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Nutrient Effects upon Embryogenesis: Folate, Vitamin A and Iodine

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Introduction

The period of human 'embryogenesis', the foundation of this chapter, is generally taken to include the initial 8-week period of human development, from fertilization through organogenesis. Knowledge of the effects of nutrients upon the normal development of the embryo during this period typically has been acquired by observation of the effects that accompany some perturbation of the delivery of a given nutrient; therefore this chapter will focus upon the results of 'perturbed' delivery of folic acid, vitamin A, and iodine.

While a developmental defect may occur at virtually any time during gestation, only perturbations that occur during embryogenesis can produce major anatomical malformations of organs that develop from the neural tube and the neural crest. Defects of the neural tube and neural crest are the most common and the most devastating in terms of mortality and morbidity, stillbirths, and spontaneous abortions. These include neural tube closure defects such as spina bifida, orofacial defects, and conotruncal heart defects [1–3]. Based upon these data, it may be argued that the most important nutrient effects during embryogenesis are those that impact upon the development of the neural tube and the neural crest; therefore, these effects will be the principal topic of this chapter.

Cells of the neural tube and neural crest essentially are identical during embryogenesis [4], therefore a nutrient that is essential for neural tube development is essential for neural crest development as well. This relationship is demonstrated by both folic acid and vitamin A: a deficiency of either during embryogenesis is related to an increase in the occurrence of

all of the members of the unholy triad of neural tube, conotruncal, and orofacial defects (see discussion below). Growing evidence indicates that an adequate supply of maternal thyroid hormone also may be essential during embryogenesis [5]. Thus, deficiencies of each of the three micronutrients that have been selected for this discussion may be related to the occurrence of major developmental abnormalities, and such deficiencies are widespread [3].

Folate

Recent data, discussed below, imply that perinatal folate supplementation will reduce the rate of essentially all neural tube and neural crest abnormalities. For example, Oakley [6] suggested that such supplementation would result in a significant worldwide reduction in spina bifida and anencephaly. However, the protection afforded to the embryo by perinatal folate supplementation is not necessarily related to its overcoming a maternal deficit that is detected by conventional or routine measures of maternal folate status.

Key Functions of Folate in Embryogenesis

Folates are synthetic and natural pteroylglutamic acid derivatives that act as cofactors in one-carbon metabolism [3, 7]. Embedded within this extraordinarily complex metabolic maze of one-carbon metabolism are two cycles with a high level of significance during embryogenesis that may be extracted and cited separately: the 'DNA cycle' and the 'methylation cycle' [7]. Cell division has an absolute requirement for sufficient amounts of reduced polyglutamylated folate to support the formation of thymidylate for DNA synthesis in the DNA cycle [7]. Methylation is the principal means for epigenetic regulation of gene expression in the vertebrate embryo, and is a key to early differentiation events.

Folate and Developmental Defects

Consistent with its key role in cell division and differentiation, maternal folate deficiency is associated with an increased incidence of a variety of developmental abnormalities [8], and perinatal folate supplementation has a highly specific and dramatic effect in improving the outcome of neural crest/neural tube development [9, 10]. This rescue effect obviously may be taken as evidence for a folate deficiency that is remedied by the supplement in some cases, and clearly demonstrates the importance of folate in early development. However, in the same populations where folate supplementation was extraordinarily effective, conventional indicators of folate status detected few cases of folate deficiency among women whose offspring had neural crest and neural tube abnormalities [11]. We have tested two

hypotheses that were designed to rationalize this apparent ambiguity, as summarized below:

Folate supplementation may rescue embryos when it overcomes a deficiency that is indigenous to the embryo that cannot be detected by analysis of maternal *folate status.* To test this hypothesis, transgenic mice were prepared with a defect in the synthesis of folate receptor Folbp1 [12]. Folbp1 −/− mouse embryos showed a high rate of neural tube/neural crest defects although the dams were folate-replete, supporting the hypothesis. These defects may be related to alterations in methylation of embryonic genes [13]. Because the folates passively cross the plasma membrane even in the absence of a functional receptor, a high concentration of folinic acid provided by injection at exactly the right time during embryogenesis prevented these defects in folate-replete dams, again supporting the hypothesis.

Folate supplementation may rescue embryos when it reduces the concentration of homocysteine, independent of the role of folate in DNA synthesis and gene methylation. Homocysteine is an amino acid in the folate-dependent methionine resynthesis cycle [7] whose serum concentration can be abnormally elevated even in folate-replete individuals [14]. Elevated homocysteine is an independent risk factor for abnormal development of derivatives of the neural tube and neural crest [15]. Experiments designed to test the teratogenicity of homocysteine showed that it caused a delay in neural tube closure [16], and induced neural crest and neural tube defects in a dose-dependent fashion [17]. Our research group has shown that homocysteine may disrupt development by acting as an antagonist of a glutamate receptor [18], by disrupting vitamin A synthesis [19], and by inhibiting synthesis of vascular endothelial growth factor [20].

In summary, folate is a cofactor in two metabolic processes that are vitally important to normal embryonic development, DNA synthesis and gene methylation. These processes may be impaired in embryos with a folate deficit, whether that deficit results from abnormal maternal nutrition, or from a genetically determined abnormal uptake of folate in the embryo. In addition, reduced maternal folate may contribute to abnormal development during embryogenesis by exposing the embryo to elevated levels of homocysteine.

