Nutrition and Diet Research Progress

# Undernutrition

## Effects, Causes and Management

Jason E. Lee
Editor

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# UNDERNUTRITION: EFFECTS, CAUSES AND MANAGEMENT

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# PREFACE

In this book, the authors present research from across the globe in the study of the effects, causes and management of undernutrition. Topics discussed include the role of early life nutrition and reproductive programming; effects of maternal undernutrition on lung growth and development in offspring; educational attainment affects on nutritional status and the long-term effects of early undernutrition on wound healing.

Chapter 1 – The concept of developmental programming implies that a stimulus or insult acting during critical periods of growth and development may result in developmental adaptations that permanently change the structure, physiology and metabolism of the offspring. Variation in the nutrient supply during fetal life in terms of both quantity, and quality (macro and micro nutrients) and especially maternal undernutrition has been highlighted as a dominant cause of programming. To date such nutritional programming effects have been largely characterized in terms of susceptibility to cardiovascular or metabolic disease. As the reproductive system and its hormonal control systems are largely established in fetal life, the arising question is if this prenatal compromise translates into any significant functional deficit in reproductive performance during adulthood. The present chapter presents the existed evidence and review the available data from numerous animal studies on the effects of early life nutritional environment on adult reproductive function. It also describes the findings of our ongoing research in this area. Human retrospective cohort studies linking early life nutritional experience, reflected by birth weight and postnatal weight gain to a number of reproductive health measures, such as pubertal onset, age at menarche, fertility and age at menopause are also presented and critically evaluated.

Specific outcomes depend on the severity, duration and stage of development when nutritional perturbations are imposed, while sex specific effects are also manifested. Apart from undernutrition, effects of relative overnutrition as well as the complex interactions between pre and postnatal nutrition is of high importance, especially in the context of our days obesity epidemic. Mechanisms underlying reproductive programming are still poorly understood. They might include altered cell proliferation/apoptosis, changes in hormone levels or receptor abundance. Epigenetic modulation of critical genes involved in the control of reproductive function and potential intergenerational effects represent an exciting area of interdisciplinary research towards the development of new nutritional approaches during pre and postnatal periods to ensure reproductive health in later life.

Chapter 2 – Numerous epidemiological data in humans and experimental studies in animals have showed that perinatal alterations, such as maternal undernutrition, increased the occurrence of chronic adult diseases. The pathophysiological mechanisms involved in the so-called "Developmental Origin of Health and Adult Diseases" are still largely unknown, but it is suggested that dysfunctions of stress neuroendocrine systems (sympatho-adrenal system (SAS) and hypothalamo-pituitary-adrenal (HPA) axis, respectively) could play a crucial role. However, the wide spectrum of experimental paradigms used (species, sex, age of the animals, severity and duration of undernutrition…) has given rise to variable, and sometimes contradictory, results that are almost impossible to interpret. To circumvent this problem, we used the same protocol of maternal perinatal undernutrition (MPU) to study the HPA axis activity and SAS in male rat at weaning and in adulthood (8-month-old), both under resting conditions and in response to stress. We have developed a maternal perinatal undernutrition experimental model (called FR50, using a 50% global caloric restriction from the last week of gestation until weaning) in rat. At weaning, FR50 pups displayed normal corticosterone plasma levels under resting conditions whereas in response to an ether inhalation stress procedure, the increase in plasma ACTH was lower than in controls. The plasma corticosterone returned to lower values than basal level 90 minutes after this stressful procedure. Noradrenergic adrenal chromaffin cells exhibited morphological alterations associated with increased catecholamine plasma levels both under resting conditions and in response to insulin-induced hypoglycemia. Adult animals still exhibited morphological alterations of noradrenergic adrenal chromaffin cells. This was accompanied by decreased catecholamines urine and plasma levels under resting conditions and augmented ones in response to fasting. In contrast, under resting

conditions FR50 male rats showed HPA axis hyperactivity with elevated glucocorticoids plasma levels that were not modified even in the presence of a severe stressor such as a 72-hour dehydration period. Together, the authors' results indicate that MPU has both short- and long-lasting consequences on the activity of neuroendocrine systems involved in the response and/or adaptation to stress in the male rat offspring. Because these systems, via the production of both catecholamines and glucocorticoids, participate to the regulation of multiple metabolic pathways, their dysfunction, in particular in chronic stress situations, may participate to the programming of several diseases from developmental origin.

Chapter 3 – Maternal undernutrition during pregnancy causes fetal growth restriction. Alterations in fetal nutritional status may result in developmental adaptations that permanently change the structure and physiology of the offspring, thus predisposing individuals to pulmonary, endocrine, and cardiovascular diseases in adult life. This phenomenon, termed "fetal programming", has led to the theory of "fetal origins of adult disease". Maternal undernutrition may have significant effects on the developing fetal lung, which undergoes rapid cellular multiplication and differentiation shortly before birth. Intrauterine growth restriction (IUGR) is an important risk factor for both early and late postnatal respiratory morbidity. Lung growth and development and later function can be affected by fetal growth restriction. Intrauterine growth restriction can be caused by maternal, placental, or fetal factors that affect the intrauterine environment. The exact pulmonary consequences linked to each of these specific causes are poorly understood. The authors have found that inadequate maternal dietary intake during late gestation altered the development of the lung structure (reduced alveolar surface area and volume fraction) and expression of lung growth factors in the postnatal period. Numerous studies have been performed to investigate the effects as well as the exact mechanism of action of maternal undernurition. The purpose of this review is to evaluate these studies in order to elucidate the harmful effects of maternal undernutrition on lung growth and development in the offspring. Subsequently, the mechanism by which maternal undernutrition induces fetal programming on lung growth will be discussed.

Chapter 4 – 24 adult Sprague-Dawley pregnant rats were divided into three groups: Control group (group C), n=8, fed *ad libitum* during gestation and lactation (until 25 days post-partum). Group underfed during gestation (group UG, n = 8), were offered only 40% of *ad libitum* rat chow intake of Control group pregnant dams. Group underfed during gestation and lactation (group UGL, n=8), were treated identically as group UG during gestation.

After parturition, litters were adjusted to either 14 (group UGL) or 8 (Control and UG groups) pups. At 2 days of age, 11 group C and 12 group UG male pups were slaughtered. Their testes were processed for standard histology and morphometrical evaluation, and for proliferating cell nuclear antigen (PCNA) immunohistochemistry (IHC) of Leydig and myoid cells. At 25 days of age, 5 group C, 8 group UG and 10 group UGL male pups were also slaughtered and their testes processed as in 2 days old pups. Semiquantitative results of PCNA IHC evaluation were converted to PCNA labeling index (LI). Body and testes weights, quantitative histological variables and PCNA LI were presented as means ± sd and analysed with anova. At 2 days of age, group UG pups had lower body weight (6.43 ± 1.07 vs. 7.63 ± 0.56 g), testes weight (0.0026 ± 0.0005 vs. 0.0035 ± 0.0005 g), gonadosomatic index (0.82119 ± 0.13162 vs. 0.92424 ± 0.08041) and total number of Sertoli cells per testis (0.50 ± 0.09 vs. 0.74 ± 0.15 x $10^3$) as compared to group C. At 25 days of age, group UGL had lower body weight (36.36 ± 3.27 vs. 61.39 ± 6,28 and 65.17 ± 5.00 g), testes weight (0.06 ± 0.02a vs. 0.12 ± 0.04b and 0.18 ± 0.03c g), seminiferous tubules diameter (196.74 ± 9.14 vs. 239.10 ± 7.68 and 244.87 ± 16.05 μm) and total number of Sertoli cells per testis (7.73 ± 2.11 vs. 12.22 ± 1.86 and 14.43 ± 2.55 x $10^4$) as compared to groups C and UG respectively. The only variable which was lower in group UGL than in group C at 25 days of age was testes weight. However, testicular weight and gonadosomatic index were higher in group UG than in both groups C and UGL, indicating compensatory growth. No differences were found in PCNA LI between groups. Present results indicate the effects of undernutrition during fetal life determine lower body and testes weight, and lower Sertoli cell numbers. Body and testicular weights, diameter of seminiferous tubules and Sertoli cell numbers in rats underfed during gestation and suckling period is lower than in their well fed controls but also lower than in rats which were underfed only during gestation. In summary, 1.- the authors confirmed the strong deleterious effect of undernutrition during fetal to postpubertal life on rat testicular development and final Sertoli cell numbers; 2.- most effects of undernutrition during gestation on testes structure, including Sertoli cell numbers, disappear at 25 days of age in rats fed *ad libitum* after birth and 3.- this kind of testicular compensatory growth in rats is accompanied by higher testicular weight than in control animals which never experienced undernutrition.

Chapter 5 – *Aim*: To investigate the prevalence of undernutrition and the impact of level of education on undernutrition among ***SAHAI*** (living below poverty line) adults from six selected districts of West Bengal, India.

*Methods:* This cross-sectional study was conducted among 600 randomly selected SAHAI families from six selected districts of West Bengal. A total of 1068 adults (> 18 years of age) were studied of whom 499 and 569 were men and women, respectively. Mid-upper arm circumference (MUAC) of each subject was recorded to the nearest 0.1 cm. Evaluation of nutritional status was based on internationally accepted MUAC cut-off values. Information on educational status of the subjects was also obtained.

*Results:* The mean (sd) ages in men and women were 40.4 years (15.0) and 38.4 years (14.6), respectively. The population had a very high rate (62.0%) of illiteracy (men = 54.5%; women = 68.5%). There was significant sex-difference in educational status (chi-square = 24.36; $p < 0.001$). There was a significant sex difference ($t = 9.71$; $p < 0.001$) in mean MUAC (men: mean = 24.2 cm, sd = 2.5 and women: mean = 22.6 cm, sd = 2.7). Results revealed that the overall sex-combined prevalence of undernutrition was 34.6%. Significantly (chi-square = 16.41; $p < 0.001$) more females (40.1%) were undernourished compared to males (28.3%). Overall, there was a very strong significant (both sexes combined: chi-square = 21.51; $p < 0.001$) association of educational status with undernutrition. In men, there was a significantly (chi-square = 8.45; $p < 0.01$) decreasing prevalence of undernutrition with increasing level of education. The highest rate of undernutrition was observed among illiterates (33.5%) while the lowest rate was observed among individuals with secondary or above level of education (18.8%). Subjects with primary level of education had intermediate level of undernutrition (23.3%). A similar trend (chi-square = 8.60; $p < 0.01$) existed among females. Among them, the highest rate was observed among illiterates (44.10%) while the lowest rate was observed among individuals with secondary or above level of education (28.21%). Subjects with primary level of education had intermediate level of undernutrition (32.14%). Logistic regression analyses revealed that educational status (independent variable) had significant impact on nutritional status (dependant outcome variable) among the SAHAI people (Wald = 20.01, $p < 0.001$). This impact was similarly strong in both sexes (men: Wald = 7.95; $p < 0.005$; women: Wald = 7.96; $p < 0.005$). These results implied that education is a significant predictor of undernutrition in this population.

*Conclusion:* Our study provided strong evidence that the level of undernutrition among adults belonging to SAHAI families was high, more so among women. These adults were experiencing severe nutritional stress which was indicative of acute nutritional deprivation. Moreover, educational attainment was strongly related to nutritional status. Urgent appropriate nutritional supplementation/intervention programs are required to reduce this

high prevalence of undernutrition. There is also a need to increase the level of educational attainment.

Chapter 6 – Child undernutrition is the most serious health problem in developing countries. It is a serious public health challenge that continues to be a primary cause of ill-health and premature mortality among children in these countries. However, there exists scanty information of the prevalence of undernutrition among Muslim school children in India. The present cross-sectional study investigated the prevalence of undernutrition among 6-16 years old Bengalee Muslim school children from a rural area of eastern India. The children (304 boys and 334 girls) were randomly selected from different schools and Madrashas (government schools) of Chapra Block Nadia District, West Bengal, India. Significant ($p < 0.001$) age difference existed in mean height and weight in boys (height: $F = 202.66$; weight: $F = 129.00$) as well as girls (height: $F = 40.12$; weight: $F = 45.03$). Overall (age and sex combined) prevalence of thinness was 56.70%. Overall prevalence of thinness (age combined) were 58.77% and 56.70%, among boys and girls, respectively. Significant sex (age combined) difference in different grades of thinness were observed among the subjects (chi-square = 77.12, $p < 0.001$). In conclusion, the authors' study provided strong evidence that these children were under acute and chronic nutritional stress in form of thinness. There is a requirement for the implementation of immediate appropriate public health nutritional intervention programmes.

Chapter 7 – Epidemiological studies have revealed that protein-calorie malnutrition is a widespread problem among infants and young children of developing countries, and in poverty areas of the industrialized nations, and a moderate degree of undernutrition is more common among children, generally associated with negligence and carelessness from the parents. Malnutrition is still a problem in the Brazil, and studies have pointed out that 31% of younger children (up to 5 year old) may present moderate or severe undernutrition. Considering these facts, the authors think it is very important to understand the effects of undernutrition on wound healing, particularly after recovering from the undernutritional state.

Chapter 8 – The impact of malnutrition on oral cavity, especially of protein deprivation, is considered an important morbidity that significantly decreases the quality of life. Poor protein intake predisposes the oral mucosa to various diseases and affects the dental structure. Recently, the effects of protein deprivation on jaw bones, including the temporomandibular joint (TMJ), have been discussed mainly due the high frequency of protein malnutrition in hospitalized and immunosuppressed patients, as well as in

patients with chronic diseases. In the case of bone fracture, these patients frequently exhibit poor bone repair, and this situation may be present in fractured TMJ. In this chapter, the authors discuss the effects of protein deprivation on fracture repair of TMJ, including the role of low concentrations of proteins and vitamins in the delay of repair in TMJ structures. Some mechanisms of malnutrition on TMJ bone and cartilage cells are emphasized, as well as on hematopoietic marrow of mandibular condyle and temporal bone, and on the TMJ masticatory muscles. Additionally they comment on the effects of malnutrition on the TMJ growth, including the problems of malnutrition on infancy that involves the jaw bones. The themes discussed in this chapter have been analyzed by their research group using experimental models in animals with TMJ fracture and protein deprivation. The authors observe that an ingestion of hypoprotein and isocaloric diet provokes delay on TMJ repair, impairment on bone cells in some periods of repair process, serum biochemical alterations, mainly in albumin and calcium, and muscle atrophy after the consolidation of TMJ fracture. They also detect poor formation of cartilage tissue and, in some cases, important anatomic alteration of TMJ, such as disappearance of articular disc, malformation of head condyle, and pseudoarthrosis. These alterations are extensively described in this chapter, focusing the most susceptible structures of TMJ when they are exposed to malnutrition. In conclusion, the authors alert the surgeons in general and the dental professionals in particular with respect to the importance of malnutrition diagnosis during the treatment of TMJ trauma.

