

Critical Periods of Development in Teratology

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Abstract

Several years ago Wilson (1973) postulated his principles of teratogenesis, which have been revisited previously. However, there is a strong need to evaluate critically and summarize recent advances in the area of susceptible periods in teratology. This review focused on three model teratogens, namely ionizing radiation, congenital rubella, and retinoids. Continued presence of rubella virus in the placental and fetal tissues suggests an extended period of susceptibility to abnormalities of lens and otocyst differentiation beyond the narrow window of embryonic period. The currently understood critical period for maternal radiation exposure-related microcephaly in the offspring has been derived from Hiroshima and Nagasaki experience. Data on pregnancy complications of radiation-exposed childhood cancer survivors is alarming. Cumulative use of diagnostic CT imaging and the associated ionizing radiation burden are reported to have a lifetime attributable cancer risk. This coupled with experimental data on radiation effect on neurogenesis in the cerebellum and that on global gliogenesis in the CNS is a reason to revisit the critical period definition for microcephaly in the offspring of mothers exposed to radiation. Both retinoic acid and valproic acid studies in experimental animals have demonstrated unequivocal evidence for the existence of more than one susceptible period for skeletal defects and cleft palate. It is important to realize the distinction between the “critical period for a malformation” and the “critical period for an organ as a whole.” Vascular disruption and post-closure neural tube defects have been observed to occur far beyond the embryonic period of development. It is now clear that the textbook description of the embryonic period as the window of susceptible period is rife with problems. In the light of newer advances in teratology, a second look at our definition of teratogenic manifestations and sophistication of methods of ascertainment of abnormal development will help us develop effective preventative strategies in the field of birth defects.

Keywords: Teratology, Critical periods, Ionizing radiation, Congenital rubella, Retinoids, Microcephaly, Cataract, Therapeutic radiation, Childhood radiation, Neurogenesis, Gliogenesis, Vascular disruption, Post-closure neural tube defects

1 Introduction

According to CDC (Center for Disease Control, Atlanta) congenital malformations are “common, costly and critical” [1]. About 1 in 33 births in the USA is affected by birth defects which amount to 120,000 babies. The European Surveillance of Congenital Anomalies [2] estimates the prevalence rate of all congenital malformations in Europe, excluding chromosomal anomalies as 219.7 per 10,000 births (2.2 %) for 2003–2007. In certain parts of Africa where antenatal care is unavailable the prevalence rate is reported to be as high as 14 % [3]. Congenital malformations and chromosomal

anomalies are of serious public health issues because they cause infant mortality, childhood morbidity, and long-term disability in survivors. They are costly. They are a burden on the economy, families, and community as well as a leading cause of years of potential life lost [4]. Congenital malformations, such as neural tube defects (NTD), are not only highly prevalent, particularly in lower and middle income countries, but they also cost significant amounts of money to the communities that are already economically strained. Yi et al. [5] reviewed studies on economic burden of NTD covering the period 1976–2010, evaluated the direct and indirect costs of care required by affected individuals, and concluded that the benefits of prevention of NTD with maternal folic acid supplementation far outweighed the cost of care required by NTD patients. A recent study looked at 37 previous studies on the burden of NTD among middle and lower income countries and analyzed data on live births, still births, and terminations and estimated that a total of 190,000 neonates were added each year to the NTD burden in those countries [6]. The cost of raising a child with spina bifida from birth to the age of 18 years is estimated to be about \$120,000 in the USA. Even greater amounts of money are spent in caring children with open spina bifida in developed countries in Europe [7]. Although not all NTD could be prevented, folate fortification and periconceptional folate supplementation have significantly reduced the incidence of NTD and improved cost saving in developing countries [8]. About a third to one half of all pregnancies are estimated to be unintended [9, 10]. It is not possible to assume that this section of women are on supplemental folate or would take care not to consume alcohol or refrain from smoking. Intended or unintended pregnancies are costly. Pregnancies complicated by situations requiring surgical procedures, preterm births, multiple births, intrauterine growth restriction (IUGR), etc. cost significantly more. Now that the beneficial effects of folate is known, there is however a concern that the probability of vitamin B12 deficiency might go unnoticed due to the masking effects of folate on B12 deficiency-related anemia and the consequent neurological damage to the patients [11]. Maternal smoking during pregnancy is known to cause orofacial clefts affecting about 6 % of births. If smoking during pregnancy is prevented, it can result in an estimated saving of \$40.4 million for each annual cohort of 430 averted cases [12]. Other pregnancy outcomes that are of interest to teratologists include preterm births and small for gestational age (SGA) births. Complications associated with preterm births are attributed to be responsible for 35 % of the world's 3.1 million neonatal deaths estimated annually [13]. Preterm birth rates are increasing in economically affluent nations. Both SGA and preterm births account not only for significant proportions of perinatal mortality and morbidity but they are also a major challenge for maternal and perinatal care globally [14]. Preterm birth rates are

reported to be increasing in developed countries [15]. Teratologists should also be equally concerned about the high prevalence rates of intrauterine growth retardation (IUGR) which is often complicated by accompanying congenital malformations. Growth restriction may be primary predisposing the fetus to malformations or secondary to the malformation or share a common etiology with the coexisting physical anomaly [16]. There is a strong body of evidence supporting the association between stillbirth, IUGR, and congenital malformations. In fact IUGR is clinically employed for the stillbirth prediction. Stillbirth and IUGR are reported to have overlapping etiology and risk factors [17, 18]. Individuals who are growth restricted in utero are now known to develop obesity, type 2 diabetes, and/or cardiovascular diseases later during adult life possibly due to hypomethylation of DNA and other epigenetic modifications [19–22].

