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Mini Review

Alcohol effects on embryonal bone growth

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Abstract

BACKGROUND:The possible teratogenic and lethal potencies of ethanol on the developing foetal bones especially when ingested during pregnancy is now well established; but the actual mechanisms of these actions are yet elusive and this had since stimulated unending interests and numerous researches particularly in the field of teratology. Are there yet any other possible mediators / agents to be implicated in the potentiation of ethanol toxicity? Are they existing or would there be possible solutions for assaults of alcohol abuse?

***METHODS*:** A retrospective and contemporary literature compilation was done in an attempt to further explore this subject and bring out more detailed information useful to answering these and other such lingering questions.

RESULTS: The incidence of congenital bone malformations associated with maternal consumption of ethanol during conception are now recognised to be due to its toxic effects on the developing tissues and impairment of normal cellular activities particularly by depriving them of essential vitamins such as folic acid, a useful precursor for the synthesis of proteins and DNA.

CONCLUSION: This is a situation where the most successful preventive measures could possibly be instituted, with folic acid supplementation, to alleviate ethanol toxicity, if total abstinence is not possible.

Key words: Alcohol; Bone; Folic acid

The fundamental aspects of teratogens

Alcohol is a well - recognized teratogen. Wilson considered factors that could affect the action of a teratogen into three basic categories : i, Dosage of the agent ii, Maternal-embryonic exchange and iii, The genotype of the foetus[1]These factors may be further classified under two broad headings as extra-embryonic and extra embryonic. The first two factors ; dosage of the agent and the maternal-embryonic exchange could be classified as extra embryonic while the third factor, is embryonic. The fetal genotype as it affects its response to a teratogen could be regarded as the inherent susceptibility of an embryo to a given agent during embryogenesis

The stage of development of the embryo when the drug is administered to the pregnant animal intercepts a dynamic series of particular biochemical events of qualitative and quantitative nature giving differential susceptibility to injury of molecules, cells, tissues and organs. Its noted that during cleavage and what would correspond to blastula stage in mammals, there is typically no teratogenic response to even high doses of agents that are quite effective at later stages of development[3-5]. This could be explained by the fact that destruction or damage to a certain percentage of cells can be tolerated by the yet undifferentiated pluripotent cells of the blastocyst, and those lost cells can be compensated for by these highly mitotic blastula cells. Thus, there is no teratogenic scar left on the organism at birth. But there seems to be a critical limit beyond which the damaging of these yet non-specialized cells can not be tolerated, if this limit is exceeded, death and resorption of the embryo results[6].When organogenesis is completed the embryo enters the fetal period, which is characterized principally by growth and functional maturation. At this stage, strict teratogenesis does not occur because embryonic processes can no longer be interrupted or diverted. Hence, any agent sufficiently potent to affect the foetus would either retard growth or cause what has been termed "Congenital Pathology" rather than malformations[5]. The developing rat enters this "Refractory Period" at about the seventeenth day post conception and this