Vitamin A

Vitamin A or retinol is required for normal embryonic development in all vertebrates. Retinoic acid (RA) is the retinoid that is most significant for the regulation of pattern formation during embryogenesis [21]. RA regulates essentially all of the keys to developmental success: apoptosis, proliferation, differentiation, and migration [22]. The mechanisms by which RA carries out

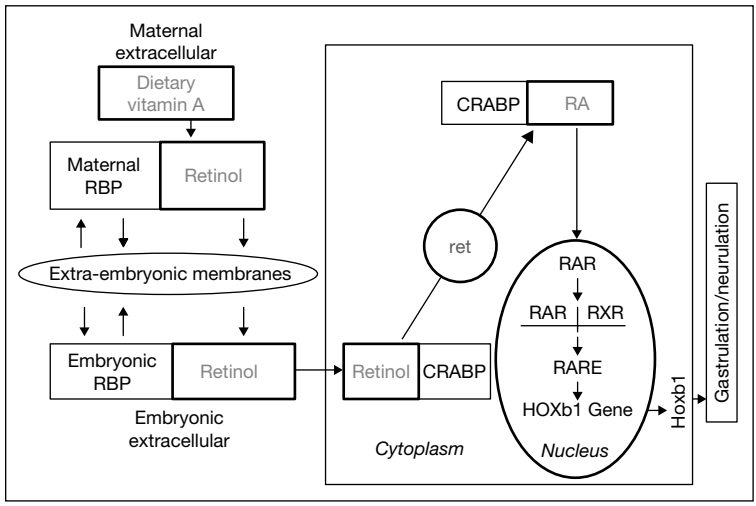

Fig. 1. The route of vitamin A or retinol from the maternal diet, across the placenta, and ultimately into a typical neural crest/neural tube cell of the embryo during embryogenesis. In this case, vitamin A as retinoic acid (RA) is shown to be involved in activation of the homeobox gene Hoxb1, which is a key regulator of the processes of gastrulation and neurulation. CRABP = Cytoplasmic retinoid-binding protein; RAR = retinoic acid receptor; RARE = retinoic acid response element; RBP = retinol-binding protein; ret = retinal; RXR = retinoid X receptor.

these vital functions have become known in the past 10 years, with rapid advances in molecular genetics.

Key Functions of Vitamin A in Embryogenesis

The correct timing and distribution of retinol is absolutely essential for normal patterning of the embryo, including such fundamental features of development as establishment of the chordate body plan. The biological activity of retinol is regulated by a complex combination of receptors and coactivators [7]. These key elements show a remarkable functional redundancy among the receptor subtypes, so that natural or experimental mutation of one subtype may have no measurable consequences during embryogenesis [23]. The developmentally significant interactions are summarized below, and shown graphically in figure 1.

Retinol-Binding Proteins. Retinol derived from the maternal diet is transported as all-*trans* retinol, bound to retinol-binding protein (RBP) [24]. RBP is essential for access of retinol to the embryo [25], and a complex system of RBP gene expression and protein secretion facilitates the transfer of retinol from the maternal serum through the extra-embryonic membranes to the embryo [26]. Retinol uptake in the embryo may be facilitated by a

cell surface receptor for RBP, but receptor-independent uptake of retinol also takes place [24].

Cytoplasmic Retinol-Binding Protein (CRBP I and II). Within the cells, all-*trans* retinol becomes attached to CRBP. Retinol may accumulate in regions of the embryo that express CRBP and the areas of the embryo expressing CRBP are uniquely susceptible to abnormal development when the availability of vitamin A is compromised [27].

Enzymes. The CRBPs present vitamin A to the appropriate enzymes, retinol dehydrogenases and retinal dehydrogenases, for its ultimate conversion to RA. The temporal/spatial distribution of RA in the embryo and consequent developmental events are sensitively dependent upon the presence of these key enzymes [21].

Cytoplasmic Retinoic Acid-Binding Protein (CRABP I and II). Retinol that is converted to all-*trans* or 9-*cis* RA is bound to CRABP. After binding, the CRABPs stabilize RA to help maintain a steady intracellular supply of this potent morphogen during development; they also may transport RA into the nucleus, where it forms its crucial affiliation with RA receptors (RARs) and retinoid X receptors (RXRs; see below) [24]. Cells in the embryo that express high levels of CRABP, such as those of the limb-bud mesenchyme, are highly sensitive to hypervitaminosis A [22].

Retinoic Acid Receptor (RAR-α, β, γ)/Retinoid X Receptor (RXR-α, β, γ). Within the nucleus, all-*trans* RA is tightly bound to one or more of the three RARs; 9-*cis* RA is tightly bound to one or more of the three RXRs. RARs and RXRs are members of the nuclear receptor superfamily that also includes steroid, vitamin D, and thyroxin receptors [24]. An RXR activated by RA binding may form a dimer with any one of the following: another RXR; an RAR; a nuclear thyroid receptor, or a nuclear vitamin D receptor [24]. Other heterodimeric interactions of the RXRs are probable as well.