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*Chapter 1*

# REPRODUCTIVE PROGRAMMING: THE ROLE OF EARLY LIFE NUTRITION

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## ABSTRACT

The concept of developmental programming implies that a stimulus or insult acting during critical periods of growth and development may result in developmental adaptations that permanently change the structure, physiology and metabolism of the offspring. Variation in the nutrient supply during fetal life in terms of both quantity, and quality (macro and micro nutrients) and especially maternal undernutrition has been highlighted as a dominant cause of programming. To date such nutritional programming effects have been largely characterized in terms of susceptibility to cardiovascular or metabolic disease. As the reproductive system and its hormonal control systems are largely established in fetal life, the arising question is if this prenatal compromise translates into any significant functional deficit in reproductive performance during adulthood. The present chapter presents the existed evidence and review the available data from numerous animal studies on the effects of early life nutritional environment on adult reproductive function. It also describes the findings of our ongoing research in this

area. Human retrospective cohort studies linking early life nutritional experience, reflected by birth weight and postnatal weight gain to a number of reproductive health measures, such as pubertal onset, age at menarche, fertility and age at menopause are also presented and critically evaluated.

Specific outcomes depend on the severity, duration and stage of development when nutritional perturbations are imposed, while sex specific effects are also manifested. Apart from undernutrition, effects of relative overnutrition as well as the complex interactions between pre and postnatal nutrition is of high importance, especially in the context of our days obesity epidemic. Mechanisms underlying reproductive programming are still poorly understood. They might include altered cell proliferation/apoptosis, changes in hormone levels or receptor abundance. Epigenetic modulation of critical genes involved in the control of reproductive function and potential intergenerational effects represent an exciting area of interdisciplinary research towards the development of new nutritional approaches during pre and postnatal periods to ensure reproductive health in later life.

## INTRODUCTION

The pioneering studies of David Barker and colleagues in the late 1980s suggested an association between birthweight and rates of adult death from ischemic heart disease (Barker and Osmond 1986, 1987; Barker et al., 1989). Since then increasing evidence has replicated and extended these findings to establish inverse relationships between birth weight and increased risk for coronary heart disease, type 2 diabetes and dyslipidaemia, leading to the "fetal origins of adult disease hypothesis" (Barker, 1995). This implies that adverse environmental factors, acting *in utero* program the development of fetal tissues and lead to dysfunctions and diseases in adults. Such programming reflects the action of a factor during specific developmental periods to exert organizational effects that persist throughout life. This first hypothesis turned later to 'developmental origins of adult health and disease' hypothesis in order to encompasses all molecular, cellular, structural and functional responses that occur after exposure to different environmental stimuli during critical periods of development, leading to long term health consequences (Gluckman and Hanson, 2004). Unbalanced maternal nutrition is one of the major environmental factors suggested to drive fetal programming and although the main focus of developmental programming has been the impact of poor fetal nutrition, fetal overnutrition is gaining an important role in the context of our

days obesity epidemic (Armitage et al., 2004; Ojeda et al., 2008). It is also well accepted that environmental factors that act during periods of postnatal developmental plasticity can also drive programming effects (Armitage et al., 2005). It has been reported that the association between birth phenotype and adult disease extends across the normal range of birth size, suggesting that even subtle influences, as reflected in the normal range of birth weights are sufficient to alter the propensity of adult disease (Glukman, 2001). Direct evidence supporting the developmental origin of health and disease hypothesis has been derived from experimental animal studies, using controlled maternal food supply during key developmental windows and precisely defining the specific outcomes related to different nutritional regimens or to other triggering factors such as maternal stress or glucocorticoid exposure (McMullen and Mostyn, 2009). The most widely used animal models in developmental programming studies have been rodents and sheep. Although rodents offer significant advantages with respect to their short gestation period and the excellent availability of molecular tools, sheep studies provide power for translation to human pregnancy, as sheep has the advantage of a long gestation period, enabling targeting of specific developmental windows and produces a fetus comparable in size to humans. A number of several key questions are the target of current research. What is the signal(s) that affects the fetus? Which is the critical window of development during which the signal acts? Which is the nature of the underlying mechanisms? Is the programming state irreversible? Are there trangenerational effects? What's the role of epigenetic influence? Compelling evidence exists that the major, non communicable diseases of our days are cardiovascular and metabolic, both susceptible to "developmental programming". There have been outstanding reviews that summarize the existed evidence and provide background on the developmental origin of cardiovascular disease, insulin resistance and type-2 diabetes, obesity and metabolic syndrome. (Armitage et al., 2004; McMillen and Robinson 2005; Ojenda et al., 2008). However, the developmental origin of health and disease approach with respect to programming of reproductive function and health has received little attention. The present chapter presents the existed evidence and review the available data from numerous animal and human studies on the effects of early life nutritional environment on adult reproductive function.

## PROGRAMMING OF HPG AXIS

### Animal Studies

As the reproductive system and its hormonal control system are established during fetal life, hypothalamo-pituitary gonadal axis (HPG) represents a target for developmental programming. In female rats maternal food restriction imposed during late gestation and lactation or solely during lactation resulted in a decrease in uterine and ovarian weights, along with disturbed folliculogenesis, reflected by a greater number of antral follicles of small size and a reduced number of graafian follicles of large size. This disruption was also accompanied by increased FSH levels at weaning (Leonhardt et al., 2003). Disturbed follicular growth, presented as lower number of follicles at vaginal opening has also been reported in intrauterine growth-retarded rats, whereas postnatal food-restricted rats have a normal number of follicles, but impaired follicular maturation (Engelbregt et al., 2000). A similar pattern of impaired follicular maturation in offspring was also evident following maternal undernutrition during lactation (da Silva Faria et al., 2008). This reduced number of primordial follicles could impact on subsequent fertility, since the follicular reserve determines fertility and duration of reproductive function, and in this regard one year-old rats undernourished during lactation were exhibited an increase in the estrus cycle length and reduced fertility (Guzman et al., 2006). The findings that the major effects on reproductive function result from nutrient restriction imposing during lactation is in agreement with the fact that the major part of cell division and organ growth is taking place during the postnatal period in this altricial species. Data on male rats are limited. Maternal food restriction during pregnancy reduces the testicular growth in offspring, whereas when it is imposed during both gestation and lactation or only during lactation results in a drastic reduction in gonadal weight and structure, indicating a potential influence on later reproductive function. (Leonhard et al., 2003) Indeed, earlier reports on reduced sexual activity following undernutrition during gestation and/or lactation (Menendez-Patterson et al., 1985), were verified by more recent data showing that exposure to low protein diet during gestation reduce sperm count and influence male's ability to impregnate female rats in the $F_1$ male offspring (Zabrano et al., 2005).

In sheep oogonial meiosis and follicular development were found to be delayed in fetuses undernourished during early gestation, indicating that undernutrition imposed even before differentiation of the ovary can

compromise subsequent follicular development (Rae et al., 2001). Effects of maternal undernutrition on the rate of cell atresia are supported by the study of Borwick et al. (1997), which showed a delay in the foetal germ cell degeneration. In a separate sheep model of intrauterine growth retardation caused by over nutrition more compelling reductions in the size of the ovarian follicular pool were reported in female offspring (Da Silva et al, 2002, 2003) The mechanism mediating such effects may involve changes in apoptosis-regulating genes that alter subsequently the balance of apoptosis and proliferation in the developing follicles and surrounding ovarian cells and could lead to a reduced number of follicles in the ovaries postnatally (Lea et al., 2006). Food-intake restriction of pregnant ewes from mating to midgestation resulted in increased DNA damage in the fetal oogonia (Murdoch et al., 2003) and recently maternal undernutrition from early to midgestation was reported to decrease late gestation fetal ovarian vascular development, which could impact follicular quality (Grazul-Bilska et al., 2009). Results are less clear with respect to male testis development. Maternal undernutrition in early gestation in sheep led to increased expression of steroidogenic acute regulatory protein (*StAR*) mRNA in the fetal testes, and increased plasma testosterone concentrations (Rae et al., 2002a). Data regarding the number of Sertoli cells are controversial and range from no effects in fetal testis weight and Sertoli cell number after nutritionally mediated placental growth restriction (Da Silva et al., 2003), to 20% reduction in the number of Sertoli cells in newborn lambs undernourished in utero (Bielli et al., 2002). Sertoli cells could provide a target for programming, as their number per testis is the most important factor that determines the ceiling of sperm production and output (Orth et al., 1988). Effects on hypothalamo-pituitary axis have also been reported in fetuses from undernourished mothers. In male sheep fetus maternal undernutrition has been shown to influence the pituitary response to GnRH challenge (Rae et al., 2002b). Altered pituitary sensitivity has also been observed in 55 days old lambs, born to mothers undernourished from 30 days of gestation to term (Deligeorgis et al., 1996).

The majority of studies addressing the impact of maternal undernutrition on HPG axis, using sheep as a model, have been mostly limited to the late gestation fetus or young lambs. However, it is well accepted that alterations in the developmental process of the HPG axis generally are perceived only at puberty or in adult reproductive life (Pereira, 2003) and more likely after the gonadal feedback is set up (Kotsampasi et al., 2009a). This is also the case with the reproductive consequences of early glucocorticoid or androgen exposure which are only evident after puberty, when many of the sex-linked

differences in developmental programming appear for the first time with the onset of gonadal steroidogenesis (Grigore et al., 2008). Moreover, early perturbations could be of significance only if long lasting effects are considered. Studies on pituitary function extended to pre and post pubertal age reported conflicting results. A lack of an effect of maternal undernutrition on basal gonadotrophin secretion and on pituitary sensitivity in terms of the LH response to GnRH challenge was reported in adult offspring born to mothers undernourished either after day 100 of gestation (Borwick et al., 2003), or from mating to 90 day of gestation (Rae et al. 2002c), although in the latter study an apparent reduction in the ovulation rate in young adult female offspring was detected, most likely through a direct effect on folliculogenesis during intrauterine development. In contrast, more recent results from our laboratory on lambs undernourished during two critical developmental windows (0-30 and 30-100 days of gestation) have shown a window-of-exposure and gender specific effect of undernutrition on pituitary responsiveness to GnRH challenge. In particular in male sheep undernutrition during the first month of age did not affect pituitary response to GnRH at 10 months of age, whereas when it was imposed during mid to late gestation (30-100 day) resulted in an enhanced LH and FSH response and increased basal FSH levels (Kotsampasi et al., 2009b). This response may have been mediated through a direct effect of nutritional insult on the neuronal circuit that controls gonadotrophin release or on pituitary stores for FSH, especially if one considers that the gestational window when undernutrition was imposed coincides with GnRH neuronal maturation (Brooks et al., 1995). However, in this same cohort of animals a striking decrease in the number of Sertoli cells along with reduced seminiferous tubule diameter was observed, thus raising the possibility for a direct gonadal effect. Indeed, numbers of Sertoli cells are determined largely by their rates of proliferation during foetal and neonatal periods and in male sheep fetuses Sertoli cell proliferation peaks during late fetal life with mitotic divisions being more numerous before birth than afterwards (Hochereau- de Riviers et al, 1987). So, this interval is of critical importance in establishing the complement of Sertoli cells that populates the adult testis and to this respect the observed effects could have been a result of a reduction in FSH levels during replication, as FSH is considered the most important factor determining Sertoli cell proliferation (Sharpe et al., 2003). On the other hand, the increased FSH response it is likely to reflect an attenuated feedback signal on the pituitary, mainly from inhibin, as a result of the reduced Sertoli cell number. However, as data on sperm quality are lacking it is not clear whether this reduced number reflects a lower spermatogenic potential.

Opposite effects have been observed in females in which an enhanced pituitary sensitivity in terms of FSH response was detected in adult offspring undernourished during the first month of gestation, which were also presented with an increased number of small follicles and decreased number of corpora lutea in their ovaries (Kotsampasi et al., 2009a). Thus, an early exposure to feed restriction may alter the central/peripheral FSH regulation and consequently higher FSH response in these animals may be associated with an attenuated feed back signal on the pituitary, mainly from inhibin. Alternatively, as uniform pituitary precursor cells proliferate to expand the population before differentiation initiates (Zhu and Rosenfeld, 2004), early undernutrition may affect the transcriptional control of this process, as nutritional effects can be mediated by alterations in gene expression (Taylor et al., 2003). In addition, the lower number of corpora lutea detected in these animals may potentially affect the establishment of pregnancy, since corpus luteum provide steroid hormonal support essential for the establishment and maintenance of early pregnancy (Niswender et al. 2000; Wathes, 1992). To this respect, previous studies reported an increased embryonic loss in ewes undernourished as fetuses (Gunn et al. 1995; Rhind et al., 1998). Regarding potential mechanisms underlying the above effects it is interesting to note that ewes undernourished during early gestation exhibited higher cortisol levels and following a CRH challenge their off springs exhibited an enhanced response in terms of ACTH and cortisol levels (Chadio et al., 2007). Glucocorticoids are proposed to act as intermediary factors that transcribe the developmental programming sequale of maternal nutrient restriction (Langley-Evans et al., 1996). A growing body of evidence shows that prenatal stress may alter gonadal function in adult offspring and support a role of glucocorticoids in programming of the HPG axis.