corresponds to the end of the eighth week of intra-uterine life in human embryos.[5] Thus, a narrow period lies between these two developmental stages, which could be described as "Critical Period"[7], when embryonic tissues are most prone to teratogenic influences. The onset of this period is marked by the time the germ layers are being formed which is at about the eight day of gestation in rats[5]. It appears that an appropriate dosage of a known teratogen administered at the appropriate time of development in a given species would cause developmental disturbances. Generally, embryonic age at the time of teratogenic action is an important determinant of which tissue is most susceptible[5]. Most organs have a particular period when they are most vulnerable to teratogenic agents, and this coincides with the time of critical developmental event in the organ, which for bones in the rat is about the 10th day of gestation[8,9]. This is the period of cellular condensation / differentiation in osteogenesis. Considering the first factor that is, the dosage of an agent, not all dosage levels of a known teratogen are teratogenic. According to Wilson[10], there are lower (sub-threshold) dosages, which apparently do not affect the normal development of an embryo, and a higher (lethal) dosage, which kills all the embryos and even the mother. Between these two extremes is a narrow 'teratogenic zone' in which dosage is sufficient to interfere with specific development and without destroying the whole embryo. Also the frequency of administration of a teratogenic agent determines more of less the extent of its action. For instance Hicks[3] observed in a study on mice exposed to 100rads of x-rays on the 9th day of gestation in which many types of malformations in almost all the fetuses was observed. The same treatment on day 10 caused deformities in 75% of the off-springs whereas further treatment on the 11th day produced no malformations. In another study, vitamin A deficient diet fed to pregnant rats on the 10th day of gestation caused mainly cardiac abnormalities whereas when administered up to the 15th day, it resulted in ocular, aortic, lung and diaphragmatic defects[6]. It would seem that an appropriate dosage of a known teratogen administered at the appropriate time of development in a given species would cause developmental disturbances. Thus the same sort of determinants that gave individuals, strains and species their distinctive similarities in normal structure and function probably give them varying degree of susceptibility to adverse influences[1].To this end it has been suggested that the occurrence of anomaly is in part a measure of the inability of genetic and other regulatory mechanisms to overcome localized sensitivity of embryos to an external intrusion[11]. This could be explained from the fact that chromosomal aberrations induced in somatic cells are the cause of malformation following exposure to teratogens. It is not quite clear whether it is the genetic component of the mother or that of the foetuses that is more important in determining the extent of the effect of a teratogen. It could therefore be concluded that an appropriate dosage of a known teratogens administered at the appropriate time of development in a given species will cause developmental disturbances. /Cell death observed in teratogen-treated embryonic organs destined to be malformed must occur selectively and within a critical period of time for a birth defect to result. Low doses of cytotoxic agents may produce levels of cell death that can be replaced through restorative hyperplasia of surviving cells, resulting in the formation of small but morphologically normal foetuses. High doses that cause damage to too many cells and organ systems to be compatible with life results in embryo lethality. Cells dying from teratogenic treatment must be replaced by proliferation of surviving cells within a critical period of time to avoid dysmorphogenesis. A cytotoxic drug such as hydroxyurea (HU) kills mesodermal cells in the limb buds. Surviving cells attempt to replace the cellular deficiency by restorative proliferation. If the replenishment occurs by the critical time when digits are formed by mesenchymal condensation, then limbs with normal amounts of digit are formed; if not ectodactyly or missing digits occur[12].Some other factors influencing the action of teratogenic agents include nutritional status of the mother, route of administration of the agents and animal species of strain differences. The role of maternal nutrition in potentiation of the toxicity or teratogenicity of an agent cannot be undermined. Investigations have shown that maternal nutritional deficiency alone could induce congenital deformities in the offsprings[13]. In fact, the first experimentally induced congenital skeletal defects in rat were achieved with a nutrient deficient treatment. This discovery was rather accidental, because Warkany and Nelson[14] had intended to induce goiter in the rats. These workers fed a diet, which has been known to be goitrogenic to the pregnant rats. But in amazement, Warkany and his colleague found that they have produced mass skeletal defects instead of goiter in the rat fetuses. They later discovered that the nutrient lacked riboflavin and that the iodinated salt they added to the maternal diet prevented goiter in the mother and the young. However, an opposing view had been expressed by Poskitt[15] who, argued against nutritional deficiency as possible teratogen or a potentiator; according to the investigator, there was no evidence that the nutrition of women giving birth to infant with Foetal Alcohol Syndrome (FAS) or Foetal alcohol effect (FAE) is any worse than those women whose infants do not have these problems even though their mothers drank equally heavily. However, it appears that enough evidence is available to establish the fact that maternal nutritional deficiency is a possible teratogenic agent or a potentiator. This had been demonstrated on bones in the rats.[16-19] Although for a long period of time, the association of malnutrition of the mothers with malformations of the foetuses was subjected to debate. Exposure to a drug or chemicals for a brief period of time, which affected or deprived the