Biological Bases for Abnormal Development with Hypo-Vitaminosis A

The RAR/RXR heterodimer is the ultimate facilitator of the biological activity of RA, acting upon RA response elements (RAREs) to regulate the expression of a panel of targets that may involve 200 or more genes. Included in this set are genes of conspicuous importance in early development, including Bmp2, Bmp7, Wnt [28], GATA-4 [29], bax, bcl-2 [30], and the majority of the more well-known homeobox (Hox) genes [31], a highly conserved set of genes that are uniquely important during embryogenesis. The following is a list of Hox genes that have been shown to be regulated by one or more RAR/RXR heterodimers, and the relevant embryonic structure [24, 32]:

Hoxa1 = neurogenic neural crest
Hoxa4 = cervical vertebrae
Hoxb1 = neurulation/neural tube closure
Hoxb2 = neural crest

Hoxb3, Hoxb4 = hindbrain
Hoxb5 = neural tube

Hoxc8 = neural tube and forelimb

Hoxd1 = forelimb
Hoxd4 = hindbrain

It is obvious from the above list that disruption of RA signaling is likely to cause abnormal development of neural crest and neural tube derivatives including the heart and the central nervous system. Development of the posterior hindbrain (Hoxd4), for example, is highly sensitive to retinoid status and fails to develop during vitamin A deficiency [33], while under the same conditions neural crest cells (Hoxa1, Hoxb1, Hoxc8) undergo apoptosis [28]. Neural crest cells generally appear to be 'particularly sensitive to changes in retinoid status' [23], therefore RA deficiency leads to abnormal development of the face, head [28] and heart [23]. The embryonic cells effected by acute RA deficiency vary with the time during embryogenesis when the deficiency occurs. Other early-acting genes also may be regulated by vitamin A including Pax-3 [34, 35], but the Hox family remains the most prominent in this context.

While vitamin A deficiency remains a public health problem for much of the world, the delivery method and the amount of vitamin A that is most appropriate for alleviation of this problem remains a controversial issue, in part because hyper-vitaminosis A is also profoundly dangerous to the developing embryo.

Biological Bases for Abnormal Development with Hyper-Vitaminosis A

Abnormal development occurs in the presence of high concentrations of various retinoids, in the embryos of humans and those of all non-human species that have been used for experiments in this context [7, 19]. A review of 40 years of the appropriate literature show that at least 50 different major and minor developmental defects have been found to arise as a consequence of exposing embryos to high concentrations of retinoids at different stages of development. However, the defects that occur most frequently with retinoid exposure are those that involve perturbed development of the neural tube and neural crest; as may be predicted from the discussion of retinoids and the Hox genes, above.

The mechanism of retinoid teratogenesis is still debated, however a logical conclusion is that higher levels of retinoids can inappropriately activate one or more of the genes that are regulated by the RAR/RXR heterodimer [24, 36], resulting in a loss of the well-controlled developmental hierarchy that characterizes normal embryogenesis.

In summary, by a complex interactive set of receptors, RA regulates the expression of early-acting genes that are fundamental to normal development,

including especially the Hox genes. This cascade of events is summarized in figure 1. By altering the expression of these genes, both hypo- and hypervitaminosis A can disrupt the elegant hierarchy of events that is required for normal development, especially of the neural crest and neural tube.

Iodine

The only physiological function of iodine in humans is its role in the synthesis of thyroxin (tetraiodo-*L*-thyronine or T_4) and the bioactive form of thyroxin, triiodo-*L*-thyronine (T_3) [3], thus the adverse developmental effects of iodine deficiency are the result of hypothyroidism. Iodine deficiency may effect 1/3 of the world population, therefore development perturbations that result from hypothyroidism are alarmingly common [37, 38]. The most well-known of these is cretinism, a developmental disorder defined by the presence of severe mental retardation and associated defects. Recent evidence indicates that cretinism is but one point on a continuum of disordered brain development that results from iodine deficiency/hypothyroidism [37, 39]. These well-known fetal iodine deficiency disorders are the result of the perturbation of thyroxin-dependent processes that occur during a long developmental era that begins at about week 15 of gestation and ends during the third postnatal year [3]. Key developmental processes during this period that are known to be thyroxin-dependent include gliogenesis, myelination and synaptogenesis [37, 39].

Unlike the results of deficiencies in folic acid or vitamin A, fetal iodine deficiency disorders are not generally cited as a cause of major malformations of the neural crest and neural tube. However, there is growing evidence that an adequate supply of maternal thyroid hormone may be essential during embryogenesis.

Functions of Thyroxin during Embryogenesis

Maternal thyroxin has a direct action upon embryos [39], and human and other vertebrate embryos express thyroxin receptors during early embryogenesis [39–41], before they have any thyroid function [39, 42]. Experimental models have shown that the thyroid hormone receptors $T_3\alpha$ and $T_3\beta$ are expressed early in embryogenesis [40], and either increased [40] or reduced [5] T_3 exposure during embryogenesis can induce major structural defects of the neural tube derivatives. However, the mechanism by which maternal thyroxin might contribute directly to the regulation of early developmental events is not known. Candidates that merit investigation may be indicated by the role of thyroid hormone in the processes of neurogenesis, synaptogenesis and gliogenesis [39] that require strict regulation of the expression of proteins of the cytoskeleton [43] and extracellular matrix [39, 44].

Although the mid- to late-gestation impact of the iodine/thyroxin axis has been more thoroughly studied and is better understood, it may be concluded that the role of iodine deficiency and maternal hypothyroidism during embryogenesis, the first 8 weeks of gestation, is a topic that is worthy of further investigation.

Selected Interactions

While these nutrients have been discussed separately, and their roles in early development cannot be separated, these myriad metabolic and regulatory interactions are too complex and extensive to be described comprehensively. However, some selected interactions between folate and vitamin A, and between thyroxin and vitamin A, are discussed here, as they relate to specific perturbations of development that may occur during embryogenesis. A summary of key interactions among these nutrients is shown in figure 2.