Taking together the above data point out to sex specific effects of developmental programming and further emphasize the significant influence of timing, type and duration that imposing insult could exert on the programming of the hypothalamic-pituitary-gonadal axis.

## Human Studies

In humans small weight at birth may be arise from maternal undernutrition or reduced nutrient delivery to the fetus due to different placental insufficiency. The majority of studies addressing the impact of early life nutritional history on HPG axis function have focused on females and in these

studies birth weight was used as a proxy for fetal development. A number of studies by Ibanez and coworkers reported that girls born small for gestational age (SGA) were presented with a marked reduction in ovarian and uterine size both during neonatal life and in adolescence, (Ibanez et al., 2000a) and showing also a strikingly low ovulation rate (Ibanez et al. 2002a). Furthermore, prenatal growth restraint was found to be followed by elevated serum FSH concentrations in infant girls and boys. **(**Ibanez L., 2002b), probably reflecting reduced fractions of granulosa and Sertoli cells within the gonads. A reduction in the number of primordial follicles was evident in growth restricted girls, compared to appropriately grown ones (de Bruin et al., 1998). The effect of pre- or postnatal nutrition on the rate of folliculogenesis probably are mediated through alterations in central/peripheral FSH regulation, since in a number of the above mentioned studies disrupted folliculogenesis was accompanied by increased FSH levels. Moreover, as the synergistic action of estrogen is required for complete follicle differentiation changes in the ovarian expression of gonadotropins, estrogen and androgen isoform receptors may present another mechanism, through which early malnutrition affect follicular development (da Silva Faria et al., 2008). However, as adolescent girls born small for gestational age has been shown to experience hyperinsulinism and hyperandrogenism often accompanied by central adiposity and dyslipidemia, it is more likely that reduced ovulation is a secondary effect and this suggestion is strengthen by the fact that treatment with metformin induced ovulation and normalize both abdominal fat and lean body mass (Ibanez et al., 2002c).

Regarding males, results on long term effects of intrauterine growth retardation on the HPG function are controversial. In boys low birth weight is associated with hypospadia, (Weidner et al., 1999), cryptorchidism and testicular cancer (Brown et al., 1986), an entity known as testicular dysgenesis syndrome (Skakkebaek et al., 2001). A reduction in testicular volume along with lower testosterone and higher LH levels was detected in males born small for gestational age (SGA) (Cicognani et al. 2002), indicating a different setup of the HPG axis with a tendency to hypogonadism in the SGA subjects. These results, with increased levels of gonadotropins, are similar to those in females, pointing out a peripheral partial insensitivity to gonadotropins (Ibanez et al., 2000b) Increased estrogen levels and aromatase activity was reported in SGA men and according to authors this increase could be part of the explanation to testicular dysgenesis syndrome (Allvin et al., 2008).

However, data from a prospective study in adolescent men found no association between smallness at birth and adolescent male pituitary-testicular

function as no significant differences for testicular volume or the secretory pattern of gonadotrophins between SGA and AGA males were observed. (Jensen et al., 2007).

A common finding among studies in rodents, sheep and humans is the disrupted follicular development following maternal feed restriction. As it is well documented that effects on oocyte number will determine the span of female reproductive life these effects are most likely to impact on subsequent fertility.

As the majority of studies in humans have been performed in adolescence, little information exist as to whether these changes persist into adulthood. Therefore it is of significant importance to determine if this prenatal compromise in the development of reproductive axis translates into any significant functional deficit in subsequent reproductive performance and particular fertility, representing the main outcome of reproductive function.

Follow up studies of historical cohort of Dutch famine women do not support a detrimental effect on fertility of women exposed to famine *in utero* during the World War II. A number of fertility markers, such as age at first pregnancy, completed family size and inter-pregnancy interval were not different between exposed or non-exposed to famine women (Lumey and Stein 1997; Lumey, 1998). A French cohort study also demonstrated no association between birth weight and fertility of both men and women and, furthermore, although subjects born SGA were more insulin resistant than AGA ones, yet no evidence of any relation between insulin resistance and reduced fertility was observed (Meas et al., 2010). Therefore, data from epidemiological studies do not led support to the impact of early life nutritional perturbations, reflected by weight at birth, on subsequent fertility. Conflicting data also exist on the onset of reproductive senescence or menopause in women, reporting either no effect on the timing of menopause (Creswell et al., 1997) or a U-shaped relationship between birth weight and time of menopause (Tom et al., 2010). This latter study also highlights the significance of growth rate rather than prematurity in the occurrence of earlier menopause. Early natural menopause is a risk factor for a number of health outcomes, notably stroke and cardiovascular diseases (Mondul et al., 2005; Ossewaarde et al., 2005).

However, retrospective studies are prone to various biases and often lack of control of confounding factors and there is also a difficulty in comparing data due to different fertility markers used. Therefore, the functional significance of the reported effects of maternal undernutrition and/or birth weight on HPG axis development and function in terms of fertility and reproductive potential should be elucidated further, before definitive

conclusion can be drawn. This necessity is enhanced further if one considers that studies with sheep, used as a model for human pregnancy, support at least a moderate effect of early nutrition on adult fertility. Ewes born to nutritional restricted mothers during mid to late gestation showed decreased progesterone concentrations at the first and second year of life, in line with the fewer corpora lutea detected in our study with females undernourished as fetuses during the same period. (Kotsampasi et al., 2009a) and most importantly they produced fewer lambs than normal fed ewes (Long et al., 2010). Reduced progesterone could have been the cause for the reduced lambing rate, because reduced progesterone levels have been shown to lead to elevated embryo mortality as seen in sheep and cows (Inskeep, 2004; Pope, 1988). Using a large sheep cohort Gardner et al., (2009) reported a curvilinear relationship between fecundity and early-life events, with apparent decreasing litter size at either end of the birth weight range. A U–shaped relationship has also been reported to exist between birthweight and increased risks of developing metabolic disorders later in life (Curhan et al., 1996). This kind of relationship may explain why many studies looking at extremes report no effect of birth weight on reproductive performance. However, after adjusting for body fatness during adolescence, the reduction in litter size associated with reduced birth weight was lost, indicating that the effects of small size at birth on reproductive performance may be secondary to reduced body fatness during adolescence (Gardner et al., 2009). By contrast, the negative relationship between large size at birth and reduced litter size appears to be largely independent of adolescent body composition.

It is widely accepted that in metabolic programming the catch up growth which follows *in utero* growth restriction underlies many of the adverse effects occurring during adulthood (Singhal and Lucas 2004; Cameron et al. 2005). The impact of catch-up growth on fertility in humans, however, is largely unknown, but it certainly complicates interpretation of the effects of nutrient restriction during pregnancy *per se* on physiological function in the offspring. Evidence exist that early life effects may be amplified or ameliorated due to postnatal nutrition. According to Predictive Adaptive Response hypothesis the risk for disease is a consequence of the degree of match or mismatch between pre and postnatal environment (Gluckman et al., 2005) and this hypothesis may also be applied regarding reproductive outcomes. Although experimental and epidemiological data link the low birth weight to postnatal adverse effects, it is also apparent that programming effects may be expressed in the absence of any changes in birth weight. Following compensation, birth weight may be within the normal range or only slightly decreased, as composition, receptor

numbers and organ structure may be changed in the presence of a relatively normal weight (Nathanielsz, 2006). A number of studies, in which programming effects were detected without any change in body weight confirm that reduced size at birth following maternal undernutrition is not required for altered postnatal physiology and this was also the case with our recent studies in which reported effects on hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-gonadal (HPG) axes were expressed without any change in birth weight. (Chadio et al., 2007; Kotsampasi et al., 2009a; Kotsampasi et al., 2009b).

The vast majority of studies investigating the relationship between early nutrition and reproductive outcomes focus on food restriction, while effects of overnutrition on reproductive function and performance have obtained little attention. With respect to metabolic programming, epidemiological and experimental evidence indicate that overnutrition during early development can contribute to an increased risk of obesity and cardiovascular and metabolic disease in later life (Armitage et al., 2005). It has also been postulated that data on the effects of overnutrition on later reproductive outcomes are limited. Overnutrition of *n*−6 polyunsaturated fatty acids has been shown to advance puberty onset in rats (Hilakivi-Clarke et al., 1997) and in a more recent study an enhanced ovarian function, reflected by higher progesterone levels was evident in maternal high fat exposed offspring (Sloboda et al., 2009). Studies in sheep also indicate that apart from small size at birth, large size is also associated with lower fertility (Gardner et al., 2009). Therefore, in our days obesity epidemic and increased prevalence of metabolic diseases in childhood and adolescents (Heerwagen et al., 2010) it is of high importance to determine the impact of maternal obesity or overweight on offspring's fertility and reproductive health.

## PROGRAMMING OF THE TIMING OF PUBERTY

### Animal Studies

Evidence from epidemiological and experimental studies support the concept that the perinatal period is a critical time period for the maturational process of puberty. A number of studies have addressed the impact of maternal undernutrition during pregnancy and /or lactation on the pubertal onset in offspring.

In a two model study in rat, one with intrauterine nutrient restriction induced by uterine artery ligation or postnatal undernutrition induced by increasing litter size, a delayed onset of puberty and impaired testicular function in male rats of both models was reported (Engelbregt et al., 2000). Maternal protein restriction during two specific developmental windows, pregnancy and lactation also resulted in a delay in puberty onset in offspring restricted for the whole period of pregnancy and lactation or only during lactation, along with a drastic reduction in plasma leptin levels (Leonhardt et al., 2003). A delayed puberty onset and accelerated reproductive ageing was observed in female progeny of dams protein restricted during lactation, in line with the well accepted idea that the major part of cell division and organ growth is occurred during this period in rats (Guzman et al., 2006). Recent results indicate that the delay in sexual maturation may be mediated through kisseptin action, as a delay in the timing of vaginal opening was observed in under nourished rats, which also exhibited significantly lower hypothalamic Kiss1 mRNA expression (Iwasa et al., 2010). The neuropeptide kisspeptin (KISS1) and its receptor (KISS1R) have been identified as an essential part of the hypothalamic circuits that govern the initiation of puberty (Navarro et al., 2007) and KISS1/KISS1R signalling plays a critical role in regulation of the GNRH pulse generator (Li et al., 2009). However, opposite effects were reported recently, with maternal undernutrition resulting in an advanced attainment of puberty, but a reduction in progesterone levels in later life (Sloboda et al., 2009). The reasons for this contrasting results are unclear but it appears that in rats lactation period is more critical in determining a delay in puberty onset. However, from a history strategy perspective this accelerated maturation may represent an adaptive response, trough which the organism trades body size and longevity for earlier reproduction (Sloboda et al., 2009).

In sheep, moderate maternal undernutrition was not detrimental to the onset of puberty (defined as first ovulation) in female lambs, (Rae et al., 2001) but in males experienced placentally mediated fetal growth restriction a delay in the onset and magnitude of sexual activation was detected (Da Silva et al., 2001). In contrast, our results on sheep undernourished during two selective windows during pregnancy found no difference on the timing of endocrine puberty, which occurred at a similar age, in both normal fed and *in utero* undernourished males, thus emphasizing the importance of critical body weight for the onset of sexual maturation (Kotsampasi et al., 2009a; 2009b). However, it should not be ignored that although sustainable increase in testosterone levels may be an indication of the onset of puberty, attainment of

spermatogenesis is a more precise indice of sexual maturation in male and data on spermatogenesis after perinatal restriction are very scarce.

Collectively, it must be emphasized that early undernutrition exerts sex – and window of exposure specific effects on the attainment of sexual maturity in offspring. The severity as well as the duration of the insult may also affect the outcome and most importantly an interplay between pre and postntal nutrition may be a significant determinant of the timing of the attainment of puberty.

## Human Studies

A secular trend towards an earlier age at menarche was documented during the last decades, both in US and Europe (Chumlea et al., 2003; Karlberg et al., 2002; Papadimitriou et al., 2008). On the other hand growing evidence is accumulated on the relationship between early life events and an increased risk of premature adrenarche, early puberty and associated fertility problems. To this respect, early life events and particular weight at birth have been reported to affect sexual maturation and a number of excellent reviews summarize the existed evidence (Hernandez and Mericq, 2008; Sloboda et al., 2010). Data on the relationship between birth weight and age at menarche are controversial, possibly because of the heterogeneity in the study designs. A number of cohort studies in UK (Cooper et al., 1996) Spain (Ibanez et al., 2000c) and Israel (Lazar et al., 2003) reported an association between low birth weight and timing of puberty or menarche, suggesting an effect of birth weight per se, but others have pointed out that an interplay between low birth weight or accelerated weight gain in infancy is more important in determining the timing of puberty. Menarche was found to occur earlier in girls who were long light at birth and presented with a higher fat mass and circulating IGF-I levels in childhood (Tam et al., 2006). These results further strengthen the concept that body adiposity, possibly through hyperleptinemia, and insulin resistance are key contributors to the normal variation in the timing of menarche. Also, a more recent Australian cohort study demonstrated that both lower BW combined with higher BMI during childhood predict early age at menarche (Sloboda et al., 2007). An additive effect of birth weight combined with accelerated postnatal growth on age at menarche has also been reported in those girls who were long and thin at birth and experienced an earlier menarche, and that this effect of birth size was potentiated by rapid growth in the first 6 mo postnatal (Adair, 2001). In contrast, dos Santos Silva et al.

(2002), initially found opposing effects of prenatal and early postnatal growth on the timing of menarche, but these effects disappeared once growth in childhood was adjusted for. Furthermore, rapid postnatal growth potentiates the effects of size at birth and is related independently to earlier pubertal maturation. Indeed, results from a large longitudinal German Study, the DONALD (Dortmund Nutritional and Anthropometric Longitudinally Designed Study) showed that in both boys and girls a relatively low birth weight and rapid weight gain between birth and 24 months, were independently associated with an earlier age in onset of puberty (Karaolis et al., 2009). However, rapid weight gain from ages 4 months to 1 year and 1 year to 7 years were shown to be the most significant factors in determining earlier menarche in women enrolled in the New York site of the US National Collaborative Perinatal Project (Terry et al., 2009). This rapid growth would also be associated with changes in leptin, insulin and other growth factors, all of which contribute to earlier menarche (Dunger et al., 2006). Thus, the timing of menarche appears to be influenced by both pre and postnatal nutrition and more likely in opposing directions, findings that could explain, in some degree ethnic differences and secular trends in the age of menarche (Tam et al., 2006). However, it could not be ignored that results from epidemiological studies are difficult to compare due to various methodologies, definitions, follow-up periods, and inclusion criteria involved and they must be treated with conscious taking into account the heterogeneity of children born small for gestational age with respect to the intrauterine insult that they experience, as pointed out by (Hernández and Mericq, 2008).