To eliminate or minimize the huge economic burden of congenital malformations, prematurity, and IUGR to the affected individuals, their families, and the health care system, efforts need to be directed at prevention. Planning preventative strategies in dealing with the issue of congenital malformations, small for gestational age (SGA), and IUGR requires a thorough understanding of the normal processes of development and the causes, molecular, cellular, and morphological mechanisms and both anatomical and functional manifestations of abnormal processes of development. Historically speaking, people have responded to the birth of a malformed baby with fear, awe, and deep concern. The literature on the history of congenital malformations includes descriptive accounts often stemming from mythology with etiology attributed to maternal impressions during pregnancy, the position of stars, divine retribution for wrong doing, etc. However, concurrently there has always been some form of scientific inquiry attributing congenital anomalies to various causes including fetal distress, arrest of embryonic growth and interference with development by chemical substances, and mechanical factors.

William Harvey (1561) (cited by Wilson [23]) first advanced his theory of developmental arrest meaning that developmental anomalies observed in newborns represented the appearance of the organ primordia at the time point when development was interfered with by an environmental agent. Etienne Geoffroy Saint-Hilaire (1772–1844) and his son Isidore Geoffroy Saint-Hilaire (1805–1861) studied embryology, comparative anatomy, and experimental teratology in an effort to explain congenital malformations. They designed experiments on chick embryos to alter development by various physical manipulations including high and low temperatures. This was possibly the first recorded scientific inquiry into environmental effects on embryonic development. The results of their studies helped shift the focus from mythology to an environmental etiology of abnormal embryonic development and thus teratology became a “special branch of the greater science

of organization” [24–26]. This work was extended by Camille Dareste’s experiments in which developmental stage-dependent monstrosities were induced in chick embryos by mechanical stimulation with heat, cold, shock, etc. during incubation [27]. Dareste believed that his experimental manipulations somehow slowed down or caused arrest of the normal developmental processes. Dareste’s work appears to have deeply impacted Stockard [28] who experimented with the common minnow (*Fundulus heteroclitus*) embryos by subjecting them to variable temperatures or variable concentrations of O₂ and CO₂, thus causing development of the embryos to slow down (by moderately low temperature, for example) or to stop (by very low temperature, for example) for given periods of time after which restoring them to the normal environment. Depending on the developmental stage at which the environment of the embryos was first altered, embryos with too slow rates of resumption were found to result in significant malformations while stoppage was followed by high mortality. Some periods (“moments”) of development when by experimental manipulation developmental arrest or interruption could be caused without embryonic death or malformations were classed as *indifferent moment*. “*Moments of supremacy*” were when primordia of organs were forming; these were developmental stages which could not be arrested without causing serious effects on subsequent development. Such sensitive periods constituted “*critical moments*” for Stockard. He claimed that all types of malformations not of hereditary origin were simply the result of developmental arrest. This belief led him to the following general principles (“or propositions”) of abnormal development (quoted from Stockard [28]):

- “All types of monsters may be caused by the one and the same experimental treatment
- Any one type of monster, such as cyclopia may be produced by a great number of different experimental treatments
- All effective treatments tend primarily to lower the rate of development
- The type of monster induced depends upon the particular developmental moment or moments during which the developmental rate was reduced”

Stockard’s propositions and other theories of birth defects, such as “teratogenic termination period” (of Schwalbe) and “critical moments” (periods) of Stockard [28] were possibly deeply influenced by Dareste’s postulates of teratogenesis, which are (quoted from Jelineck [24, 28]) as follows:

- “Identical defects can be induced by administering different agents
- Particular embryos react to an adverse condition in dissimilar ways

- The dissimilarity is caused by unequal combinations of inherited gifts (genes) and extrinsic influences
- Type of defect depends upon the strength and time of action of adverse impulse
- The smaller the defect, the later it will become apparent”

For these reasons Dareste is regarded as the founder of experimental teratology, although Dareste himself bestowed that distinction and honor upon Etienne St Hilaire. There is a short but excellent summary account of the history of teratology in the first half of 1900 [29].

Although Mendel postulated his theory of inheritance in as early as 1865, it was only in 1900 his postulates became widely known when Hugo de Vries, Carl Correns, and Erich von Tschermak-Seyenegg also arrived at the same conclusions independently and unaware of Mendel’s work [30]. Soon the medical community started easily attributing almost all birth defects to a genetic cause. Subsequently the discovery that maternal exposure to ionizing radiation, maternal rubella infection, and maternal dietary excess and deficiency of micronutrients during pregnancy could cause congenital anomalies in the offspring convinced the scientific community beyond doubt that malformations might arise as a result of several non-genetic causes. The situation began to change rapidly and extensively when Warkany and his group published results of a series of experiments they conducted in laboratory animals showing that maternal nutritional deficiencies could induce predictable frequencies of fetal malformations. The thalidomide embryopathy reported in 1960s [31, 32] was an unexpected catastrophe which took the teratologists around the world by surprise. It brought to light the possibility of “innocent” prescription drugs also being highly teratogenic to human embryos. It led to rapid expansion and greater scope of the field of teratology and reproductive toxicology. Some knowledge of prenatal development is important in understanding the concept of embryonic susceptibility to develop malformations.