Vitamin A and Folate

Limpach et al. [19] from our research group have shown recently what may be an indirect effect of folic acid deficiency upon RA metabolism during embryogenesis. As described above, the availability of folate is inversely proportional to the concentration of homocysteine, and hyperhomocysteinemia is a risk factor for neural crest and neural tube defects [15, 17, 45]. In an experimental model, homocysteine was shown to interfere with the conversion of retinal to RA by β-galactosidase [19]. The resultant homocysteine-induced RA deficit was manifested in a reduction in the expression of RAR-β and an increase in major heart defects. There was a partial rescue from abnormal development when embryos exposed to high concentrations of homocysteine were given vitamin A at the same time [19].

Vitamin A and Thyroxin

Vitamin A and thyroxin are interactive as they are transported to the cells, and in the nucleus via their related receptors. RA is transported in the plasma attached to RBP as discussed above, and most RBP is complexed with transthyretin, a larger protein that also binds thyroid hormones, as its name suggests [24]. A great deal has been published about the potential role of transthyretin during various stages of pre- and postnatal development. For the present discussion, it is significant that RBP and transthyretin are both expressed specifically in heart-forming areas of the very early embryo, suggestive of a potential interactive role in cardiogenesis; however the nature of that role is not yet clearly defined [46].

Thyroid hormone regulates genes by interacting with its receptors (TRs) in the nucleus; TRs are members of the same nuclear receptor superfamily as

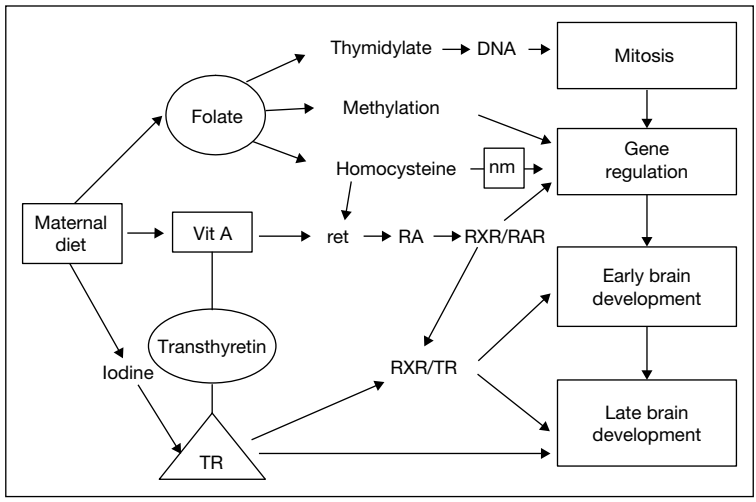

Fig. 2. Mechanisms for the regulation of developmental processes by folate, vitamin A and iodine; and interactions among these key nutrients. This figure highlights brain development during embryogenesis, when neural tube closure occurs (early brain development), and after week 15 of gestation (late brain development), when neurogenesis, cell migration and synaptogenesis are the dominant processes. Folate metabolism may impact upon retinoic acid (RA) synthesis when elevated homocysteine interferes with the processing of retinal [20]. Elevated homocysteine also may provoke dysregulation of genes in early development via its effect upon the calcium channel of the N-methyl-*D*-aspartate type of glutamate receptor (nm) on neural crest or neural tube cells. Vitamin A (Vit A) is transported in the serum bound by transthyretin in common with thyroid hormone. In the cell nucleus, a retinoid X receptor/ triiodothyronine heterodimer (RXR/TR) may regulate gene expression related to both early and late brain development. RAR = Retinoic acid receptor.

the RXRs described above, and they may form the heterodimer RXR/TR [24]. For the many and varied gene-regulatory functions of TR, RXR/TR heterodimerization may be required for TR-related gene activation; it may serve to enhance or facilitate TR activity, or it may be irrelevant, depending upon the nature of the *cis*-acting TR response element [47]. When the RXR/TR dimer is relevant and essential, there is the obvious possibility that variations in retinoid concentration could disrupt development, not only by the more well-known RAR/RXR signaling route, but by inducing loss of TR function via perturbed RXR/TR heterodimerization.

Conclusion

Deficiencies in folic acid, vitamin A, and iodine are widespread, and are the basis of a substantial proportion of major developmental anomalies

including neural tube closure defects, orofacial defects, and conotruncal heart defects. Each of these key nutrients plays a unique and critical role in the regulation of early development. Folate is a cofactor in two metabolic processes that are vitally important to normal embryonic development, DNA synthesis and gene methylation. Vitamin A is required for pattern formation during embryogenesis, and it regulates the expression of early-acting genes that are fundamental to normal development. Iodine is essential for the synthesis of the thyroid hormones, and there is growing evidence that an adequate supply of maternal thyroid hormone is essential during embryogenesis.

In addition to their separate effects upon early development, folic acid, vitamin A and thyroid hormone may interact in complex ways to maintain normal developmental potentials in the early embryo. Conversely, a reduction in the availability of one of these key nutrients may produce an unexpected impact upon the availability or synthesis of another.

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Discussion

Dr. Yajnik: In India many people are vegetarian and there is a substantial prevalence of B_{12} deficiency. There is little folate deficiency as measured by circulating levels. The circulating homocysteine levels are much higher than reported in the literature. For example, the European median level is 9 μmol while in Pune it is 21 μmol and 80% of people have hyperhomocysteinemia. Recently we measured homocysteine, B_{12}, folate and methylmalonic acid in pregnant women and found that 70% of our women in villages are B_{12}-depleted, that is a level below 150 μmol/l [1]. Only less than 1% had folate levels which could be classified as low by the international standards. Methylmalonic acid concentration is very high. Median total homocysteine concentration at 28 weeks of pregnancy is 8 μmol. Homocysteine is a strong predictor of small for gestational age (SGA) babies so that the level of >8 μmol increases the risk of SGA 3.5 times. The second thing we found is an interaction between B_{12} and the folate status of the mother and offspring size. If the mother has high folate levels but low B_{12} levels, then the baby is heavier because of higher adiposity rather than higher muscle. At 6 years of age these children were the most insulin-resistant. Therefore we are now actually looking at the interaction of B_{12} deficiency and folate status in driving fetal growth, body composition and the future risk of insulin resistance. It is perhaps relevant that the National Supplementation Programme in India concentrates on iron and folic acid, B_{12} has never been part of that equation. This is surprising because 70% of the population is vegetarian and B_{12}-deficient. We need to fit B_{12} deficiency in this.