Increasing accumulating evidence (Ibanez 1998, 1999; Ong et al., 2002) strongly indicate that postnatal adrenal androgen secretion may be programmed during fetal and early postnatal development. Control studies in Spanish girls reported a relationship between low birth weight, early adrenarche and precocious pubarche, followed by subsequent ovarian hyperandrogenism, central obesity, hyperlipidemia, and insulin resistance after puberty (Ibanez et al., 1998; Ibanez et al., 2002c). In a large population-based study of boys and girls in the UK (The Avon Longitudinal Study of Parents and Children (ALSPAC) both lower birth weight and larger current body weight independently predicted higher adrenal androgen levels and furthermore children showing rapid postnatal weight gain between 0–3 yr had higher adrenal androgen levels at 8 years of age (Ong et al., 2004a). Data from the same cohort study indicated that a continuous inverse relationship exists between birth weight and DHEAS levels through the range of birth weights. Small infants who gained weight rapidly during early childhood had the

highest levels of adrenal androgen at age 8. Also, in case control studies girls born SGA and having accelerated postnatal growth have been reported to exhibit features of PCOS (Ibanez et al., 2007) and metabolic syndrome (Ibanez et al., 2006a; 2006b). In line with these observations, Ong et al. (2002; 2004b) reported the long-term risk for central obesity and insulin resistance in low birth weight infants who gained rapid weight during the first two years of life and who became insulin resistant in childhood. It has been shown that central and total adiposity is increased in children born small but gaining weight rapidly (Ong et al., 2000), which are also presented with higher IGF levels and reduced insulin sensitivity (Ong et al., 2002). Consequently, the increased IGF-I and insulin levels after low birth weight and rapid infancy growth could lead to the development of higher adrenal androgen production and to earlier or more pronounced adrenarche. Indeed, it has been reported that IGF-I and insulin levels are higher in girls (Silfen et al., 2002) and boys (Denburg et al., 2002) with premature adrenarche than in control children. These very early changes in fat distribution and insulin sensitivity could have important consequences on the subsequent tempo of growth and possibly on the initiation of earlier pubertal development. Thus, elevated insulin-like growth factor I concentrations and insulin resistance as well as higher leptin levels following rapid infancy weight could contribute to the trigger for earlier pubertal development, by promoting the activity of the gonadotropin-releasing hormone pulse generator, thereby influencing the timing of puberty (Dunger et al., 2006). Results from a Chilean survey of healthy girls reported no differences in DHEA levels between SGA and AGA girls, but SGA girls of this cohort had higher leptin levels and insulinogenic index at the beginning of puberty (Hernández et al., 2006). According to authors suggestions the relationship between the levels of androgens and exaggerated adrenarche/premature pubarche may be related to the prevalence of predisposing genetic variants of insulin and androgen sensitivity and infancy weight gain during childhood more than the characteristic of being SGA.

Conclusively, one can argue that the higher incidence of early menarche in low birth weigh girls reflects a disruption of the adipoinsulinar axis, thus linking early growth restrictions, post natal adiposity and reproductive development. Small for gestational age children exhibited a higher risk for developing metabolic syndrome in their adult live (Jaquet et al., 2005). A central role for early weight gain and insulin resistance and its association with earlier pubertal development has been confirmed by studies where treatment of low birth weight girls with metformin resulted in a delay in their pubertal

development up to menarche along with a decrease in leptin and IGF levels (Ibanez et al., 2006 b; 2008a).

It has been reported that precocious adrenarhe and premature pubarhe augment the risk of developing PCOS, (Ibanez et al., 2007). Although the concept of "prenatal androgen excess" is a widely accepted hypothesis on the origins of PCOS (Abbot et al., 1998; Xita and Tsatsoulis, 2006) the fact that PCOS development in low-birth weight girls can be prevented by metformin treatment (Ibanez et al., 2006b) started before and given across puberty led support to the recently proposed "adipose tissue expandability "hypothesis (Virtue and Vidal-Puig, 2008). According to this hypothesis it could be postulated that that the small for gestational age children which also develop hyperinsulinemic androgen excess (Ibanez et al., 1998), share a key feature in early life, namely a reduction in the expansion of sc adipose tissue, (Garg., 2006; Ibanez et al., 2008a; de Zegher et al., 2009), more likely leading to diminish the subsequent capacity to store lipids sc. So, hyperinsulinemic androgen excess may commonly be driven by the exhaustion of the capacity to expand sc adipose tissue in a metabolic safe way (de Zegher et al., 2009).

In conclusion, early life nutritional history and/or size at birth seem to play a critical role in determining age of sexual maturation. However, as the majority of studies clearly indicate that the onset of puberty and menarche may be linked both to intra-uterine and postnatal growth pattern, more detailed data on pre- and postnatal growth are required in order to evaluate the complex interactions between size at birth, infancy growth trajectory and timing of sexual maturation, especially when considering the association of pre and postnatal interactions to the later onset of metabolic disease (Hales and Ozanne, 2003). Change in the timing of puberty presents an area of great concern for public health, as early puberty has been associated with the metabolic syndrome (Frontini et al., 2003), overweight (Wattigney et al., 1999; Adair and Gordon-Larsen, 2001) and breast cancer, (Rockhill et al., 1998), but it also impact social policies, as it may result in earlier onset of sexual activity (Wyatt et al., 1999) and a number of teenage problems, such as depression, eating disorders and poor school performance (Posner, 2006). Thus unravelling the possible pathways through which these factors might operate would allow for early interventions towards normalization of pubertal attainment and prevention of late onset diseases, associated with early puberty onset.

## CONCLUSION

The impact of early nutritional environment on postnatal health outcomes, including reproduction, has been a target of an ongoing research activity and a considerable number of animal studies lent support to the developmental programming concept. There in now increasing accumulating evidence that reproductive development and performance are influenced by early life nutrition. The way through which environmental insults, such as nutrition contribute to the onset of later detrimental outcomes likely involves a complex interaction between the maternal environment, placental changes, and epigenetic programming of the embryo. However, the mechanisms through which early life events are transmitted to the target organs are complex and still poorly understood. They may include structural changes, altered cell proliferation/apoptosis, changes in hormone levels and receptor abundance. Early nutrition may affect a number of developmental, metabolic and endocrine pathways and these effects are dependent on sex, the nature and duration of the insult, the critical developmental window during which insult is imposed and the rate of postnatal growth. Maternal endocrine milieu is crucial in mediating the effects of nutrition, as hormones can directly or through changes in placenta phenotype act on the fetal tissues to alter cell growth and differentiation, consequently affecting their function later in life (Fowden et al. 1998; 2006). Endocrine programming has recently been highlighted as mediating such effects, since undernutrition disrupts a wide range of endocrine pathways, including HPG axis, resulting in long term effects on offspring's health (Harding et al., 2010). Epigenetic modulation of gene transcription provides the most plausible mechanism through which fetal nutrient supply can alter gene expression in the developing fetus, leading to later permanent effects. Fowden and Forhead (2010) highlighted the potential role of hormones as epigenetic signals in determining the phenotypical outcome of environmental cues acting during intrauterine development, as hormones signal the type, severity and duration of the environmental cue to the developing feto-placental tissues. There have been outstanding reviews on how the concept of epigenetics have been applied to the developmental programming hypothesis and such an approach can potentially contribute to better understanding of the mechanisms underlying programming of reproductive function as well (Junien and Nathanielsz, 2007; Waterland and Michels, 2007). Such evidence comes from the field of reproductive medicine where results from studies on reproductive outcomes after in vitro fertilization (IVF) (Mukhopadhaya and Arulkumaran, 2007) strongly support the well

recognized concept that alteration of biochemical and biophysical conditions at conception and during early embryonic life associated with ARTs may result in changes in epigenetic processes and lead to short and long term effects on development and health (Wadhwa et al., 2009).

The gender specific effects observed for several programming outcomes as a result to early nutritional perturbations could also be explained in the light of the well documented sexual dimorphism in environmental epigenetic programming. As dimorphic genes expression might be under the control of sex-specific epigenetic marks, environmental factors, including nutrition, can influence, in a sex-specific manner these flexible epigenetic marks, mainly during critical windows of development (Gabory et al., 2009). The concept of epigenetic modulation underlying sexual dimorphism is further enhanced by recent results showing that both promoters of androgen and estrogen receptor genes, and the expression of their target genes, are regulated by epigenetic mechanisms (Foecking et al., 2008).

One of the most significant feature of developmental programming is the evidence that adverse consequences of altered intrauterine environments can be passed from first generation to second generation offspring and recent evidence suggest that environmental epigenetic programming could be transmitted to the next generations in a sex specific manner and lead to trangenerational effects (Gabory et al., 2009) Epigenetic modulation of critical genes involved in the control of reproductive function and potential intergenerational effects represent an exciting area of interdisciplinary research towards development of new nutritional approaches during pre and postnatal periods to ensure reproductive health in later life. There is no doubt that much remains to be done. However, the concept of developmental programming of reproductive function is increasingly recognized as possessing important implications for reproductive health and fertility.

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*Chapter 2*

# MATERNAL PERINATAL UNDERNUTRITION PROGRAMS STRESS NEUROENDOCRINE SYSTEMS IN THE MALE RAT

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## ABSTRACT

Numerous epidemiological data in humans and experimental studies in animals have showed that perinatal alterations, such as maternal undernutrition, increased the occurrence of chronic adult diseases. The pathophysiological mechanisms involved in the so-called "Developmental Origin of Health and Adult Diseases" are still largely unknown, but it is suggested that dysfunctions of stress neuroendocrine systems (sympatho-adrenal system (SAS) and hypothalamo-pituitary-adrenal (HPA) axis, respectively) could play a crucial role. However, the

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wide spectrum of experimental paradigms used (species, sex, age of the animals, severity and duration of undernutrition…) has given rise to variable, and sometimes contradictory, results that are almost impossible to interpret. To circumvent this problem, we used the same protocol of maternal perinatal undernutrition (MPU) to study the HPA axis activity and SAS in male rat at weaning and in adulthood (8-month-old), both under resting conditions and in response to stress. We have developed a maternal perinatal undernutrition experimental model (called FR50, using a 50% global caloric restriction from the last week of gestation until weaning) in rat. At weaning, FR50 pups displayed normal corticosterone plasma levels under resting conditions whereas in response to an ether inhalation stress procedure, the increase in plasma ACTH was lower than in controls. The plasma corticosterone returned to lower values than basal level 90 minutes after this stressful procedure. Noradrenergic adrenal chromaffin cells exhibited morphological alterations associated with increased catecholamine plasma levels both under resting conditions and in response to insulin-induced hypoglycemia. Adult animals still exhibited morphological alterations of noradrenergic adrenal chromaffin cells. This was accompanied by decreased catecholamines urine and plasma levels under resting conditions and augmented ones in response to fasting. In contrast, under resting conditions FR50 male rats showed HPA axis hyperactivity with elevated glucocorticoids plasma levels that were not modified even in the presence of a severe stressor such as a 72-hour dehydration period. Together, our results indicate that MPU has both short- and long-lasting consequences on the activity of neuroendocrine systems involved in the response and/or adaptation to stress in the male rat offspring. Because these systems, via the production of both catecholamines and glucocorticoids, participate to the regulation of multiple metabolic pathways, their dysfunction, in particular in chronic stress situations, may participate to the programming of several diseases from developmental origin.

**Keywords**: maternal undernutrition, HPA axis, sympatho-adrenal system, male rat, stress response, perinatal programming



## INTRODUCTION

Several epidemiological studies have revealed a robust association between poor growth of the fetus/neonate and the increased propensity to develop chronic adult diseases such as type 2 diabetes, obesity, hypertension and cognitive disorders. These findings have led to the developmental origin

of health and adult disease (DOHAD) hypothesis, which states that an adverse perinatal environment, such as undernutrition, permanently changes morphology, physiology and metabolism of the offspring, thereby predisposing individuals to metabolic, endocrine, cognitive, and cardiovascular diseases in adult life [1-4]. Although the physiological mechanisms involved in such perinatal programming remain largely unknown, the development of several animal models has allowed study of these alterations. In particular, animal models mimicking an overexposure of the fetus to glucocorticoids, such as prenatal stress or dexamethasone, a synthetic glucocorticoid, injections during gestation have been shown to be more sensitive to the subsequent development of metabolic and cognitive diseases [5-14]. Since glucocorticoids play a crucial role in the adaptation and the response to stress as well as in metabolic regulations [15], it has been proposed that perinatal stress from diverse origin (nutritional, viral or bacterial infection, cognitive or other) might play a crucial role in the genesis of chronic adult diseases from developmental origin.

The adrenal gland plays a major role in the response to stress via two neuroendocrine systems: the sympatho-adrenal system (SAS) and the hypothalamo-pituitary-adrenal (HPA) axis (figure 1). SAS is involved in fast responses whereas HPA participates to the delayed responses to stress. When activated SAS leads to the local delivery of noradrenaline on target tissues, and also induces, via adrenal medulla stimulation by cholinergic nerve fibers, a massive release of catecholamines into the blood circulation. As depicted in figure 1, both glucocorticoids and catecholamines are involved in various metabolic regulations, suggesting a close link between adaptation to stress and metabolism. In humans, Cushing's syndrome, a rare disorder characterized by a mortality rate four times that of the general population, is a unique model to elucidate the physiological consequences resulting from exposure to chronic stress-level elevations of endogenous cortisol. Interestingly, the symptoms frequently encountered in Cushing's patients follow those observed in adults previously exposed to a perinatal stress i.e. hypertension, type 2 diabetes, obesity and cognitive disorders (16-22). Furthermore, patients suffering from pheochromocytomas, that are rare tumors secreting high levels of catecholamines, are subjected to serious metabolic and cardiovascular complications [23]. In view of these data, it seems clear that perinatal perturbations during critical time-windows of HPA axis and/or SAS development might contribute to the development of metabolic and cognitive adult disorders observed in perinatally stressed individuals.