normal nutrient for only that period of time might be expected to have relatively little impact since severe reduction in protein and caloric in takes during the first 10 days of pregnancy in the rat followed by a return to an adequate diet prolonged the gestation, although weight of the new born were normal . The only deficit noted from early deprivation of protein during gestation was a deficit in cerebral protein content at birth. Moreover, some workers had argued against nutritional deficiency as a possible mediator or potentiator of teratogens. According to these investigators, there was no evidence that the nutrition of women giving birth to infants with FAE or FAS is any worse than those women whose infants do not have these problems even though their mothers drank equally heavily. However, it appears that enough evidence is available to establish the fact that maternal nutritional deficiency is a possible teratogenic potentiator. This had been amply demonstrated in the rats[20,21]**Cellular development of bone** Broadly, the mode of bone development is classified into two: direct and indirect osteo-genesis[22]. In direct osteo-genesis, bone cells differentiate, that is, progressively specialize from precursor cells directly without involvement or replacement of any other tissue. Such osteo-genesis is usually referred to as intra-membranous ossification. Examples of these are the flat bones of the skull and the first bone that surround the shaft of the long bones such as the femur. In indirect osteo-genesis, the bone that is deposited replaces a pre-existing tissue that act as model or template for bone formation. In the humans, that template is usually cartilaginous, and this process is known as endochondral ossification[23]. The occurrence of bones in vertebrates has been attributed to three main reasons[24,25]. Firstly, evolutionary, that is, bone grows to adapt the organism to its environment over a long period of geological time; secondly, functional, that is, bone grow to best fit the organism to its life style; and thirdly, biophysical or biochemical reasons, that number of factors, chiefly among these are: (i) the number of cells that migrate to the site(ii) local, paracrine factors for the promotion of cell division at the site of condensation(iii) production of autocrine factors that promote cell division by cells within the condensation itself(iv) the length of the time between the initiation of the condensation and the deposition of bone.Variation in any of these factors will affect the final size of the bone. In fact, many evolutionary biologists believe that this factor account for differences in the location, number, size and shapes of bones among species[22,28]. Previous investigations have indicated that the condensation stage triggers off the next stage of development known as differentiation. Differentiation is the appearance of specialized bone forming cells. Differentiation is initiated by synthesis of growth / differentiation factors by the aggregated mesenchyme. Initially, these condensed cells cannot be identified as precursor cells either as pre-osteoblasts or pre-chondroblasts, since they show none of their characteristic features[30].Initially calcium influx into the cells alters leading to the is bone growth follows physical principles so as to minimize stress per unit area imposed upon it. Basically, skeletal development is in three phases, which occur during prenatal or is carried forward into post – natal life[25]. They are: (i) differentiation, morpho-genesis into new shapes and patterns and growth. Each of the phases occurs sequentially and initiation of a proceeding step depends on the successful completion of the previous and any of the phases could be affected by either intrinsic (genetic) or extrinsic (environmental) factors. Migration of the bone - forming cells most usually in a pre-osteo-genic form is an essential step in the earliest stage of development of some bones, especially in the head region; cells that become osteo-blasts do not arise where bone will form. These arise from cranial neural crest cells in the developing brain[22]. Similarly, the cells that form the vertebrae do not arise around the spinal cord but from segmental blocks of mesoderm, that is, the somites. These cells need to migrate from their original location to surround the spinal cord and then develop into the vertebral column. This phenomenon led the famous biologist, F.W. Spemann[27], to remark that ‘we are walking with parts of our body, which we could have used for thinking if they had developed in another position in the embryo.’ Sequel to cell migration in certain bones, generally skeletal tissue development is preceded by aggregation or accumulation of like cells (mesenchymal cells) which marks the first visible organization of skeletal tissue stem cells[28]. This is the stage that the great geneticist, Hans Grunerberg[29] referred to as ‘membranous skeleton’. This phase is most prone to any assault and the sooner such influence, the more pronounced would be the consequences. Several cases had been reported in which skeletal elements fail to form if a condensation does not reach a critical size resulting in bradypodism, phocomelia and brachycephally in mice and chicks[28,29]. More over, according to Hall[22], the size of a condensation depends on a synthesis of either mRNA type I collagen for bone or mRNA type II collagen for cartilage[28]. The growth / differentiation factors are small peptides with specific actions on responsive cell, that is, cells with receptors for growth factors. These factors have emerged as major candidates over the past few years as differentiation signals in osteogenesis[31]. Furthermore, development of bones involves morphogenesis, which is the process by which shape is generated and this had been classified into two, that is, the first and the second order[32]. The first order of morphogenesis involves the assumption of fundamental shape of the bone; for example, the long bone such as femur, flat or plate shaped bones of the skull or the irregular (vertebral) bones. However, this aspect of bone is largely controlled by intrinsic factors and is least affected by external factors[30]. The second order of morphogenesis is the appearance of ‘minor’ architectural features of bones such as knobs, bumps, foramen or tubercles. These features are functionally induced, for instance, by the attachment of muscles, ligaments or the passage of nerves and vessels. These