Dr. Rosenquist: B_{12} deficiency has also been shown in a number of studies, at least in part, through homocysteine metabolism involved in an increase in the rate of occurrence of these kinds of defects. The relationship between B_{12} and hyperhomocysteinemia of course and folic acid is the topic of a number of studies. We have not specifically tested the relationship between B_{12}; that is not to say that we don't know that it is extraordinarily important in homocysteine metabolism, because it certainly is.

Dr. Yajnik: Theoretically would it have the same effect as folate deficiency because B_{12} deficiency would drub folate?

Dr. Rosenquist: If in theory B_{12} insufficiency caused an elevation in homocysteine that was equivalent to the elevation that one gets with folate, then there is absolutely no doubt that one would have the same negative effect because we have shown in

the presence of sufficient folate that homocysteine as a teratogen causes the defect. So if homocysteine increases then it can be predicted. It has been shown to be predictable in our experiments and in some epidemiologic studies from Holland that anything that causes homocysteine to rise is associated with an increase in these kinds of defects.

Dr. Uauy: What about the excess; if people take an excess of any of these nutrients? Of course obstetricians are very concerned about vitamin A, but what are the mechanisms for the adverse effects on the potential interaction between them?

Dr. Rosenquist: I think it is fairly well known that hypervitaminosis A is associated with an increase in the occurrence of those kinds of defects. When I say those kinds of defects I am referring to neural tube, neural crest defects. So I won't say much more about that. There seems to be some debate, and nutritionists would certainly know better than I about the nature of that debate, on what level of vitamin A is considered to be too much. My look at the literature was quite interesting and the range that is considered to be too much vitamin A is quite dramatic. Those kinds of defects are well known, but rare. I think it is rare; I think having too much vitamin A in a diet is an exception. Some drugs of course are known to cause these effects; some people eat large amounts of animal organs and may have hypervitaminosis A, but it is rare. As far as too much folic acid is concerned, and I have never said this in public before and it has never been published, so I am saying this for the first time in the realization that it is going to be published: in our experiments with the chicken embryo model we wondered about this issue and we added folate until in fact we began to see some abnormalities with folate at very high levels. I don't remember the exact level, but we could titrate back to normal embryos with zinc. Our conclusion was that there was a binding of zinc with very high levels of folate. Now it has been suggested to me by the proponents of folate as a preventor of defects that this is a dangerous thing to discuss. It seems that therefore it might not be advisable to supplement with folate. All I can tell you is that the amount of folate that we use to bind the zinc was very, very high indeed. So I think there is no evidence that elevated folate in the human is unsafe. But I must honestly say that we did this experiment and found that very high levels of folate did chelate zinc, and we think it was zinc chelation that was responsible for the problems.

Dr. Uauy: Retinoic acid is presently sometimes used for skin problems. Is there any information on how bioactive retinoic acid is versus vitamin A, and the potential for toxicity under these conditions?

Dr. Rosenquist: I don't know the answer to that. I don't know how much passes through the skin, but I think we know the danger of that kind of treatment.

Dr. Kramer: You grouped these 3 groups of defects together as neural crest defects for good reasons. I am familiar with the California Birth Defects Monitoring studies of conotruncal defects and clefts associated with maternal folate intake [2]. But in the randomized trials of folate supplementation, where the purpose was to prevent neural tube defects (NTDs), I don't recall whether the investigators actually looked at conotruncal defects or clefts, and if so, what they found. At least for clefts, the incidence is in the same order of magnitude as that of NTDs. So I am asking you what they found in those trials, and if they didn't find a reduction in clefts or conotruncal defects, why not? Is it simply a sample size problem or some other explanation?

Dr. Rosenquist: I don't have the reference here but one of the papers by our collaborating investigator Dr. Shaw indicated a decline in conotruncal defects. Was that the question?

Dr. Kramer: That is the case-control study, the one on clefts. My question is why the randomized trials on pre-conceptional folate supplementation didn't find a reduction in clefts or conotruncal defects?

Dr. Rosenquist: I think the initial papers published about that do not show that. I can't answer that. I don't actually know how the basic science extrapolates the epidemiology.

Dr. Uauy: I would like to comment on this because the CDC [3] now has a registry in the US relative to folate supplementation and of course NTDs are first, but there are already statistical differences in heart defects and also in clefts. This is not case-control but this is actually the supplementation policy paralleling the changes in folate levels. Of course sample size is a problem because NTDs are the most common; not all clefts, but clefts are also affected by this.

Dr. Kramer: That is interesting, but I am just wondering why there isn't evidence from the trials?

Dr. Rosenquist: I think one of those questions we can never answer is why doesn't something happen?

Dr. Waller: I wanted to comment on your question, Dr. Kramer. There is some evidence: the very large field trial on folic acid that was done in China which, as you know, showed a huge reduction in NTDs in the north [2]. Berry (personal commun.) is about to publish the data on oral clefts, and he told me that they did not see any reduction in cleft palate only, but for cleft lip with or without cleft palate they did observe a decrease.

