproductive Toxicology.

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