2 Developmental Periods

Clinically pregnancy is commonly divided into three sequential trimesters, each lasting 3 months. Developmentally the transition from one trimester to the next is not abrupt but a continuum. Some embryologist would divide development into three periods, namely the period of implantation, embryonic period, and fetal period, though clinicians who also pay greater attention to developmental-stage-specific morphogenetic processes would divide development into (1) blastogenesis—up to 2 weeks postconception, (2)

organogenesis—end of week 2 (or beginning of week 3) to end of week 8 postconception, (3) fetogenesis—early (9–15 weeks), mid (16–25 weeks), and late (26–38 weeks) fetal periods [33]. The highlights of the first trimester include events such as implantation, differentiation of the inner cell mass into three germ layers, craniocaudal and lateral foldings of the embryo, embryonic body formation, organogenesis, fetal and placental membranes development, and establishment of a functional circulatory system. During the second and third trimesters, the organ primordia continue to grow and elaborate and differentiate in complex ways so that the embryonic organ systems attain a certain level of functional maturity. The fetus as a whole grows in size and with time attains definitive body proportions. Keeping pace with the fetus, the placenta grows in size, differentiates further, and secretes a myriad of substances that are of major metabolic and developmental significance. The placenta plays an active role in materno-fetal exchange processes and supports fetal growth. Although short lived, in terms of its structural complexity and functional diversity the placenta is possibly the most versatile of all organs. Intrauterine development and growth are tightly controlled both spatially and temporally by a series of morphoregulatory processes but remain subject to epigenetic modifications [34, 35]. Developmental control mechanisms are not invincible to environmental influences. The sensitivity of the embryo to the modifiers appears to differ at different time points during development and is species specific [36, 37].

3 Principles of Teratogenesis

Based on his own observations in his experimental studies and critical evaluation of the literature of his time Jim Wilson, one of the founding fathers of Teratology Society, first formulated his five Principles of Teratology in 1959 [38], which he subsequently revised and restated in his monograph *Environment and Birth Defects* in 1973 [23] and in other publications. For the purpose of understanding of our readers, Wilson's Principles of Teratology [23] are stated here:

1. "Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors
2. Susceptibility to teratogenesis varies with developmental stage at the time of exposure to an adverse influence
3. Teratogenic agents act in specific ways on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis)
4. The access of adverse influences to developing tissues depends on the nature of the influence

5. The four manifestations of deviant development are embryonic death, malformations, intrauterine growth restriction and functional deficit
6. Manifestations of deviant development increase in frequency and degree as dosage increases from the no effect to the totally lethal level”

Since Wilson (1973) enunciated the Principles of Teratology [23], the field of Teratology has had significant advances in keeping pace with the progress in genetics, molecular biology, and developmental biology and we have come to realize how the recent advances in the realm of teratogenic mechanisms have only validated and strengthened further the scientific bases of Wilson’s Principles of Teratology. This review focuses on developmental periods of embryonic susceptibility to exposure to adverse influences.

Charles Stockard (C R Stockard 1879–1936) made some milestone contribution to developmental biology, aging, alcohol effect on embryonic development, etc. However, his most outstanding contribution to teratology is the concept of “critical moments” which is based on the results of his experiments with fish embryos [28, 39]. Although many of us teratologists who belong to the post Warkany-Wilson era do not agree with all that Stockard published, there is no denial that Stockard is a milestone in the ontogenesis of the “Principles of Teratology” and that he will continue to be cited in teratology literature. At a time when there weren’t electron microscopy and PCR, simply by subjecting the eggs of minnows and trout to cold temperatures Stockard [28] produced a variety of cyclopic and two headed embryos and observed that the window of opportunity to produce these anomalies was limited to the first 24 h after fertilization of the egg. If the exposure was delayed beyond 24 h nothing remarkable happened. He called these moments “moment of supremacy” and “moment of indifference,” respectively, which later became referred to as “sensitive/critical period” and “refractory period,” respectively. The concept of “critical moments” now widely referred to as “critical periods” and Schwalbe’s theory of “teratogenic termination periods” possibly inspired Wilson [23], thus leading to his postulate #2 “Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.”

4 Validating the Principle of Critical Period

It is outside the scope of this review to discuss critical periods in relation to all teratogens. It will discuss critical periods in relation to *a physical teratogenic agent*—ionizing radiation, *an infectious teratogenic agent*, rubella, and *a chemical teratogenic agent*—the retinoids.

4.1 Ionizing Radiation

Ionizing radiation is possibly the earliest and extensively researched example of human teratogens [40, 41] thought to be in support of the concept of Wilson's postulate on developmental stage-dependent susceptibility to teratogenesis of human embryos. Radiation-affected pregnancies of the Nagasaki-Hiroshima atomic bomb explosion episodes highlighted for the first time the deleterious maternal and fetal complications of large doses of radiation. Congenital malformations and fetal growth restriction observed subsequently in numerous pregnancies exposed to radiation for diagnostic and therapeutic reasons were described to be development stage dependent [42–44]. Humans are exposed rather continuously to low doses of radiation and at low dose rates. The data of vast majority of reports published over the past 100 years on reproductive outcome of radiation-exposed human pregnancies come from Hiroshima and Nagasaki involving high doses emanating directly from atomic bomb explosions. Otake et al. [45] reviewed this literature and suggested 8–15 weeks of post-fertilization period as the most sensitive period for severe mental retardation in the offspring of women exposed to radiation when they were pregnant. Several investigators seem to tacitly approve a low-dose radiation-sensitive period of 8–15 weeks after conception too [33, 46]; however, they also suggest that accumulation of radiation effects via repeated-diagnostic procedures in a pregnant woman could result in microcephaly and severe mental retardation in the offspring. Epidemiologic and laboratory animal studies with an acute dose of 1 Gy [47, 48] suggest an “all or none effect” during preimplantation period, malformations, growth restriction, and microcephaly for organogenesis period and only IUGR and microcephaly for fetal period. Epidemiological studies commonly report microcephaly and mental retardation occurring in children following maternal exposure to radiation. The critical periods referred to in these studies vary extremely (gestation weeks 4–19; 8–15; 16–25, etc.; see for references De Saints et al.[47]), primarily because the doses and frequency of radiation exposures were neither consistent nor comparable across studies possibly dictated by individual patient needs. Surveying volumes of literature as well as 27 cases of pregnancies irradiated for pelvic diseases, Dekaban [44] drew a tentative timetable of abnormalities showing the period of 3.5–20.0 weeks of gestation as the critical period of radiation-induced fetal malformations and IUGR. He noticed that with advancing gestational age, there was a progressive decline in the incidence of irradiation-related malformations and reduction in severity of IUGR. Apparently this timetable is in sharp contrast to the developmental stage- and dose-dependent organ system-specific malformations he observed in a mouse study he did with 200 R dose of irradiation administered on each consecutive day between 7th and 18th days of gestation. The data from this study are similar in many respects to those of numerous experimental