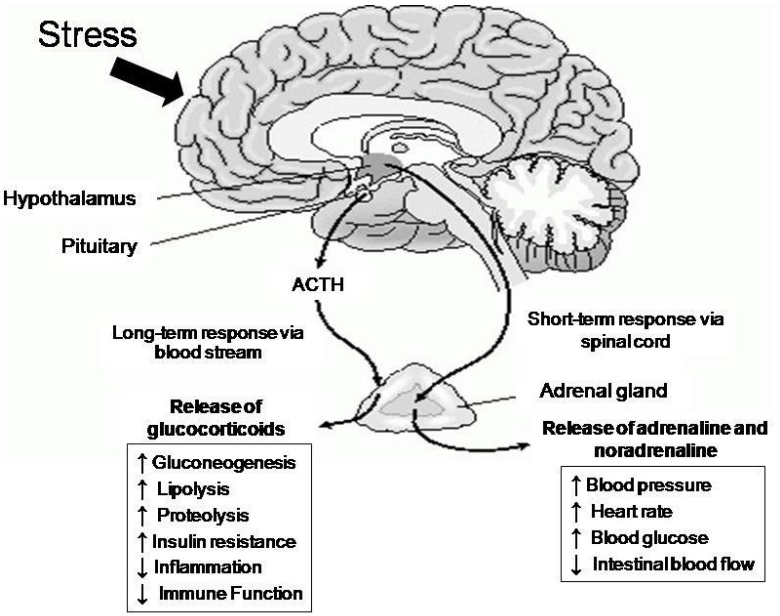


Figure 1. Main physiological and metabolic effects of sympatho-adrenal system (right side) and hypothalamo-pituitary-adrenal axis (left side) in response to stress.

## ONTOGENY OF STRESS NEUROENDOCRINE SYSTEMS

### Sympatho-Adrenal System

Adrenal medullary chromaffin cells (CC), which produce catecholamines, are derived from sympathoadrenal pluripotent stem cells originating from the embryonic neural crest, aggregate at the dorsal aorta and subsequently migrate into a ventrolateral direction to colonize the adrenal glands. These cells acquire their sympathoadrenal characteristics, such as tyrosine hydroxylase (TH) expression and the consequent noradrenergic phenotype, during their migration. Subsequently, sympathoadrenal progenitors differentiate into sympathetic neurons and small intensity fluorescent (SIF) cells in the ganglia or continue their migration to the adrenal gland. Finally, these chromoblasts colonize the cortical primordium around embryonic day (E) 15 and differentiate into neuroendocrine CC or medullary neurons. The final step of CC differentiation is characterized by the appearance of a large number of adrenaline-producing cells resulting from the induction of

phenylethanolamine-N-methyl transferase (PNMT) gene expression by glucocorticoids (24). Some neuronal markers, such as growth-associated protein 43 (GAP43), that are present in chromoblasts and in intra-medullary sympathetic neurons are repressed in adrenergic CC after birth, notably by glucocorticoids, and persist only in differentiated noradrenergic CC [25]. Functional connections between splanchnic nerve endings and adrenal CC are absent or incomplete at birth. Splanchnic neurotransmission becomes effective at the end of the first postnatal week [26]. Several data suggest that the final structural and biochemical maturation of adrenal medulla results from complex interactions between several growth factors produced by CC, neuronal inputs, and neurotransmission from the splanchnic nerve [25, 27, 28]. Thus SAS is supposed to be fully functional at the end of the first week of postnatal life, suggesting that perinatal environmental perturbations taking place between E15 and postnatal day (PND) 7 may profoundly affect SAS activity.

## Hypothalamo-Pituitary-Adrenal Axis

During the second half of pregnancy, the fetal HPA axis is essential for the development, maturation and homeostasis of the fetus, and also to prepare to the extra-uterine life [29]. In the rat, fetal HPA axis development is taking place during the second half of gestation ([30], figure 2). The regulation of fetal corticosterone secretion by adrenocorticotropic hormone (ACTH) appears at E16-E17. Then, fetal plasma corticosterone levels rise, reaching a peak at E19. Adenopituitary proopiomelanocortin (POMC) gene expression, the ACTH precursor, is first detected at E15, rises between E17 and E21, and parallels pituitary ACTH content [31]. At this stage, the secretion of ACTH is already regulated by hypothalamic corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH mRNA is first detected at E17 in paraventricular nucleus (PVN), then levels of CRH increase until E19 before decreasing until E21 [31]. AVP mRNA, first detected at E18, augments from E18 to E21 [30]. The negative feedback control exerted by corticosterone is not effective before E18, since pharmacological fetal adrenalectomy does not modify CRH mRNA levels in the PVN of E17-E18 fetuses [32]. These data indicate that the third week of gestation constitutes a particularly sensitive period during which environmental perturbations might alter HPA axis development.

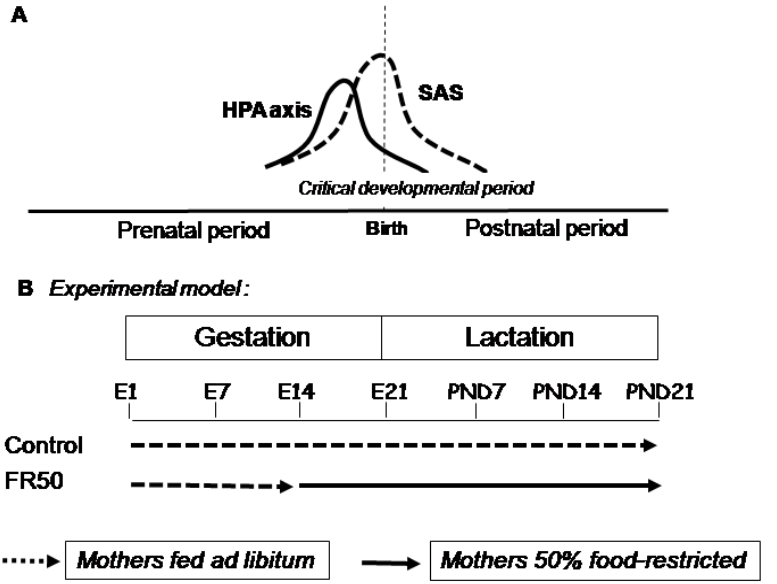


Figure 2. (A) Comparison of the periods of hypothalamo-pituitary-adrenal axis and sympatho-adrenal system development and maturation in the rat. (B) Schematic representation of the FR50 experimental model. E: embryonic day. PND: postnatal day. In FR50 mothers, daily food intake is reduced by 50% from the last week of gestation (E14) until the end of lactation (PND21).

## CONSEQUENCES OF PERINATAL ENVIRONMENTAL PERTURBATIONS ON NEUROENDOCRINE SYSTEMS

Numerous studies have reported that different perinatal perturbations, including prenatal (maternal undernutrition, alcohol exposure, low-protein diet, immune challenge, maternal stress) and postnatal manipulations (neonatal handling, modified maternal behavior, exposure to dexamethasone, infection, maternal undernutrition) can program HPA axis activity in several species [7, 33-54]. Although animal models also provide evidence that the sympathetic nervous system can be programmed following manipulations during gestation and/or lactation [55-60], the impact of early life stress on adrenal medullary function has been poorly documented. For example, it has been shown that adult male rat perinatally exposed to maternal mild caloric restriction exhibited decreased plasma levels of catecholamines after decapitation [61]. A sex-and age-specific effect of maternal perinatal undernutrition has been reported in

adult sheep. Post-weaning undernutrition increased adrenaline output to stress in females whereas it was ineffective in 2.5 years old males [62]. Recently, it has been reported that a mild level of restriction during various time points in the perinatal period can have opposing results on the developmental programming of both HPA axis and SAS than those investigating severe restriction [61]. Taken together, these data demonstrate that both HPA axis and SAS are programmed by perinatal manipulations such as maternal undernutrition. However, the phenotype of HPA and SAS function following these manipulations depends on several parameters such as the animal species, the timing and intensity of the stress, the nature of the stressor, litter size and the gender of the fetus or neonate. The wide spectrum of experimental paradigms used has give rise to highly variable results that are often very difficult to interpret. Therefore, to circumvent this problem and to analyze the effects of maternal perinatal undernutrition on HPA axis and SAS function in the offspring, we used the same experimental model in the rat (figure 2). This model called FR50 (for food restriction 50) consists in applying a 50% maternal food restricted diet starting from the day 14 of gestation until weaning (PND21).

## EFFECTS OF FR50 ON STRESS NEUROENDOCRINE SYSTEMS IN MALE RATS AT WEANING

### Sympatho-Adrenal System Activity

At weaning the body weight as well as the weight of the adrenals, liver and thymus were reduced in FR50 animals [39]. Using immunohistochemical techniques, we have reported that maternal perinatal 50% food restriction induced alterations in noradrenergic CC aggregation and in nerve fiber fasciculation in the adrenal medulla male rat at weaning [63]. These morphological changes, which appeared as early as PND7, were associated with advanced functional splanchnic neurotransmission and enhanced activity of adrenal medulla in response to metabolic stress [64]. Clusters of noradrenergic cells were smaller but more abundant as well as more widely distributed in the medulla of FR50 compared to controls (table 1). Since it has been reported that predictive noradrenergic CC form few large clusters as early as E16.5 (65], it is reasonable to think that small clusters observed in PND21 FR50 adrenal medulla could correspond not only to a delayed settling

of chromaffin tissue but also to a remodeling of this structure. Although the molecules and mechanisms involved are not known, putative candidates such as GAP43 and SLIT2 have been shown to be overexpressed in adrenal glands of PND21 FR50 male rats [63]. Because GAP43 is associated with synaptic plasticity, its presence in chromoblasts and its persistence only in noradrenergic CC suggests a possible role in the migration and/or aggregation of CC into the adrenal medulla. During development, GAP43 is also required for selective fasciculation to maintain topographic organization of axons [66]. In rat adrenal, the expression of GAP43 decreases during the second postnatal week to a level comparable to that observed in adults reflecting the definitive organization of the medulla [65]. It is thus possible that maternal FR50 changes gene expression in the adrenal medulla, which would provoke the remodeling of the structure of CC and intramedullary neurons in rat pups at weaning. These modifications may be in turn responsible for a plastic response as a compensatory and/or adaptive process to permit aggregation of CC and settlement of innervation. Accordingly, an increased expression of Slit 2 mRNA level was observed in FR50 adrenal gland [63]. Slit 2 acts via Roundabout receptors and plays important roles in neuronal, glial and neural crest cell migration [67-70], as well as in axon elongation/branching [71], and provides repulsive cues to migrating non neuronal cells [72]. SLIT2 could also be involved in the remodeling of CC aggregates and/or nerve fiber fasciculation observed in the FR50 adrenal medulla at weaning. Our results suggest that overexpression of genes implicated in morphogenesis and plasticity may reflect an adaptive or delayed settling of adrenal medulla.

At the functional level, although TH and PNMT mRNA levels as well as the adrenal content in catecholamines were unaffected, maternal undernutrition led to enhanced circulating concentrations of adrenaline and noradrenaline after decapitation of PND21 rat pups ([63], table 1) and in response to insulin-induced hypoglycemia (unpublished data, table 3). After decapitation, the proportional relationship between adrenal and circulating adrenaline/noradrenaline ratio in both control and FR50 animals strongly suggests that noradrenaline levels in plasma are mainly due to production from the adrenal medulla at this stage of development. This is in line with the fact that genes such as chromogranin B, a polypeptide precursor involved in secretory granules biogenesis [73], and $Ca^{2+}$-dependent actin-binding proteins exhibited higher level of expression in PND21 FR50 adrenals. These observations suggest that the adrenal medulla of FR50 pups remains at a high level of activity during postnatal life, presumably to allow adaptation to neonatal adverse environment elicited by maternal undernutrition.

**Table 1. Effects of FR50 on parameters of SAS under resting conditions in postnatal day (PND) 21 and in 8-month-old male rats**

| | PND 21 | 8-month-old |
|---|---|---|
| Adrenal weight | | |
| absolute (w) | ↓ | ↔ |
| relative (w/Bw) | ↑ | ↑ |
| Adrenal content | | |
| A | ↔ | ↔ |
| NA | ↔ | ↔ |
| A/NA ratio | ↔ | ↓ |
| Noradrenergic CC clusters | | |
| relative area | ↓ | ↓ |
| relative number | ↑ | ↑ |
| Adrenergic CC clusters | | |
| relative area | ↔ | ↔ |
| relative number | ↔ | ↔ |
| Plasma catecholamines | | |
| A | ↑ | ↓ |
| NA | ↑ | ↔ |

↓ decrease; ↑ increase; ↔ not modified. W, weight; Bw, body weight; A, adrenaline; NA, noradrenaline; CC, chromaffin cells.

## Hypothalamo-Pituitary-Adrenal Axis Activity

Although FR50 offspring displayed normal total plasma corticosterone levels (table 2) their plasma ACTH (table 2) and CBG levels were diminished under resting conditions [39]. In the hippocampus, FR50 pups exhibited increased MR and GR gene expression (table 2). After an ether inhalation stress, the increase in plasma ACTH was attenuated when compared to controls ([39], table 4) while the augmentation in plasma corticosterone returned to values lower than basal level 90 minutes after this stressful procedure. These results are in agreement with another study that has demonstrated that a severe maternal undernutrition during the entire gestational period diminished the ACTH secretory response to an insulin injection [33]. Together, these experiments suggest that perinatal maternal food restriction reduces the HPA axis activity in offspring at weaning. Interestingly, there was a discrepancy between ACTH and corticosterone plasma levels (table 2), suggesting that the developing adrenal gland is more sensitive to the stimulatory effect of pituitary ACTH or that corticosterone secretion is partly independent of ACTH stimulation.