Dr. Luo: I think there is a collaborative study with the United States. Is that the study you are talking about?

Dr. Waller: The large field trial for folic acid.

Dr. Luo: I haven't read the new one; I just know the old one published in the *New England Journal of Medicine* [4] sometime ago.

Dr. Waller: Do you know whether they published on oral clefts?

Dr. Luo: I am not sure.

Dr. Waller: I believe he told me that, and in Texas we looked at the prevalence. We have a large birth defects registry in Texas, one of the largest in the nation now. We looked at the prevalence of oral clefts before and after fortification and saw just a non-significant decrease in cleft palate only, but not in cleft lip, and it was only a 13% decrease. So we didn't really think that we were seeing any evidence for the effect of folate supplementation. So for oral clefts, which is the one I know best, the evidence even with case-control studies is very inconsistent. There is certainly not the large drop in oral clefts that you see with NTDs. We should be able to detect it readily with oral clefts because we don't have the problem with prenatal diagnosis and abortion, so we detect all of them. There is the problem that we are not ascertaining a lot of them but we are ascertaining all oral clefts, and there may be some effect of folate on them but it is certainly not to the same degree as NTDs. The hearts are more complex, and I leave them to the pediatric cardiologists.

Dr. Kramer: I think those are important comments because they suggest that even though neural crest embryology puts the three kinds of defects together, the epidemiologic evidence isn't quite as strong for clefts and heart defects as it is for the NTDs.

Dr. Bleker: You spoke very clearly about the significance of early pregnancy and early embryogenesis. Could you speculate a minute about the significance of this early stage of pregnancy with respect to disease in later life?

Dr. Rosenquist: As you may know, the US National Institutes of Health are very interested in this issue. It is obvious that there are cardiovascular diseases for which one can extrapolate a potential relationship between early embryogenesis and later life. In fact atherosclerosis in later life is one them, and heart defects as well. My speculation, and it is purely speculation, is that when these studies are fully funded and operational, that there will be a good correlation between early embryogenesis in a

number, particularly I am thinking of cardiovascular and brain functions. I am certain that those will be the two key areas.

Dr. Lönnerdal: I was interested in the observations you made in the knockout mouse model, they were very convincing. I just wonder, of course it will be very difficult to assess embryonic folate transport in the human, but is there any indication that folate-binding protein in the human could be defective or suboptimal? Is there a polymorphism in the gene or anything that has been discovered?

Dr. Rosenquist: The evidence so far is that there is very little polymorphism in humans and that in fact this particular kind of folate-binding protein is potentially not highly significant in humans. We are looking at other kinds of folate-binding protein now.

Dr. Waller: I wanted to address what you brought up about low levels of maternal thyroxin possibly being associated with birth defects. I don't know whether you are talking about animal studies or human studies. I looked at this a few years ago and I know that it is very hard to identify women who have low levels of thyroxin in the period of embryogenesis. We can identify women who take thyroxin, most of them take it for hypothyroidism, but presumably if they comply their levels aren't low, and those women don't seem to have an excess of birth defects from the studies I saw. So I am just wondering how we can even identify women who have low levels of thyroxin because they would basically have undiagnosed hypothyroidism?

Dr. Rosenquist: In fact I was talking about experimental models. I don't know that there is any evidence and I am glad you said something about it, but I am not familiar with any evidence.

Dr. Uauy: The data on hypothyroidism at the population level show that there is increasing fertility and increasing wastage [5]. Moreover I think it is very relevant that thyroid hormone from the mother feeds brain development in the baby for the first 20 weeks. This is actual passage of the intact hormone, one of the few cases where you have intact passage. Then there are data on premature infants which in fact show that, especially the supplementation trials from the Netherlands [6], that before 30 weeks of gestation, giving this to the baby has a positive effect both at 2 and 7 years in terms of mental development and school performance. Later on apparently there is an adverse effect because you have the tradeoff of maturation versus cell replication. So somehow, later in the picture, thyroid hormone enhances maturation at the expense of cell replication. The interaction of thyroid hormone in the baby has a positive effect early on and then an adverse effect later on. Especially with premature babies there is insufficiency of the neonatal thyroid to actually provide this, and it is not only about thyroid hormone but also the deiodinized receptor system which is tissue-specific and time-specific, so it is a complicated relationship. By the way I have the Eriksson data from the CDC. If you would like to see the full range of defects from the CDC I can actually set it up in a minute.

Dr. Duan: You are actually talking about epigenetics. Could you highlight the recent advances for the connections with nutrition? I mean epigenetics in a broader sense, not only related to folic acid, vitamin A and iodine, but other factors related to epigenetics and fetal malformation.

Dr. Rosenquist: The general topic of gene-nutrient interaction is relatively new and is a growing area. Certainly the National Institutes of Health in the US is very interested in this particular issue. There are two areas of growth from which we can expect a great deal of information in the next 10 years. There are more and more well-defined knockout mice, like the one I was talking about, in relation to receptors, transport, proteins, enzymes and metabolic cycles of the various micronutrients, we are only beginning to touch that now. The second thing regarding not necessarily genetics but modern molecular biology is to look at structural biology: the structure of surface

receptors and the way they interact with nutrients. That is beginning to get a little more interest. The entire idea of micronutrients as key issues in human health is growing very rapidly, so I am very optimistic.