studies reported in the literature. A common inference from these experimental animal studies and human clinical studies is a warning that the application of the concept of critical period for radiation-induced malformations is rife with problems and pitfalls. In the development of the nervous system, neurogenesis of the cerebellum and dentate gyrus of the cerebrum as well as global gliogenesis in CNS in particular are events that are largely late gestational and early postnatal in timing; these observations are reinforced by robust data from experimental studies that substantiate the fact that proliferative and migratory cell populations are vulnerable to environmental and nutritional interferences. Robust experimental data are available showing that low-dose radiation of late gestational and young postnatal animals gives rise to CNS morphological and behavioral abnormalities [49–53] and therefore careful interpretation of the structural and functional consequences of irradiation at different time points in life of the organism and our definition of what constitutes a teratogenic outcome (spontaneous abortion or resorption in lab animals, gross anatomical malformation, growth restriction, or functional disorders) [54]) will help us define “sensitive period/critical period or teratogenic termination period vs. period of organogenesis.” Any conclusions on critical period for radiation would only make sense if one takes into account the dosage—acute vs. chronic exposure, maternal and fetal genetic makeup, concurrent exposure to prescription and nonprescription therapeutic agents, maternal nutritional status, etc. Also important to realize here is the distinction between the “critical period for a malformation” and the “critical period for the organ as a whole” [54]. In the case of brain open defects of the neural tube such as anencephaly might occur over a narrow window of time during neural tube closure, whereas neuronal/glial cell deficits might result from influences that interfere with cell proliferation, differentiation, and/or migration that characterize longer windows of growth spurts later in development [55]. Defining such differential susceptibility in humans is complex. Recognition and appreciation of this critical vulnerability as an essential aspect of development will benefit significantly if data from well-controlled animal experiments are taken advantage of for comparison with human development as described by Clancy et al. [56, 57].

One another body of data that has not received much attention of the teratologists is the time- and activity-dependent metabolic/functional maturation of the different regions of the brain. Studies of cerebral glucose utilization with positron emission tomography (PET) have revealed the existence of progressive metabolic maturation process that starts at 2–3 months of age and persists until ages of 16–18 years. The periods of neuronal and glial proliferation, progressive synaptogenesis, production of neurotransmitters, and various receptors are followed by postnatal periods of selective apoptosis. Surviving neurons also go through a pruning phase

characterized by selective synaptic elimination and repeated activity-dependent stabilization of certain circuits, thus molding an optimum cytoarchitecture of the CNS appropriate for the future needs of the individual [58, 59]. Patients subjected to radiologic evaluations or treated with radiation for neoplastic diseases become exposed to amounts of radiation that are commonly assumed to be safe [60]. Computer tomography (CT) scans, by virtue of their ease of acquisition of serial multiple images in quick succession and high levels of diagnostic capabilities, have revolutionized medical practice throughout the world. However, a small percentage of obstetric and gynecologic physicians have always suspected that abdominal scans use unrealistically high levels of radiation that might harm pregnancy. For example, a chest CT scan uses about 100 times greater amount of ionizing radiation than a lateral or AP chest X-ray. Although CT only accounts for about 17 % of all medical imaging, it contributes to about 50 % of clinical imaging-derived radiation burden [61]. High-speed multiphase image acquisition results in increasing amounts of ionizing radiation exposure. Abdominal and pelvic CTs outnumber CTs of other regions of the body. More recently there has been an alarming numerical increase in the use of CT in patients of age 1–18 with a lifetime attributable cancer risk [62]. Increased risks of infertility, miscarriage, stillbirth, intrauterine growth restriction, and preterm birth have been reported in studies that looked at pregnancies of radiation-exposed childhood cancer survivors [63, 64]. These deleterious later effects of childhood exposure to radiation strongly indicate the need for further modifications of the concept of susceptible periods in teratology to include reproductive outcomes of childhood and pre-pregnancy exposures.

4.2 Maternal Rubella Infection

Maternal infections during pregnancy are attributed to be causally related to about 3 % of total malformations observed during the first year of life [65]. This figure has to be cautiously interpreted in view of the fact that rate of intrauterine infections during pregnancy is estimated to be as high as about 14 % when appropriate laboratory investigations are used for diagnosis [66] and that syphilis and rubella infections are associated with a high incidence of congenital anomalies in the offspring; in fact about 64 % women report to have had one or more infections during their pregnancies [67]; some congenital abnormalities, such as deafness and eye defects due to maternal infections, are not detected until the second year of life or even later [68]. Two German physicians (de Bergean and Orlow) are credited to be the first to describe the clinical manifestations of rubella [69]. Although popularly known as German measles based possibly on this German connection, there was some confusion about the relationship of rubella to measles and to scarlet fever. It was yet another German physician, George de Maton, who for the first time characterized rubella in 1814 as a distinct disease

entity, called “rotheln” [69–71]. Henry Veale, a British Army surgeon, is said to have named the disease rubella (from the Latin for “reddish things,” the rashes) in 1866. He thought the original word “*rotheln*” sounded “harsh and foreign to our ears.”