**Table 2. Effects of FR50 on parameters of HPA axis under resting conditions in PND21 and in 8-month-old male rats**

| | PND 21 | 8-month-old |
|---|---|---|
| Hippocampus | | |
| MR mRNA | ↓ | ↑ |
| GR mRNA | ↓ | ↓↓ |
| Hypothalamus | | |
| CRH mRNA | ↓ | ↔ |
| AVP mRNA | nd | ↑ |
| Adenopituitary | | |
| POMC mRNA | ↔ | ↑ |
| Plasma | | |
| ACTH | ↓↓ | ↔ |
| corticosterone | ↔ | ↑↑ |

↑increase; ↓ decrease; ↔ not modified; nd not determined. Double arrows indicate a drastic modification. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; CRH, corticotrophin-releasing hormone; AVP, arginine vasopressin; POMC, proopiomelanocortin; ACTH, adrenocorticotropin hormone.

**Table 3. Effects of stress on parameters of SAS in control (C) and FR50 PND21 and 8-month-old male rats**

| | PND 21 | | 8-month-old | |
|---|---|---|---|---|
| | insulin-induced hypoglycemia | | 72-h fasting | |
| | C | FR50 | C | FR50 |
| Adrenal content | | | | |
| A | ↓ | ↓ | ↔ | ↔ |
| NA | ↔ | ↔ | ↔ | ↔ |
| A/NA ratio | ↓ | ↓ | ↔ | ↔ |
| Plasma catecholamines | | | | |
| A | ↑ | ↑ | ↑ | ↑↑ |
| NA | ↔ | ↑↑ | ↔ | ↑↑ |

↑increase; ↓ decrease; ↔ not modified. Double arrows indicate a drastic modification. A, adrenaline; NA, noradrenaline.

According to the latter hypothesis, FR50 may alter POMC processing, and generate peptides such as Lys-γ3-MSH that has been shown to potentiate the steroidogenic activity of ACTH both *in vivo* in adult rats [74] and *in vitro* in humans [75]. Interestingly, in the developing rat Lys-γ3-MSH potentiates the effect of an acute ACTH injection on corticosterone secretion [76]. Such a "CNS-like" POMC processing naturally occurs between the second and third

postnatal weeks in rat corticotrope cells [77], suggesting that maternal FR50 would delay the maturation of this adenopituitary cell type. Together, our results suggest that maternal perinatal undernutrition delays or definitely alters the development of HPA axis in male rat pups at weaning.

## EFFECTS OF FR50 ON STRESS NEUROENDOCRINE SYSTEMS IN 8-MONTH-OLD MALE RATS

As reported above, maternal perinatal undernutrition programs both HPA axis and SAS in male rat neonates, but it is important to determine whether the alterations observed at weaning persist in adult FR50 rats. In young adults (4-month-old), we had reported that maternal perinatal undernutrition induced subtle modifications of both SAS and HPA axis when compared to controls [49, 78]. Since several studies have shown that the aging process promotes HPA axis hyperactivity [79-81], we hypothesized that the effects of maternal food restriction on neuroendocrine stress systems would be more pronounced during late rather than early adulthood. Thus, we decided to investigate the consequences of maternal perinatal undernutrition on stress neuroendocrine systems in 8-month-old male rats.

### Sympatho-Adrenal System Activity

As previously observed in neonates, FR50 8-month-old adult male rats had increased relative adrenal weight under resting conditions, indicating that adrenal growth is maintained within an optimal range presumably to ensure metabolic adaptation. Interestingly, morphological alterations observed in noradrenergic clusters at weaning persist in FR50 adult rats, suggesting that perinatal programming of adrenal medulla is definitive ([82], table 1). In contrast, architecture of adrenergic clusters did not seem to be modified in FR50 rats. However, in the offspring from undernourished mothers, the circulating levels as well as urine concentrations of adrenaline were reduced under basal conditions while noradrenaline concentrations were not modified (table 1). The impaired adrenaline release in FR50 was associated with a reduction of adrenaline/noradrenaline ratio in the adrenal gland [82], indicating that maternal undernutrition has long-term consequences on the biosynthesis of catecholamine. In addition, adrenal medullary response to a

72-h fasting was differentially regulated in FR50 rats. FR50 CC exhibited hypersecretion of both adrenaline and noradrenaline despite no change in catecholamine content whereas adrenergic CC were selectively stimulated in control rats (table 4, [82]). This result is consistent with the preferential activation of preganglionic sympathetic neurons regulating adrenergic CC that occurs in response to the glucopenia [83]. These data also indicate that functional alterations persist in adult FR50 rats, and suggest that the remodeling and/or innervations of noradrenergic CC could be associated with adrenal noradrenaline secretion. However, it can not be ruled out that noradrenaline may provide from sympathetic neurons, although this seems unlikely since fasting is known to reduce the activity of sympathetic nervous system [84]. The denser innervation of the adrenal medulla, measured by cholinesterase activity, observed in FR50 rats [82] suggests that an increase synaptic release of acetylcholine from splanchnic nerve terminals may contribute to improvement of the stimulus-secretion coupling efficiency in the adrenal medulla of FR50 rats.

**Table 4. Effects of stress on parameters of HPA axis in control (C) and FR50 PND21 and 8-month-old male rats**

| | PND 21 | | 8-month-old | |
|---|---|---|---|---|
| | ether inhalation | | 72-h dehydration | |
| | C | FR50 | C | FR50 |
| Hippocampus | | | | |
| MR mRNA | nd | nd | ↓ | ↔ |
| GR mRNA | nd | nd | ↓ | ↑ |
| Hypothalamus | | | | |
| CRH mRNA | nd | nd | ↓ | ↓ |
| AVP mRNA | nd | nd | ↑ | ↔ |
| Adenopituitary | | | | |
| POMC mRNA | nd | nd | ↑ | ↓ |
| Plasma | | | | |
| ACTH | ↑↑ | ↑ | ↑↑ | ↑ |
| corticosterone | ↑↑ | ↑↑ | ↑↑ | ↔ |

↑increase; ↓ decrease; ↔ not modified; nd not determined. Double arrows indicate a drastic modification. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; CRH, corticotrophin-releasing hormone; AVP, arginine vasopressin; POMC, proopiomelanocortin; ACTH, adrenocorticotropin hormone.

At the gene expression level, we observed that the kinetic expression of several PACAP-sensitive mRNAs was different between weanling and adult animals in the FR50 and control groups [82]. Among the 384 genes studied, 32 genes exhibited an increased expression while 3 of them showed decreased mRNA levels in the adrenal glands of control animals. In contrast, no gene increased their mRNA levels in FR50 adrenals whereas 116 genes showed a decreased expression from weaning to adulthood. We do not know if this discrepancy is responsible for functional consequences, but it could be speculated that tissue-specific epigenetic mechanisms may be involved in these long-term consequences of maternal perinatal undernutrition on gene expression. In line, we have shown previously that FR50 adrenals in weanling animals showed an overexpression of DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3a), an enzyme involved in DNA methylation [63]. From weaning to adulthood, the gene expression of DNMT3a was drastically reduced only in the FR50 group, suggesting that epigenetic regulation is distinct in control and FR50 adrenals during the aging process. Epigenetic dysregulation with age is highly tissue specific and represents a major factor in the pathophysiology of aging-related diseases [85, 86]. In addition, recent studies have shown epigenetic modifications in response to adverse intrauterine environment such as undernutrition exposure in a variety of tissues including adrenal gland [87, 88]. Thus, it could be argued that epigenetic mechanisms might account, at least in part, for the global pattern of down-regulation observed in FR50 adrenals. Together our data demonstrated for the first time that maternal undernutrition has long-term programming effects on the adrenomedullary function and gene expression, and modifies its secretory responsiveness to metabolic stress.

## Hypothalamo-Pituitary-Adrenal Axis Activity

As hypothesized, numerous alterations that were not observed in young adult appeared in mature rats from perinatally undernourished mothers (table 2, [50]). Under resting conditions, plasma corticosterone values were very high in spite of normal ACTH levels suggesting either that the adrenal cortex is hypersensitive to the ACTH stimulation or that corticosterone secretion is, partly independent of ACTH action. Using perifusion experiments, we have shown that, in absence of ACTH, FR50 adrenals secrete huge levels of corticosterone while ACTH did not substantially stimulate corticosterone secretion (unpublished data). These experiments strongly suggest that the

excessive corticosterone secretion observed in FR50 animals under resting conditions does not require the action of ACTH, and that adrenal factors might be responsible for this oversecretion of glucocorticoids. In addition, in spite of high levels of corticosterone, adenopituitary POMC mRNA was elevated when compared to controls, indicating the negative feedback usually exerted at the pituitary level is altered in 8-month-old FR50 male rats (table 2, [50]). Interestingly, although GR binding was not affected in FR50 pituitaries, both binding to MR and MR mRNA expression were strikingly increased, suggesting that MR overexpression may impede glucocorticoids action on GR binding elements present on POMC promoter [52]. Finally, the high POMC mRNA level observed in FR50 adenopituitary was not correlated with ACTH plasma levels suggesting an altered POMC processing. Interestingly, we demonstrated that prohormone-convertase 2 (PC2), which is involved in processing ACTH into smaller peptides such as α-MSH and CLIP, mRNA expression is significantly enhanced in FR50 adenopituitary. Although it has not been demonstrated, our results suggest that maternal undernutrition might be responsible for a shift in anterior pituitary POMC processing similar to that observed in melanotrope cells and in pituitary corticotrope adenomas [89].

As expected, dehydration stress hugely stimulated HPA axis activity but only in control animals (table 4, [50]). Although increased ACTH secretion was observed in dehydrated FR50 animals, no modification of corticosterone plasma level was noticed. However, FR50 rats had basal corticosterone levels comparable to those observed in controls after water deprivation, suggesting that, in FR50 rats, basal secretion is maximal and thus could not be increased even in presence of elevated plasma ACTH levels. Together, our data demonstrate that mature FR50 male rats exhibited HPA axis hyperactivity under resting conditions but were not able to cope with the dehydration stress.

## CONCLUSION

The adrenal gland plays a pivotal role in the adaptation to stress and is also involved in several metabolic regulations. Within the adrenal gland, the adrenomedullary and adrenocortical systems are closely linked both at functional and anatomical levels [90]. For example splanchnic innervation, in addition to regulate the adrenal medulla, also influences adrenocortical function, and adrenocortical growth as well as diurnal variations in adrenal steroidogenesis [91]. These interactions are important for a fine-tuning control of the adrenal gland and for the regulation of its function in situations of stress

and diseases. In humans, epidemiological studies have reported HPA axis dysregulation and SAS hyperactivity in patients suffering from a metabolic syndrome [92, 93], suggesting that dysfunction of stress neuroendocrine systems may play a role in metabolic programming.

Our results indicate that maternal perinatal undernutrition has long-term consequences on stress neuroendocrine systems in the male rat. This "memory" of perinatal life concerns both the morphology, the function as well as gene expression in male rat offspring from undernourished mothers. However, in spite of these modifications, FR50 animals exhibit modest metabolic disturbances such as a mild hypertension and a transient hyperglycemia after an oral load of glucose (unpublished data). Since HPA axis and SAS present opposite activities both under resting conditions and in response to stress, it is tempting to speculate that the uncoupling of both axes may represent adaptive mechanisms contributing to protect and/or limit adverse cardiovascular and metabolic alterations induced by perinatal malnutrition. However, it seems clear that under severe stressful conditions such as chronic stress or in presence of a hypercaloric diet, the fragile equilibrium observed in offspring from undernourished mothers might be disturbed, and that this allostatic load may contribute to program metabolic diseases.



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*Chapter 3*

# EFFECTS OF MATERNAL UNDERNUTRITION ON LUNG GROWTH AND DEVELOPMENT IN THE OFFSPRING

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## ABSTRACT

Maternal undernutrition during pregnancy causes fetal growth restriction. Alterations in fetal nutritional status may result in developmental adaptations that permanently change the structure and physiology of the offspring, thus predisposing individuals to pulmonary,

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endocrine, and cardiovascular diseases in adult life. This phenomenon, termed "fetal programming", has led to the theory of "fetal origins of adult disease". Maternal undernutrition may have significant effects on the developing fetal lung, which undergoes rapid cellular multiplication and differentiation shortly before birth. Intrauterine growth restriction (IUGR) is an important risk factor for both early and late postnatal respiratory morbidity. Lung growth and development and later function can be affected by fetal growth restriction. Intrauterine growth restriction can be caused by maternal, placental, or fetal factors that affect the intrauterine environment. The exact pulmonary consequences linked to each of these specific causes are poorly understood. We have found that inadequate maternal dietary intake during late gestation altered the development of the lung structure (reduced alveolar surface area and volume fraction) and expression of lung growth factors in the postnatal period. Numerous studies have been performed to investigate the effects as well as the exact mechanism of action of maternal undernurition. The purpose of this review is to evaluate these studies in order to elucidate the harmful effects of maternal undernutrition on lung growth and development in the offspring. Subsequently, the mechanism by which maternal undernutrition induces fetal programming on lung growth will be discussed.