Dr. Uauy: So the data from the CDC cover 1968–1980, looking at traditional spina bifida, anencephaly and relative risk. As you can see choanal atresia and preaxial polydactyl in fact have increased, but atrial and ventral septal defects, cardiac malformation, and both cleft lip and palate are very significant. The size of the effect as you see in relative risk is quite small, so this is putting together a huge amount of data. These are all CDC data. In fact they are now trying to have larger trials to be able to pick this up in prospective control trials. These are the whole CDC data from Dr. Eriksson. What is interesting though is that there may be a selection of other defects, but choanal atresia shows that we may also be having a potentially adverse effect on other malformations.

Dr. Hornstra: Let's go back to vitamin A for a moment again please. There are large parts of the world where there is a low consumption of β-carotene, resulting in hypovitaminosis A. My own experience is from Kenya where a large part of the country has an extremely low intake of β-carotene. Do you know of observational studies with respect to perinatal problems and do we have results of supplementation studies in these parts of the world?

Dr. Rosenquist: Actually I don't. My review of the literature indicates that the answer would be no; it doesn't look as though there are accessible studies that have been done to address that issue.

Dr. Yajnik: There are studies in Nepal [6] and Bangladesh done by West and his group of the Johns Hopkins Institute on vitamin A supplementation. It reduces maternal mortality and perinatal problems. Therefore they think ethically we cannot withhold vitamin A supplementation in future trials.

Dr. Hornstra: That was supplementation with vitamin A or with β-carotene?

Dr. Yajnik: Vitamin A.

Dr. Hornstra: Is anything known about β-carotene supplementation? There is a representative here from Kenya I think. There are very good natural sources of β-carotene and it shouldn't be too expensive to insist on increasing that particular component of the diet.

Dr. Yang: I have a comment about this. In studies done in China the ratio of β-carotene and vitamin A is generally very low compared with other studies because the Chinese diet is different from other diets. These studies used the stable isotope, and perhaps we should have data from studies on children using the stable isotope labeled in vegetables to see what the ratio to the vitamin A is.

Dr. Hornstra: It may be true that conversion is low as far as synthetic β-carotene is concerned, but as I explained there are excellent sources of natural β-carotene also in less developed countries. Palm oil for instance is an excellent source of β-carotene and it would be relatively inexpensive to promote the intake of this oil to increase the consumption of β-carotene and prevent hypovitaminosis A [8–11].

Dr. Wasunna: You made some comments about the deficiency studies that have been done in Kenya. We do not really have a register for defects as such, and most of the nutrient deficiency studies have more to do with child growth patterns than defects. So the data that we have are perhaps not a direct answer to this topic. We do not have proper data to answer this question, but it would be interesting to look at this in retrospect, and see whether the areas with high deficiency levels may also be registering a few more defects. A problem we have, as in many developing countries, is record keeping and documenting the various defects seen at birth, because a large number of births take place in health institutions and therefore keeping accurate data on that will be difficult.

Dr. Luo: You showed very interesting data on the knockout model. How does folate work? Do you completely knockout the receptor and show dose-response phenomena?

Dr. Rosenquist: Folate will cross the cytoplasmic membrane in the absence of a transporter by simple mass action, but the folate transporters and folate receptors facilitate the process by orders of magnitude. So in the absence of sufficiently functional receptors, it is possible to get sufficient folate across the membrane but it requires a tremendously high level of supplementation. So the answer is it crosses the membrane by simple mass action.

Dr. Luo: The other question: can you comment on how the status of these 3 important substances, folic acid, vitamin A and iodine, can be used to monitor the status of a deficiency?

Dr. Rosenquist: That probably is way out of my area of expertise. You mean by monitoring in the population?

Dr. Luo: Yes.

Dr. Rosenquist: I ask my colleagues to comment. I don't know the answer to that.

Dr. Luo: Sometimes a fortifying program can provide folic acid to all the pregnant or pre-conceptional women. But how do we know if they are deficient in folic acid or not, or vitamin A or iodine as well?

Dr. Uauy: For folate you can actually monitor both folic and homocysteine levels, they would probably be good indicators of folate status. They can be measured in red cells or plasma. Another potential way might be to screen for tetrahydrofolate reductase deficiency because that population is particularly vulnerable, and again that group may obviously have higher requirements. Some of the tests on a vast cohort have in fact actually separated groups by genotype the tetrahydrofolate reductase, since they are most sensitive to folate supplementation. So potentially there are screening tools that may be applicable to populations.

Dr. Kramer: There have been some studies in Canada both in Newfoundland and in Kingston, Ontario (Liu S, unpublished observations). Newfoundland was an area with a very high incidence of NTDs prior to fortification. The Canadian studies show that since food fortification the rates of NTDs have decreased in Newfoundland; they weren't very high in Kingston before fortification. But more importantly the serum folate levels and red cell folate levels and serum homocysteine levels have gone in the directions that you would expect with fortification. Given the concentrations of folate that would be required to be toxic, it is probably not necessary to screen a population for folate or homocysteine levels before the study, because the additional folate provided by fortification, as far as we know, does no harm, and it is probably better to supplement everybody, i.e., the entire population, than doing it selectively among those with low folate or high homocysteine.

Dr. Rosenquist: I agree with Dr. Kramer, I think that is a very important point.

Dr. Uauy: One point though is that presently the defined upper level is actually 1,000 μg which is not too high if you are talking about 40 μg being required. The reason for that is not necessarily a toxic effect of folate but the potential for masking B_{12} deficiency, and that has been complicated by people who object to folate fortification unless also fortifying with B_{12}. In fact in Chile 3 years ago we fortified at the population level and monitored this. We have not had a problem with B_{12} being masked in the surveyed population, but we are now considering adding B_{12} and we will be able to also observe the potential additional benefits of B_{12} because of the interaction with folate. So this time we might even do it in a control fashion because it is hard to do large studies, and people accept the control study if there is already evidence of a benefit. But for B_{12}, it is still the pending issue.