Rubella virus (RV), a member of the family *Togaviridae* and the only member of the genus *Rubivirus*, is transmitted via respiratory aerosols. The nasopharyngeal mucosa of the upper respiratory tract and the underlying lymphoid tissue are the initial sites where RV replication occurs. The upper deep cervical regional lymph nodes subsequently become involved. Although initially mild, and often asymptomatic, macropapular rashes appear within 2–3 weeks after exposure. The rashes appear first on the face and spread rapidly to the trunk and limbs. Patients develop mild fever, pharyngitis, lymphadenopathy, arthritis, and arthralgia with non-immunized women being affected more frequently than men. More serious complications such as progressive rubella panencephalitis have also been reported in some of the RV-infected patients.

Historically there was a worldwide epidemic rubella infection in 1940 and in 1941, Norman McAlister Gregg [72], an Australian ophthalmologist reported for the first time a high frequency of congenital cataract, reduced pupillary reaction to light, atrophic iris and congenital heart defects comprising patent interventricular foramen, and patent ductus arteriosus in newborn children [71, 72]. To his meticulously recorded history of 13 cases were added 65 cases of infants with cataract collected by his fellow physicians. Gregg and his fellow physicians thought of several etiological factors including the German measles that preceded the birth of numerous infants with a recognizable spectrum of congenital abnormalities, termed congenital rubella syndrome (CRS). Similar cases were reported subsequently from Japan, Europe, and the USA. Greg’s report [72] showed for the first time that a virus could be a human teratogen. The rubella pandemic that occurred in the USA from 1962 through 1964 resulted in approximately 12.5 million cases of clinically established rubella, 11,000 fetal demise, 20,000 live born infants with CRS, and 2100 neonatal deaths due to congenital anomalies that constitute congenital rubella syndrome (CRS). The estimated cost to the national economy was approximately \$2.0 billion [73, 74]. CRS is the cause of a preventable spectrum of malformations that comprises ophthalmic (cataract, microphthalmia, glaucoma, unusual type of nystagmus (of “course, jerky, purposeless, searching type”)), cardiovascular (atrial and ventricular septal defects, widely patent ductus arteriosus, pulmonary stenosis, and coarctation of the aorta), and neurological defects (microcephaly, mental retardation, and sensory neural deafness), and intrauterine growth restriction [75]. Other complications that become discernible later in the lives of these children include mental retardation, glaucoma, retinopathy, type 1 diabetes mellitus, thyroid dysfunction, and non-affective psychosis [76–81]. The anomalies that constitute CRS also contribute to miscarriages and infant mortality in the first 2 years of life [82].

With the implementation of an effective rubella vaccine program, CRS prevalence has become most significantly reduced in USA, continental Europe, Australia, and Japan; the reduced frequency in CRS is attributed to (1) a reduction in RV infection and (2) early termination of pregnancy when CRS is diagnosed. The other side of the issue however is that it continues to be a major health concern in countries where the rubella vaccine program is unavailable. A relatively recent estimate indicates that about 100,000 infants are born with CRS annually worldwide [83, 84]. With the advent of technological development and progressive emigration from countries without a routine rubella vaccine, there seems to be a global reemergence of the disease; however small the increase might be, CRS remains to be the most preventable congenital disease [85, 86].

4.2.1 *Susceptible Periods of CRS*

Rubella virus is known to cross the placenta [87–89]. Numerous studies have established the teratogenic effects of RV and the fact that most children with CRS have more than one defect [90–92]. The virus replicates in cell culture, disrupts cytoskeleton, impedes cell cycle, and causes mitochondrial changes and subsequently apoptosis of infected cells [93]. It infects and interacts with embryonic cells. It also slows down cell proliferation and induces apoptosis via complex mechanisms in infected organs. Both immunologic immaturity of the embryo and developmental stage are reported to influence the extent of teratogenic outcome following maternal primary rubella infection. The virus is reported to be present in the placenta during maternal viremia even before the rashes appear and the virus can be recovered from the placenta and from fetal tissues after therapeutic abortions, at birth in live fetuses and in about 10 % of cases at 1 year after birth. It causes necrosis of the trophoblast, progressive villus inflammation, and damage to endothelial cells of the chorionic vessels of the placenta [94, 95]. Microscopically vascular lesions in CRS patients are described as extensive local proliferation of tunica intima observed near the branching points [96, 97]. Rubella virus establishes persistent infection in the embryo, continuously replicating and instituting histopathological changes. Pathological modifications of fetal endothelial cells of the developing heart and great blood vessels are said to lead to cardiac septal defects, patent ductus arteriosus, and pulmonary stenosis. Intrauterine growth restriction (IUGR), congenital deafness, and neurodegenerative lesions are also described to result from RV-induced vascular insufficiency and fetal nutrient deprivation [98]. Continued presence of the virus and consequent fetal and placental vascular pathology might not only cause focal tissue damage and growth restriction [98, 99], but also complicates any conclusions drawn about critical periods of susceptibility to malformations in maternal rubella.