## 1. INTRODUCTION

Maternal undernutrition during pregnancy causes intrauterine fetal growth restriction. Alterations in fetal nutritional status may result in developmental adaptations that permanently change the structure and physiology of the offspring, thus predisposing individuals to pulmonary, endocrine, and cardiovascular diseases in adult life. Maternal undernutrition may have significant effects on the developing fetal lung, which undergoes rapid cellular multiplication and differentiation shortly before birth. Lung growth and development and later function can be affected by fetal growth restriction. Intrauterine growth restriction has been shown to associate with reduced lung function during infancy and perhaps throughout adulthood [1-5]. This phenomenon termed "fetal programming" has led to the theory of fetal origins of adult disease [6, 7]. Numerous studies have found a range of general cellular and molecular effects of IUGR on the developing lung, including reduced lung weight, DNA or protein content, and impaired alveolization. We have demonstrated that inadequate maternal dietary intake during late gestation altered lung surfactant system, lung structure (reduced alveolar

surface area and volume fraction), and the expression of lung growth factors in the postnatal period [8, 9]. IUGR has multiple causes, including maternal calorie restriction, maternal protein restriction, hypertension, anemia, placental infarction, and tobacco smoking [7]. The exact pulmonary consequences linked to each of these specific causes are poorly understood. Animal models have provided a very useful source in the clarification of the outcomes and mechanisms elicited during developmental programming. We used a maternal undernutrition model to study the effects of maternal undernutrition-induced IUGR during late gestation on lung growth and lung surfactant system and morphometry in the postnatal period. The purpose of this review is to evaluate these studies in order to elucidate the harmful effects of maternal undernutrition on lung growth and development in the offspring. Subsequently, the mechanism by which maternal undernutrition induces fetal programming on lung growth will be discussed.

## 2. OVERVIEW OF LUNG DEVELOPMENT

The development of the lungs starts in the embryo with the evagination of an avascular epithelial bud and subsequent growth into surrounding mesenchymal tissues. Following embryogenesis, the fetal lungs in all mammalian species undergo three anatomically different stages of growth termed pseudoglandular, canalicular, and saccular [10] (Table 1). The lung begins as a ventral outgrowth (laryngotracheal groove) from the wall of the foregut at approximately 4 to 6 weeks gestation in humans. Two longitudinal folds of tissue on either side of the groove grow together and fuse to form laryngotracheal tube, which distal end termed the lung bud. Approximately 28 days after fertilization, the lung bud branches to form the left and right primary bronchial buds, which will develop into the left and right lungs. Branching morphogenesis of the left and right bronchi forms specific lobar, segmental, and lobular branches. This process extends through the canalicular stage of lung development up to approximately 20 weeks gestation in humans. Dichotomous branching continues for approximately ten weeks, establishing the conducting portion of the airways. Up to 24 orders of branches are generated, the final level being the terminal bronchioles. The terminal sacs have started to dilate and differentiate into alveoli at 26 weeks. The stroma thins and brings the growing capillary network into close relationship with the immature alveoli.

**Table 1. Chronology stages of lung development in the human and mouse/rat**

| Stage | Embryonic | Pseudoglandular | Canalicular | Sacuular | Alveoar |
|---|---|---|---|---|---|
| Human | 3.5-6 wks | 6-17 wks | 15-24 wks | 24-36 wks | 36 wks-8 yrs |
| Mouse/rat | Day 13-14 | Day 15-18 | Day 18-20 | Day 19-birth | Day 4-28 |

The timing of each phase is markedly different between species although both rodents and humans have identical phases of lung development.

The cuboidal cells of the terminal sac epithelium differentiate into alveolar type II cells, which secrete low levels of surfactant. Type II phenotype juxtapose a capillary differentiate into type I cells, which flatten and expand to increase the surface area available for gas exchange. During subsequent weeks there is a rapid expansion of the respiratory portion of the lung. The composition of pulmonary surfactant is developmentally regulated. By week 30, there is a significant increase in the amount of surfactant secreted from the type II cells. The final stage of lung development occurs after 36 weeks gestation and continues into adulthood. At around 36 weeks, the first mature alveoli appear, characterized by thin walled interalveolar septa with a single layered capillary network. New alveoli are generated by a process of septal subdivision of existing immature alveoli. Lung development is a continuum from embryogenesis to early adolescence [11, 12]. Although the lungs are developed sufficiently to sustain life at birth, growth is far from complete at this point. Approximately 80% of alveoli in the human adult lung arise following birth. As the alveoli mature and the walls thin, there is a decrease in the relative proportion of stroma to total lung volume which contributes significantly to growth for 1 to 2 years after birth. By 3 years, the overall morphology of the lung has been established and subsequent expansion occurs through a proportional growth of all lung components until adulthood.

## 3. Effects of Maternal Undernutrition on Lung Growth and Surfactant System

### 3.1. Effects of Maternal Undernutrition on Body Weight, Lung Weight, and Lung Volume in the Offspring

Maternal undernutrition during late gestation is associated with poor maternal weight gain and reduced fetal body weight in rats [13, 14].

**Table 2. Body weights of control and undernourished pregnant rats during the last 7 gestational days**

| Groups | n | Length of pregnancy (day) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Control | 6 | 378 ± 12 | 394 ± 11 | 412 ± 12 | 426 ± 12 | 446 ± 11 | 468 ± 11 | 488 ± 12 |
| IUGR | 4 | 399 ± 12 | 387 ± 12 | 387 ± 12 | 390 ± 12 | 393 ± 14* | 401 ± 14* | 406 ± 15* |

The undernourished dams received 50% rations of the control food intakes during their last trimester from days 15 to 21 of gestation. Values are the mean ± SEM; *p < 0.05, compared with control rats at each time point.

**Table 3. Body weight, lung weight, and left lung volume in control and IUGR rats**

| | n | Age (days) | Body weight (g) | Lung weight (g) | Lung/body weight(%) | Lung volume (ml) | Lung volume/ body weight (%) |
|---|---|---|---|---|---|---|---|
| Control | 12 | 1 | 6.7 ± 0.1 | 0.13 ± 0.00 | 1.93 ± 0.06 | 0.42 ± 0.04 | 6.24 ± 0.54 |
| IUGR | 7 | 1 | 6.2 ± 0.2* | 0.10 ± 0.00** | 1.64 ± 0.07** | 0.28 ± 0.01** | 4.60 ± 0.15* |
| Control | 13 | 7 | 15.4 ± 0.8 | 0.28 ± 0.02 | 1.81 ± 0.03 | 0.96 ± 0.03 | 6.36 ± 0.35 |
| IUGR | 10 | 7 | 11.4 ± 0.7** | 0.18 ± 0.02* | 1.58 ± 0.05** | 0.64 ± 0.02** | 5.45 ± 0.26 |
| Control | 14 | 14 | 33.1 ± 1.1 | 0.53 ± 0.02 | 1.61 ± 0.07 | 1.60 ± 0.04 | 4.87 ± 0.19 |
| IUGR | 11 | 14 | 25.3 ± 1.4** | 0.44 ± 0.03* | 1.52 ± 0.04 | 0.88 ± 0.04** | 4.16 ± 0.33 |
| Control | 14 | 28 | 94.3 ± 2.6 | 0.69 ± 0.03 | 0.77 ± 0.02 | 2.48 ± 0.04 | 2.45 ± 0.08 |
| IUGR | 6 | 28 | 82.8 ± 4.5* | 0.65 ± 0.03 | 0.78 ± 0.01 | 1.61 ± 0.21** | 1.94 ± 0.21 |

The undernourished dams received 50% rations of the control food intakes during their last trimester from days 15 to 21 of gestation. All the dams delivered spontaneously at term and were then immediately switched back to standard rat chow. The offspring were nursed by their mothers until being weaned at 4 weeks of age. Values are the mean ± SEM; *p < 0.05, **p < 0.01, compared with control rats at each time point.

IUGR caused by placental insufficiency in sheep reveals similar findings [15, 16]. We found that maternal undernutrition (undernourished dams received 50% rations of the control food intakes) during the last week of pregnancy reduced maternal body weight (Table 2) and neonatal body weight, lung weight, and lung volume in the postnatal period (Table 3). When adjusted

for body weight, the lung weight and lung volume were significantly lower in IUGR rats than in control rats. Although the experimental dams were immediately switched back to standard rat chow after delivery, they nourished their offspring till 4 weeks of age. This consequence means that newborn rats received insufficient nutrient in the postnatal period and this event could partly contribute to the differences of body weight between older control and IUGR rats.

## 3.2. Effects of Maternal Undernutrition on Surfactant and Surfactant Protein in the Offspring

Pulmonary surfactant is composed of approximately 90% lipids and 10% proteins which functions to stabilize the lung by producing a surface-active monolayer at the air-liquid interface of the terminal airways. The surface activity properties are primarily due to dipalmitoyl phosphatidylcholine which comprises approximately 45% of surfactants by weight. Relatively few studies have examined the effect of maternal undernutrition on the development of the lung surfactant system in postnatal animal models. Total saturated phosphatidylcholine content in the lung was higher on postnatal day 1 than at any other age and fell as rats aged in control and IUGR rat lungs (Figure 1). These results are compatible with the generalization that total lung surfactant pools are higher at term birth than at any other time in an animal's life [17]. The differences in total lung saturated phosphatidylcholine content between control and IUGR rats became smaller as rats get older. Total saturated phosphatidylcholine contents were lower in IUGR rats' lungs and the value reached statistical significance on postnatal day 1 only (Figure 1). Saturated phosphatidylcholine/total phospholipid ratios in lung tissues were comparable between control and IUGR rats. These results are consistent with the findings of Lechner et al. [18], who found that prenatal starvation reduces total lung saturated phospholipids in fetal and term guinea pigs at birth. Ryan et al. administered very low-density lipoprotein intravenously over 3 hours to pregnant rats and found that saturated phosphatidylcholine content is increased in fetal alveolar pre-type II cells [19]. Based on these findings we speculated that lower lung saturated phosphatidylcholine content in IUGR rats is possibly due to reduced fatty acid substrates for the biosynthesis of phosphatidylcholine and that this effect recovers gradually when the dams were switched back to standard rat chow after delivery. Karadag utilized a rodent model of 50% maternal food restriction from gestational day 10 to term and found that lung

lipid accumulation was significantly decreased at postnatal day 1 and significantly increased at postnatal 1 month [20]. There were also significant temporal changes in the parathyroid hormone-related protein/peroxisome proliferator-activated receptor gamma signaling pathway and surfactant synthesis. They conclude that maternal food restriction alters fetal lung lipid differentiation programming by affecting specific epithelial–mesenchymal signaling pathways.

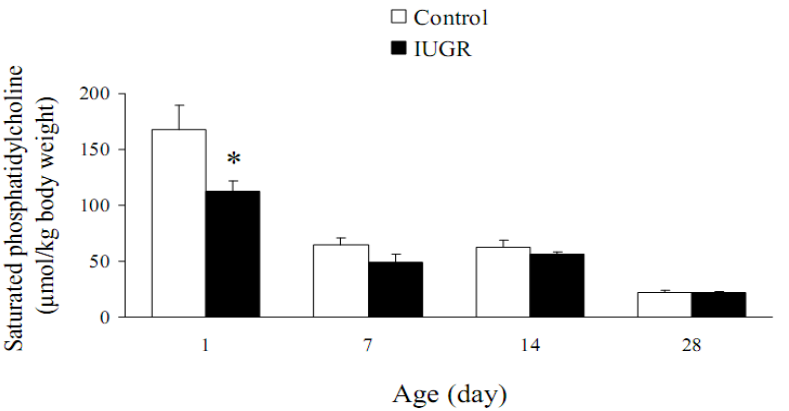


Figure 1. Saturated phosphatidylcholine contents of lung tissue in control and IUGR rats during the first 4 postnatal weeks. The undernourished dams received 50% rations of the control food intakes during their last trimester from days 15 to 21 of gestation. All the dams delivered spontaneously at term and were then immediately switched back to standard rat chow. The offspring were nursed by their mothers until being weaned at 4 weeks of age. Values are the mean ± SEM. Saturated phosphatidylcholine contents reached a peak on postnatal day 1 and decreased as the rats aged. IUGR rats had significantly lower saturated phosphatidylcholine than control rats on postnatal day 1 (*$p < 0.05$). The values were comparable between control and IUGR rats in the ensuing weeks.

Four lung specific surfactant proteins (SP-A, SP-B, SP-C, and SP-D) have been found to be associated with the surfactant [21]. They are synthesized primarily by alveolar type II cells or bronchiolar epithelial cells, and are required both for the transition between lamellar bodies and tubular myelin as well as for the spreading of tubular myelin components to the surface film [22]. Schellhase et al. investigated the ontogeny of SP-A, SP-B, and SP-C mRNAs in the developing rat lung and speculated that genes for surfactant proteins may be differentially regulated [23]. The effects of maternal undernutrition on surfactant protein gene expression in postnatal lung tissues are not well characterized and inconsistent in the literature. Gagnon et al.

reported that asymmetric fetal growth restriction caused by chronic placental damage (110-130 days' gestation) was associated with a significant increase in SP-A and SP-B mRNAs in fetal lamb lung [24]. Contrariwise, Cock et al. found that IUGR caused by umbilicoplacental embolization (120-140 days' gestation) had no significant effects on the expression of SP-A, SP-B, and SP-C mRNA [25].

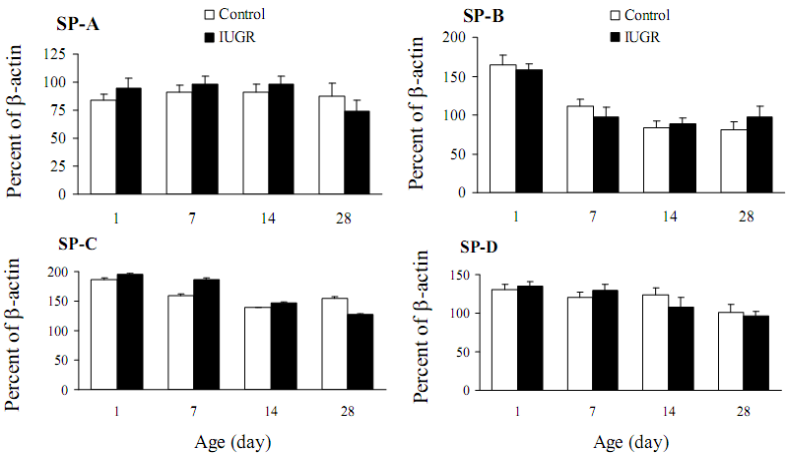


Figure 2. Effects of maternal undernutrition on mRNAs encoding SP-A, SP-B, SP-C, and SP-D in control and IUGR rat lungs. Treatment details are given in the legend to Figure 1. For each postnatal day, the results were expressed and plotted as percent of β-actin. Values are the mean ± SEM. Lung SP-A, SP-B, SP-C, and SP-D mRNA expressions measured with RT-PCR were similar between control and IUGR rats during the study period.