Dr. Yajnik: Folate 'toxicity' might be exaggerated by B_{12} deficiency. In India there is no folate fortification of food, it mostly comes from vegetables and other items

people are eating. The standard obstetric practice is to put women on the folic acid tablets early in pregnancy and the commonest brand, which is popular with obstetricians, is called 'Folvite' (5 mg or 5,000 μg). Recently we did a survey of 100 prescriptions in an antenatal clinic and showed that in the first trimester the lowest dose of Folvite prescribed was 1 tablet/day and not uncommonly 2 or 3 tablets which amount to 10–15 mg. I think we have to take notice of this. What is considered very safe may not be that safe.

Dr. Kramer: I would like to make a point about supplementing early in pregnancy. Embryogenesis occurs so early in pregnancy that it is typical for a woman not to know that she is pregnant during that critical period. In fact, major structural defects cannot be prevented by supplementing early in pregnancy. Not that you shouldn't supplement, but with respect to embryogenesis, it won't help.

Dr. Yajnik: In my hospital we see a number of women with bad obstetric history who are following up with the obstetrician before they become pregnant again. Most of these women receive Folvite before they conceive.

Dr. Waller: I would like to make the point that many of us in birth defects believe that low folic acid alone does not cause NTDs. I reviewed the literature to find reports of outcomes on women who had folic acid deficiency and I found a report from Britain in the 1950s or 1940s of 300 such women. All of these women developed megaloblastic anemia during their second trimester, which is when it comes on, so they would have been pretty deficient in their first trimester, and there were only 3 NTDs. That was the best report I could find and I am looking for other reports. But the leading paradigm, the leading thought, is that there is probably a genetic variant in a certain portion of women that causes them to need more in their embryos. Now they are saying it is in the embryo where the genetic defect really lies, but anyway it causes the pregnancy to need greatly more folic acid than would be needed otherwise; so it is a gene environment interaction. This is not proven but there is a misconception that low levels of folic acid cause NTDs and it is really not that simple. I would like to reiterate though that in China there was a recent study showing that, especially in the north, they have lower levels of folate in their blood [7] and so it is a particularly good population to use the supplementation approach across the whole population, since they are starting at a lower baseline for folate.

Dr. Rosenquist: I think Dr. Waller is saying in a different way what I said earlier, and that it is necessary to have low folate to get benefit from supplementary folate.

Dr. Duan: Is there any literature reporting on the over-dosage of folic acid and malformation?

Dr. Rosenquist: No, there is no literature about that. In fact the only time you ever heard that is here, when I just said that. It is just something that I think for the sake of full disclosure it is important to know. If you dump enough folic acid on embryos they don't respond well, it may have something to do with folic acid binding zinc. Secondly I think that the amount of folic acid that one would have to take in a population base to get the same effect would be monumental, they probably would have to eat practically nothing else.

Dr. Pencharz: I just wanted to support what Dr. Yajnik was saying about vitamin B_{12}. In 2003 in the *American Journal of Clinical Nutrition* there was a very nice editorial on the prevalence of B_{12} deficiency in vegetarians and in vegans. I was astonished at just how high it was, I think somewhere in the range of 70–80% even in vegetarians, not only vegans. So as we are worrying about this and hyperhomocysteinemia and all the rest, I think we are going to have to worry about B_{12}. I think about B_{12} as you don't have to supplement a great deal and clinically now, as far as the women with initial anemia are concerned, that is the problem with the intrinsic factor by using

about 500 μg you can use an ultimate pathway. I have shown in my patients that we can very easily correct the B_{12}, but that is not for the general population. But the point I am making is let's not forget B_{12}, and I think Dr. Yajnik was making the same point.

Dr. Uauy: One thing we think about regarding massive interventions during pregnancy in women of reproductive age is what else are we doing in addition to protecting from NTDs? Other sensitive genes are perhaps going to be favorably selected, and we are putting them in the pool considering that you have 40% embryonic wastage. Given your interest in embryogenesis, the other potential concerns regard things that are going to pop up at age 20 or later.

Dr. Rosenquist: That is a very important point and I think it is something that needs to be discussed by molecular geneticists. I think the answer is yes. A key issue in 21st century molecular genetics is how do we get to the point where we can make an appropriate prediction? Without concerted efforts to act in that particular issue, which is very important, it will take us too long to get there. So this issue needs to be addressed separately and intensely.

Dr. Duan: I have a comment regarding Dr. Waller's statement about the research in China. Actually the research was done in the northern part of China where the winter is very cold and freezing and there aren't any fresh vegetables, which is why there are a lot of patients suffering from very low folic acid levels. With folic acid fortification in that part of China we really achieved a good result by lowering the incidence of NTDs, but after a while we reached a baseline that we cannot change anymore. So I totally agree with you that there are some NTDs which are not caused by low folic acid. Taking Shanghai as an example. In this city we have a lot of fresh vegetables even in winter and the NTD incidence in that part of China is comparatively low, and by supplementing folic acid you will not achieve the great success seen in the northern part of China. As far as I know that research was carried out here in Beijing in a research center in collaboration with CDC in the United States. Now they are trying to extend the research to observe the effect of folic acid fortification and to see the incidence of cardiovascular defects and other birth defects.

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