Reviewing the literature on clinical delineations of CRS, it becomes obvious that the early reports [71, 100–102] meticulously described the epidemiology, virus-induced embryo pathology, and phenotype of the infants; but their focus was not on sensitive periods. However, subsequent studies that looked into the temporal relationships of maternal rubella infection and congenital malformations of the offspring report different periods of susceptibilities [103]. For instance, De Santis et al. [104] summarized 35 reports of CRS cases into three trimesters with semester 1 being most susceptible, semester 2 moderately susceptible, and semester 3 only susceptible to IUGR. Based on their observations on a cohort 42 full-term infants with CRS with exact LMP date and time of onset of rubella rashes of the mothers during pregnancy, Ueda et al. [105] assumed that the critical period for rubella-associated IUGR was the interval from GD 16 to 100. Three most consistent anomalies observed in individuals with CRS are cardiac malformations, cataract, and deafness. A cutoff point of 16th–17th weeks appears to be repeatedly emphasized as the termination period of RA infection-associated fetal anomalies [103, 106, 107]. For example in Miller et al.'s study involving over 1000 women with confirmed rubella infection at different stages of development, pregnancy continued in 40 % and the infants were followed up clinically and serologically for 2 years [103]. Infants infected before the 11th week had mainly heart defects and deafness, whereas 35 % of those infected at 13–16 weeks had deafness alone. Infections after the 17th week caused no defects. Some of the cardiac anomalies and deafness do not manifest until later in life [108] and therefore these gestational time points do not necessarily define the commonly perceived critical periods of developmental susceptibility described in teratology and embryology texts. Also important to mention here is the secondary disruptive effects of the virus infection on the scala vestibuli, scala tympani, stria vascularis, hair cells of cochlea, modiolus, etc. on the subsequent development of the already formed inner ear components of the auditory organ system [109]. With respect to cataract, as in the case of congenital rubella-related deafness, it is difficult to obtain accurate estimate of the timing of embryonic insult by the virus. Since RV is blood-borne, it might not be able to enter the lens vesicle after the hyaloid blood vessels degenerate and lens capsule forms and therefore the risk of cataract should decline after week 5 of gestation [110]. However, this theory has been contested by Wolf [111] who asserts that the period of vulnerability to cataract extends to week 11. Ueda et al. [112] however observe that this susceptibility extends from 26 to 57 days (week 4–8) after fertilization. In vitro experiments on eye rudiments obtained from therapeutic abortions the rubella virus failed to cause cataracts when infected after the lens capsule enveloped the closed lens

vesicles (6–10-week embryos) [113]. Munro et al. [114] report CRS in infants following infection as late as 33 weeks. Development of the eye involves the processes of acquisition of precursors from diverse sources (optic vesicle from neur ectoderm, lens placode from surface ectoderm, and the mesenchyme of neural crest origin), as well as morphogenetic processes such as cell proliferation, migration, differentiation, and apoptosis all which are tightly controlled by nuclear DNA-specific gene expression and epigenetic modifications [115, 116]. A similar complexity exists with respect to the otocyst and inner ear development [117, 118]. Therefore, it is obvious that the critical periods proposed for cataract and deafness in congenital rubella syndrome in the literature are based on often transient symptoms and/or patient history and they do not seem to take into account the chronic nature and severity of the infection and the molecular mechanisms of the embryology of the structures that are malformed. They also ignore the role of secondary effects of altered hemodynamics associated with concomitant vascular anomalies of CRS embryos. The temporal relationships between maternal rubella and fetal anomalies should be interpreted keeping in mind the progressive nature of the disease and the secondary effects that might mask any assumptions on the time of infection based on transient symptoms. What has also been overlooked further in the interpretation of the critical period in rubella teratogenesis is the fact that in addition to the developmental stage at which exposure to infectious agent occurs, there are several other factors that are contextually known to influence the teratogenic outcome (see Wilson [23]).

4.3 Retinoic Acid

4.3.1 Vitamin A Deficiency/Excess

In this review the term *retinoid* is used in place of vitamin A. The generic term *retinoid* encompasses both naturally occurring compounds with vitamin A activities and synthetic analogs of retinol with or without the biological activity [119]. Vitamin A derivatives include retinol, retinyl esters, all *trans*-retinoic acid, and its geometric isomer, 13-*cis* retinoic acid. Retinoids are known to control and maintain many biological processes such as development, normal growth of epithelial and skeletal structures, vision, and immune, reproductive, and integumentary systems [120]. Over 500 genes have been reported to be regulated by retinoic acid. Following ingestion, vitamin A is absorbed in the small intestine as a component of the chylomicrons and stored in liver parenchymal cells (80–90 %), and to a lesser extent in stellate cells and extrahepatic adipocytes as retinyl esters or converted to retinal, the precursor of all-*trans*-retinoic acid. The 13-*cis* retinoic acid, also called retinoic acid, is *isotretinoin*. Several retinoids are used as therapeutic agents [121]. All currently available retinoids are teratogens [122]. Even before Greg [71] highlighted for the medical community the viral origin of birth defects, some landmark

experiments on the effect of maternal dietary restriction of vitamin A in pigs during pregnancy were shown to cause congenital anomalies in the offspring [123]. Pigs maintained on a diet vitamin A deficient supplemented with small amounts of cod liver oil for 160 days and then bred gave birth to offspring that had developed ocular, renal, gonadal, auricular, and palatal defects. Some of the surviving blind pigs when mated with normal pigs did not result in blindness in the offspring indicating that the anomalies were not heritable.

Warkany and his associates initiated a series of experiments in laboratory rats seeking to determine the fetal outcome of maternal dietary deficiencies during gestation [124–127]. These studies showed for the first time that the maternal vitamin A deficiency could induce a predictable pattern of fetal malformations which could be prevented by exogenous vitamin supplementation. Subsequently numerous reports in humans and animal models showed that excess vitamin A and its metabolites could produce a characteristic constellation of malformations that are dose and developmental stage specific [128–133]. Retinoic acid (RA—all *trans* retinoic acid), the metabolically most active metabolite of vitamin A, is now known to be a model teratogen and a thoroughly investigated morphogen, which regulates embryonic cell proliferation, differentiation, and apoptosis [134, 135].