secondary features develop not, for instance, in tissue culture primodium where the structures are not in contact with the developing bones[32]. Further into the post natal life, bone growth continues though slowly and is finally arrested in adulthood in the "determinate" type of skeletal growth occurring in vertebrates such as man[32]. Final height is set, when all the stem cells in the growth plates of long bones are used up, hence, growth ceases. Other vertebrates such as some fishes and tortoise exhibit "in-determinate" growth in which growth continues though quite slowly through out life. Particularly in the post-natal life, bones are prone to influence such as hormonal and mechanical factors; and in this respect, the clavicle is more dependent upon biochemical factors than any other bone ever studied so far[24,33-35]. Mechanical factors also play important role by adjusting bone formation and resorption so that skeletal mass, shape and structure are optimal to resist the stress imposed upon it[22].Hormones play vital roles in skeletal growth by regulating calcium mobilization from the skeleton to maintain the calcium balance of the body. Growth hormones and somatomedin C regulate both the division and differentiation of chondrocytes in the growth plate and are therefore important regulators of developments in the growth of those bones that are formed by endo-chondral ossification[24]. The mitochondria in the osteoblasts had been reported to act as storage sites for the Ca^{2++} and PO^{4---} ions , which are gradually incorporated into newly laid down matrix (osteiod) during calcification[36].Bone matrix contains collagen and other materials. One of the major amino acid constituents of collagen, glycine, is partly liberated during the metabolism of folic acid. For instance, with calcium provided by the vesicles so as to bring about a precipitation. In addition, the vesicles provide pyrophosphates, the enzyme that destroys inorganic pyrophosphate, which would otherwise act as an inhibitor of calcification[38] The osteo-toxicity of ethanol Poor bone mineralisation is common in alcoholics who have been found to be vitamin D deficient[39]; and low vitamin D was reported to reduce the absorption of calcium[40]. Moreover, diminution in cranial and limb bone dimensions, body emaciation, low skeletal weights and decrease in size of the ethanol - treated animals had been observed[41-45] These observations are in consonance with and extend the earlier reports on the Foetal Alcohol Syndrome (FAS)[47] Depressed serum levels of osteocalcin during acute alcohol intoxication have been reported to indicate alcohol even acutely retards osteoblasts activity[48]. Osteocalcin is a vitamin K dependent protein synthesized by osteoblasts and released in to the circulation. Its concentration in the serum is elevated in states of diminished bone synthesis[39]. It had been suggested that ethanol ingestion induce agglutination or rosettes formation by the red blood cells which consequently lowers the circulating oxygen level, resulting in the impairment of normal cell functioning, particularly that of the osteoblasts[49]. Shiola and co-workers[50] examined the feto-toxic effect of ethanol and maternal hyperthamia in the following injection of tritiated glycine, radioactivity is seen in osteoblasts within a short time; soon after, it disappears from the cell but appears in the adjacent newly formed bone matrix[37]. The matrix is synthesized (conjugated) from the constituent amino acids by the osteoblasts. Vaughan[38] found that labeled glycine incorporated by osteoblasts was deposited on adjacent bone surfaces within one hour of labeling; and in a previous report, it had also been observed that osteoblasts produced two or three times its known volume of matrix during its most active period on the periosteal surface[37]. In the earliest stages of osteogenesis, fine cross-banded collagen fibrils appear near the constituent amino acid by the osteoblasts in the soluble form, that is, the collagen. They are subsequently 'exported,' and once outside the cell, tropocollagen macromolecules apparently link up and join end-to end and side-to-side to form cross-banded collagen fibrils which are insoluble at body pH 7.4. The deposition of collagen fibrils continues and gradually the thickness of the collagen fibrils increase to reach the dimension visible by light microscopy; the material produced up to this point correspond to the osteoid or pre-osseous matrix of light microscopy. alcification commences within the matrix vesicle soon after the osteoid is laid down; the matrix vesicles were first identified as minute, more or less rounded structures that range from 30nm to 1um in size, and are present in matrix of osteoid tissues undergoing calcification. These vesicles are known to exhibit a relatively high alkaline phosphatase activity and seem to serve several roles in initiating calcification[38]; that is, they accumulate calcium and their phosphatase act to bring about enzymatic hydrolysis of ester phosphate to yield orthophosphate which would react mice. They observed structural defects including skeletal and visceral malformations following combined treatment of a single dose of 25% ethanol and heat – stress in a water bath at 42^{o}C for 10 minutes on day 8 of gestation. It had been demonstrated that a relationship exist between bone strength and consumption of ethanol in rats., that is, a significant inverse correlation between the strength required to break the femur and the dose of ethanol administered.[51,52] A study had also been conducted on the effects of gestational ethanol ingestion in non-human primates. Thirty – one pregnant macaca nemestria were exposed to weekly ethanol doses ranging between 0.3 – 4.1g/kg maternal weight. Morphometric analysis performed on their cranial radiographs showed that animals exposed to high doses of ethanol had, on average, smaller cranial volume.[53] It is hereby proposed that ethanol probably has a fascilitatory effect on the actions of the calcification inhibitors. Calcification inhibitors are a family of inorganic compounds comprising the pyrophosphatases, phosphonates and diphosphonates; these act normally to prevent calcium deposits from forming in soft tissues. The calcium and phosphate ions occurring in the plasma bathing bone-forming tissues precipitate from solution and form calcium phosphate crystals in and on the collagen fibrils of osteoid. Initially, these are arranged in clusters;