5 Critical Period of Developmental Susceptibility to Retinoids

Numerous clinical case reports have reported a spectrum of craniofacial anomalies in infants following prenatal exposure to retinoids [136–143]. In the 16-month period following its release for the treatment of cystic acne approximately 120,000 women of childbearing age used isotretinoin. Since the deleterious fetal outcome became obvious, pregnancy prevention programs have been instituted for women of childbearing age group who intend to use isotretinoin. However, the drug continues to be used in pregnancy although significantly less frequently (0.32–0.95 per 1000) than before [144]. Retinoid use during pregnancy is reported to be associated with a high incidence of spontaneous abortions (about 20 %) as well as elective medical terminations to avoid birth of infants with several congenital malformations. Against a background malformation rate of 3 % in the population, isotretinoin-exposed pregnancies are reported to have a malformation rate, which is higher than that of thalidomide (35 % vs. 20 %) [145–148]. Isotretinoin-exposed pregnancies are further characterized by spontaneous abortions elective terminations [148]. The frequency of elective terminations is much higher than previously reported. There is robust evidence from clinical reports that retinoid exposure during gestation results in predictable pattern of

fetal malformations. Commonly observed anomalies include hydrocephalus, microcephalus, cerebellar hypoplasia, Fallot's tetralogy, transposition of great vessels, cardiac septal defects, aortic arch hypoplasia, thymic hypoplasia/aplasia, spina bifida, and limb defects. Whereas craniofacial malformations are suggestive of neural crest sensitivity to retinoids, several organs of non-neural origin are also affected in retinoid embryopathy. Children that appear physically normal with no gross malformations at birth are subsequently found to have mental retardation and impaired neuropsychological function [148, 149]. Children that appear physically normal with no gross malformations at birth are subsequently found to have mental retardation and impaired neuropsychological function [148]. Clinical observations have been corroborated by data from well-controlled laboratory animal experiments [131, 150–156]. Put in perspective these data would indicate that retinoic acid fulfills satisfactorily all the six Principles of Teratology that Wilson (1973) [23] enunciated. Clinical studies report that the critical period of maximum susceptibility to gross malformations in retinoid exposed pregnancies is weeks 2–5 postconception. However, one should remember that the pre-implantation period, the remaining organogenesis period, and the fetal period are not without risk [157, 158].

Retinoic acid (RA) alters embryogenesis in a developmental stage-dependent manner. It is important to note here that the amounts and periods of exposure to retinoids differ from pregnancy to pregnancy which should influence the fetal outcome differentially. Experiments in C3H/101T1/2J mice [159] indicate that all-*trans* retinoic acid (RA) administered at pre-implantation and early gastrulation stages (GD 4.5–5.5 postconception) results in supernumerary and ectopic limb induction, and limb and lower body duplications indicating that pre-implantation stages of mammalian development is susceptible to RA toxicity contrary to “all or none” refractory period concept [23, 28]. Experiments in TO mice show that doses higher than 75 mg/kg RA at pre-implantation stages result in complete failure of implantation. A lower dose (50 mg/kg) at GD 6.5–7.5 results in significant resorption and ectopic hindlimbs in the infraumbilical regions, often a single limb and absence of tail in about 4 % of the survivors (unpublished data). The susceptibility or lack of susceptibility thus appears to be dependent upon strain of the mice. In addition to the strain differences, there were methodological differences in experiments conducted in these two laboratories: whereas Rutledge et al. [159] suspended RA in DMSO and administered IP, our lab suspended RA (100 mg of RA) in a mixture of 0.5 ml ethanol plus 4.5 ml corn oil mixture and administered orally. DMSO possibly allows significantly slower rates of absorption of RA transperitoneally in contrast to the RA in ethanol-corn oil mixture via oral route, thus making RA available to interfere with differentiation of precursor cells of organ

primordia (caudal somites, hindlimb buds) which were not even present at the time of treatment. In fact RA as a teratogen is different from other teratogens in that it defies the developmental arrest theory by acting on the precursor cells that are destined to form organs whose primordia do not even exist at the point in time during development when it is administered. In the mouse, the forelimb buds appear as a thin ridge opposite somites 8–12 (late TS [Theiler Stage] 14, GD 9) when the embryo possesses about 15–20 somites and the hindlimb buds appear when the embryo has developed about 30–35 pairs of somites (TS 16, GD10) [160]. Various hindlimb malformation such as unipodia, sympodia, syrenomelia, and meromelia are induced in TO mouse embryos following a single dose of (100, 150, or 200 mg) RA administered on GD 8, when there are only up to seven pairs of somites and the limb buds are nonexistent [151]. These embryos also completely lacked tails and possessed severe malformations and/or agenesis of other caudal median structures at term [151].

Liao and Collins [161] observed hindlimb duplications in about 30 % of embryos of C57 mice following maternal treatment with RA on GD 5.5. They interpreted the anomalies in terms of the RA's ability to induce gene expression for ectopic primitive streaks, primitive nodes, and notochords as well as inhibition of endodermal and mesodermal cell migration. The visceral, skeletal, and neural structures that are affected in caudal dysplasia/regression syndrome in humans arise from *caudal eminence* (not to be confused with "tail bud" of chick embryos) first identified at Stage 9 between the cloacal membrane and neurenteric canal (Fig. 1 in O'Rahilly and Muller [162, 163]. Anomalies of the hindlimb, caudal neural tube, lower body wall, etc. observed in embryos treated with RA at GD 5.5 [151, 161] are a compelling experimental evidence that refutes the theory of developmental arrest [23] leading to malformation of an organ in which case the malformed organ would just resemble in appearance or mirror the stage when the teratogenic exposure occurred.

An additional fact that RA exemplifies is that certain organs have more than one phase of sensitivity to a teratogen. Experiments in Kochhar's laboratory on DBA/2J and ICR mice revealed two phases of maximum susceptibility to RA-induced cleft palate separated by a narrow window [164, 165]. However, this phenomenon appears to be species and strain dependent. The TO strain shows a very high incidence of cleft palate in response to RA administered on one of GD 8 through 13 lending support to a susceptibility continuum of cleft palate [166]. Although the end result (anomaly), namely cleft palate, remains the same, the pathogenetic mechanisms might be different for the same [167–169]. Retinoic acid (RA) and valproic acid (VPA) are both well-established teratogens, both in humans and laboratory animals. They both are known to induce anomalies of craniofacial structures of neural crest origin

and several organs of non-neural origin. A recent European study on maternal use of valproate monotherapy during first trimester of pregnancy reveals significant increases in risks for spina bifida, atrial septal defects, cleft palate, hypospadias, polydactyly, and craniosynostosis [170]. Short of structural malformations, VPA is also known to induce dose-dependent cognitive functional impairment in the offspring even after adjusting for common variables [171–173]. Maternal exposure to retinoic acid has a similar neuro-behavioral effect on the offspring [174]. Although such behavioral anomalies could be attributed to the ability of the drug to alter gene expression, and inhibit neural and glial cell proliferation, synaptogenesis and apoptosis as evidenced from animal experiments, the exact mechanisms in humans remain unclear for now. Prenatal exposure to RA and antiepileptic agent valproic acid (VPA) also are known to cause intrauterine growth restriction (IUGR). Both VPA and RA are structurally weak acids with limited plasma half-life. However, when it comes to the question of critical periods for congenital malformations most studies suggest the first trimester as the most susceptible period. As a fat-soluble vitamin, about 80 % of consumed vitamin A is stored in the liver; thus even if the mother discontinues in-taking retinoids, the embryo continues to be exposed from the reserves. For obvious reasons treatment with VPA of epileptic pregnant women doesn't stop abruptly. Continued treatment with therapeutic agents might cause microscopic structural changes and suboptimal growth in mid and late gestational fetuses with lasting functional consequences. Therefore the first-semester critical period suggestion must be carefully interpreted.

Experimental studies indicate that there are malformation-specific critical periods with regard to RA and VPA teratogenesis. The results of Shenfelt's painstaking experiments with hamster [132, 133] illustrate clearly this malformation-specific critical periods for maternal RA treatment during gestation. There are numerous examples in teratology that illustrate the fact that a certain malformation could be induced by different teratogens with significant differences in critical periods. For example, both RA and VPA induce vertebral and costal malformations and variations (supernumerary cervical and lumbar ribs and sternal anomalies) in mouse embryos. The vertebral anomalies include hemivertebrae, agenesis, fusion, as well as numerical reduction in presacral vertebrae [175]. The frequency and severity of these anomalies appear to be both dependent on developmental stage at teratogen administration and the strain of the mice experimented on. Thus in TO mouse fetuses a high frequency of cervical and lumbar ribs are induced by maternal treatment with VPA on GD 7 and GD 8, respectively. Fetuses with cervical ribs tend to have cervical neural arch anomalies, while fetuses with lumbar ribs often possess eight pairs of sternal ribs. In the RA experiments, the critical period for lumbar ribs is GD 8–12 while that for cervical ribs is GD 9–12. Peak incidence of

cervical ribs occurs following treatment on GD 10 and GD 11; lumbar rib incidence peaks are observed when treatment occurs on GD 8 and 11. The total number of presacral vertebrae is reduced from 26 to 25 and restricted to GD 7 (30 %) and GD 8 (18 %) of VPA treatment. The reduction is more severe (from 26 to as few as 23) in GD 8 VPA treatment. The critical period for RA-induced reduction in presacral vertebrae extends to GD 9. Thus VPA has a narrow window (GD 7 and 8) whereas RA has a longer window (GD 9–12) of susceptibility for the sternal defects. These axial skeletal malformations may be mediated by virtue of the ability of RA and VPA to alter gene expression [176, 177] or to induce histone deacetylation inhibition (HDACi) and epigenetic modification as suggested by Menegola et al. [178, 179].

6 Summary

In summary, it is apparent that birth defects are common, cause morbidity and mortality, and contribute significantly to economic burden. Preventative planning requires a thorough understanding of the pathogenetic mechanisms of birth defects. During the last 100 years the science of teratology has made remarkable progress in keeping pace in development in medical genetics, embryology, and developmental biology. Wilson postulated a set of principles of teratology which not only has stood the test of time but also deeply enhanced our understanding of teratogenic mechanisms. This paper looked at Wilson's principle of teratogenic susceptibility in the context of susceptible periods during development in relation to three model teratogens, namely ionizing radiation, congenital rubella, and retinoids. In view of the newer data in this area, it is becoming increasingly important to interpret the critical periods contextually, rather than in isolation. In other words, Wilson's six principles are interdependent. The whole is greater than the sum.

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Developmental and Reproductive Toxicology

Faqi, A.S. (Ed.)

2017, XII, 314 p. 24 illus., 17 illus. in color., Hardcover

ISBN: 978-1-4939-7206-7

A product of Humana Press