forming 'seeds' or nuclei on which crystals of bone minerals grow[54]. Impaired protein (collagen) synthesis following alcohol-induced folate deficiency, in addition to direct inhibitory effect of ethanol on bone cell activity, could possibly explain these anomalies[55,56]. Earlier reports indicate that alcohol consumption results in reduced osteoblasts activity; and this also leads to decreased bone formation[57]. As reported in this and other studies, it would be expected that almost all skeletal structures are affected following ethanol treatment, an observation, which supports the theory that alcohol induced osteodysgenesis is not site specific[20].Roles of folic acid in development The maternal deficiency of pteroyglutamic acid is remarkably potent with diverse teratogenic influences. It has been studied extensively and intensively for many years; severe folic acid deficiency in the gestationally ethanol - exposed mice fetuses had been reported. Acetaldehyde - the main metabolite of ethanol induced both the depression of intestinal absorption of the vitamin from exogenous sources and its supply from endogenous synthesis by bacteria. Folic acid is a known precursor in DNA-protein synthesis. Significant reduction both in the cellular DNA and protein synthesis following ethanol ingestion had also been observed. The importance of folic acid in prenatal development has been well documented both clinically, and dossiers of published reports on the fed diet lacking folic acid and containing 1% succinyl sulfathiazole (to reduce intestinal biosynthesis of the vitamin) are available[58-60] **Reparative growth following teratogenesis** The importance of reparative processes in the final Embryonic repairs had traditionally been regarded in term of tissues regeneration. The critical lesions, however, involve injury to individual cells. Detailed analyses of the capacity of the embryonic cells to expression of malformation after teratogenic insults has not been given adequate consideration in the field of teratology. For most teratogenic agents, a threshold dose exists below which abnormal development cannot be detected[1]. This 'threshold' changes through out gestation and there are developmental stages, that is, during organogenesis period, during which embryo is highly resistant to teratogenic insults. Implicit in this concept of a threshold dose is that the embryo possesses a varying capacity at different developmental stages to repair teratogenic damage[1]. Repairs of teratogenic insults during the organogenesis period had traditionally been viewed in terms of tissue regeneration or of restorative hyperplasia of the surviving cells to replace dead cells undergoing necrosis from teratogenic insults would be interesting to investigate. Study of the differential capacity of cells surviving teratogenic insults versus those that die to repair DNA damage may contribute an understanding to the process of cell death. Correlation of the time dependent insults with the rate of repairs of DNA damage may help elucidate the target organ specificity of certain teratogens for example, the question may be asked: are embryonic limb susceptible to teratogenic insults on day 11 of gestation but not on day 14 due to a depressed capacity of the day 11 bud to repair DNA damage? Such questions yet need to be addressed as earlier workers in this field had pointed out[1]. The processes whereby embryos cope with teratogenic insults are fundamental to understanding the mechanisms of teratology. A deleterious response may occur only after the defence mechanisms are overwhelmed[1] repairs lesions in DNA during the organogenesis period contribute to an understanding of the basic mechanism of teratogenesis

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Conclusion

The actual mechanism by which ethanol mediates its teratogenic effects particularly at the early stages of cell differentiation in the foetuses is yet to be clearly understood. It now appears that the majority of incidences of congenital malformations are associated with environmental factors. Moreso, this appears to be the category in which the most successful preventive measures could be instituted. It is for this reason that so much attention has been devoted to the study of exogenous factors or altered maternal environment of the foetus and alcohol is one such common factor

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