The discrepancy between the results of those studies may be due to the differences in the etiology, severity, and gestational timing of IUGR. Observing an extensive period of umbilicoplacental embolization (120-146 days' gestation), Joyce et al. found that IUGR lambs have similar SP-A and SP-C mRNA levels but higher SP-B mRNA levels as compared with control lambs at 8 weeks after birth [16]. Briana et al. measured serum SP-D concentrations in 20 IUGR and 20 appropriate for gestational age full-term neonates on postnatal days 1 and 4 and found significantly higher serum SP-D concentrations in IUGR fetuses and IUGR neonate born via vaginal delivery on postnatal day 1. They speculated that these results reflect increased alveolarvascular permeability and protein leakage into the circulation, intrauterine glucocorticoid exposure accelerated lung maturation, lung liquid reabsorption, and the initiation of breathing and delivery stress [26]. Orgeig et

al. have shown that IUGR induced by carunclectomy results in a significant reduction in lung SP-A, -B, and -C protein and mRNA expression in sheep fetus at gestational days 133 and 141 [27]. They suggest that placental restriction induced by carunclectomy may lead to chronic hypoxemia and hypercortisolemia that then inhibit surfactant maturation. These data suggest that IUGR fetuses are at risk of lung complications, especially if born prematurely. We described the ontogeny of surfactant protein mRNAs in the postnatal rat lung after maternal undernutrition during late gestation. The results of our study showed that maternal undernutrition did not affect the gene expressions of SP-A, SP-B, SP-C, and SP-D in the offspring's lung (Figure 2). Therefore, we suggest that IUGR does not accelerate pulmonary maturation and that surfactant protein and surfactant lipids are regulated by separate metabolic pathways.

## 4. Effects of Maternal Undernutrition on Insulin-Like Growth Factor System in the Offspring

Development of the human respiratory system involves the formation of a highly ordered airway branching system with 25,000 distinct terminations, giving rise to more than 300 million alveoli as well as the differentiation and proliferation of over 40 different cell types [11]. Several growth factors and their receptors play critical roles in cell proliferation, migration, and differentiation during this well-coordinated growth process. Insulin-like growth factors (IGFs) are small peptides that modulate cell proliferation and differentiation during embryogenesis with the cell-surface receptor, IGF receptor type 1 (IGFR-1) [28-30]. IGFR-1 belongs to a receptor tyrosine kinase family, and previous studies demonstrated that both IGF-I and -II act through IGFR-1 for mitogenic signaling in murine embryonic development [31, 32]. IGFR-2 has no tyrosine kinase activity and is important in internalizing IGF-II/receptor complexes and transporting IGF-II to lysozymes [33]. IGF-II mRNA expression is decreased in hypoplastic lungs produced by transaction of the cervical spinal cord or tracheal drainage [34], and IGF-I and IGF-II mRNA expressions are increased in the large lungs produced by tracheal ligation [35, 36]. The effects of IUGR consequences on the lung IGF system (peptides, receptors, and binding proteins) in postnatal rats were limited in the literature. We induced IUGR by giving dams 50% rations of the

control food intakes during the last week of pregnancy and demonstrated that lung IGF-I, IGF-II, IGFR-1 and IGFR-2, and the IGF binding protein mRNA expressions increased as rats aged and reached a peak on postnatal day 14. Maternal undernutrition significantly increased IGF-I and IGF-II mRNA expressions on postnatal days 1 and 28. Lung IGFR-1, IGFR-2, and IGFBP mRNA expressions were similar between control and IUGR rats during the study period (Figure 3). These results suggest that IGF-I and -II participated in the transduction of IUGR to alter lung development.

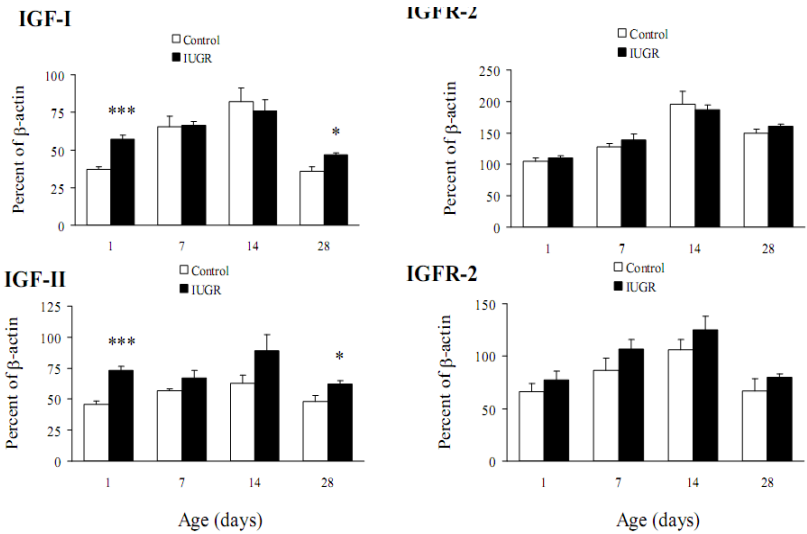


Figure 3. Effects of maternal undernutrition on mRNAs encoding IGF-I and -II and IGFR-1 and -2 in control and IUGR rat lungs. Treatment details are given in the legend to Figure 1. The results for each postnatal day are expressed and plotted as a percent of β-actin. Values are the mean ± SEM. Maternal undernutrition significantly increased IGF-I and -II mRNA expressions on postnatal days 1 and 28 (*$p < 0.05$, ***$p < 0.001$ vs. the control group at each postnatal age). Lung IGFR-1 and -2 mRNA expressions were similar between control and IUGR rats during the study period.

## 5. Effects of Maternal Undernutrition on Lung Morphometry

Maritz et al. found that in lambs fetal growth restriction induced by umbilico-placental embolization causes smaller airspace volume density at 8 weeks after birth [15]. We found that IUGR rats exhibited a significantly

lower volume fraction of airspace on postnatal days 7 and 14 and that the alveolar surface area and alveolar surface area/body weight ratio were significantly lower in IUGR rats as compared with control rats (Figure 4 and 5). These morphometric changes were similar to those observed after the antenatal administration of dexamethasone as reported by Okajima et al. [37].

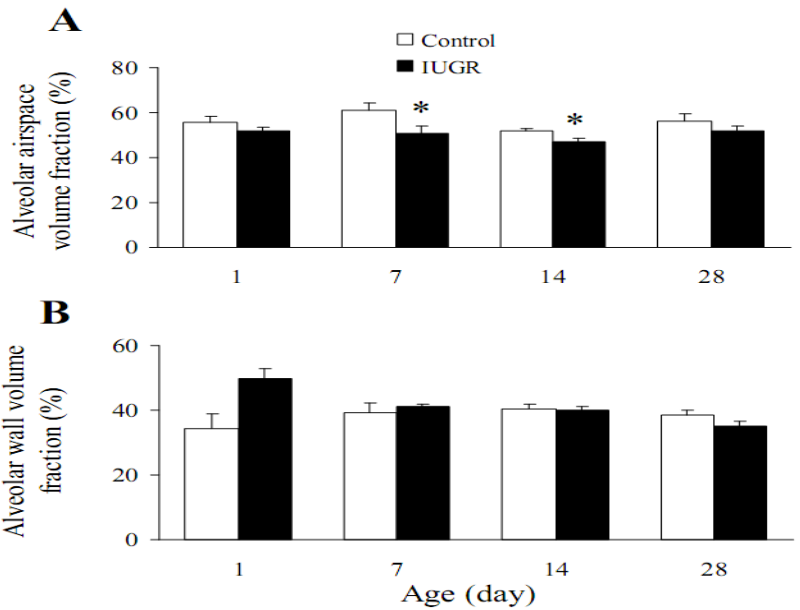


Figure 4. Changes in (A) volume fractions of the alveolar airspace and (B) alveolar wall thickness as a function of postnatal age for control and IUGR rats. Treatment details are given in the legend to Figure 1. Values are the mean ± SEM. Volume fractions of the alveolar airspace were lower in IUGR rats, and values reached statistical significance on postnatal days 7 and 14 (*$p < 0.05$). Alveolar wall thickness was comparable between control and IUGR rats.

These results further support that maternal undernutrition during late gestation increases the fetal exposure to maternal glucocorticoids [13]. Restricting calorie in adult mice reduces the alveolar surface area whereas refeeding them fully reverses this change [38]. Decreased alveolar surface area causes decreased lung compliance and increased resistance. These data suggest that decreased alveolar surface area is one of the major factors to impair pulmonary function in IUGR infants and children.

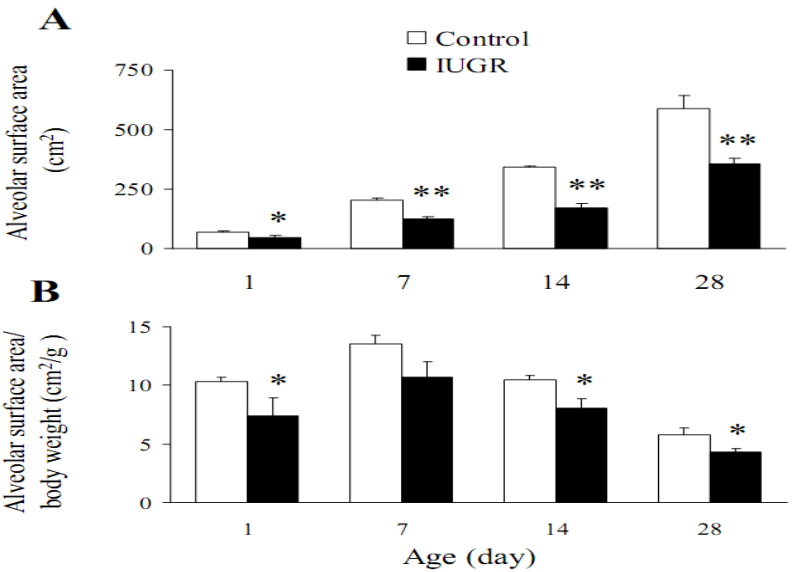


Figure 5. Changes in (A) alveolar surface area and (B) alveolar surface area/body weight ratio as a function of postnatal age for control and IUGR rats. Treatment details are given in the legend to Figure 1. Values are the mean ± SEM. The alveolar surface area increased as rats aged, and values were significantly lower in IUGR rats during the study period (*$p < 0.05$, **$p < 0.01$). The alveolar surface area/body weight ratio reached a peak on postnatal day 7 and decreased as rats aged in control and IUGR rats, and values were significantly lower in IUGR rats on postnatal days 1, 14, and 28 (*$p < 0.05$).

## 6. The Mechanisms by which Maternal Undernutrition Induces Fetal Programming on Lung Development

The plasma corticosterone level was significantly lower in IUGR rats than in control rats on postnatal day 1 only (Figure 6). In IUGR and control groups, corticosterone concentrations increased between postnatal days 7 and 28. The changing pattern was parallel to the growth curve of rat adrenal glands that had a decline for the first week followed by a rise beginning on postnatal day 8 and attains a maximum on postnatal day 25 [39]. Lesage et al. reported that maternal undernutrition during late gestation induces both IUGR and an overexposure of fetuses to maternal corticosterone, which disturbs the hypothalamo-pituitary adrenal axis [13]. Plasma corticosterone concentration

and total lung saturated phosphatidylcholine content were significantly lower in IUGR rats compared with control rats on postnatal day 1 only. These data indicate that suppression of the expected postnatal increase in corticosterone and negative feedback control exerted by high maternal corticosterone in IUGR rats and there is dissociation between lung surfactant lipid levels resulting from IUGR and endogenous corticosterone levels. Jobe et al. found that intra-amniotic endotoxin injection enhances lung maturation and does not alter cord cortisol levels in preterm lambs [40]. These results suggest that there are other potential lung maturation factors. Glucocorticoid may stimulate choline-phosphate cytidylyltransferase activity and increase phosphatidyl-choline synthesis in fetal rat lung; antenatal glucocorticoid treatment promotes lung structural maturation in preterm lambs [41, 42]. However, the incorporation of precursor substrates such as glucose, lactate, and palmitate into fetal lung phospholipids was reduced in maternal fasting during late gestation [43]. Based on these findings, we suggest that precursor substrate supplement is more important than glucocorticoid in enhancing lung maturation.

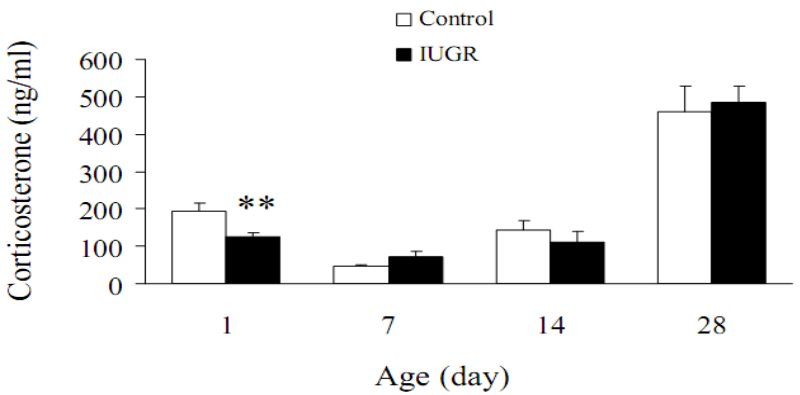


Figure 6. Plasma corticosterone levels in control and IUGR rats. Treatment details are given in the legend to Figure 1. Values are the mean ± SEM. Plasma corticosterone was significantly lower in IUGR rats than in control rats on postnatal day 1 (**$p < 0.01$). In both groups, corticosterone concentrations increased between postnatal days 7 and 28.

Klinger et al. showed that targeted disruption of the Mapk6 gene (encoding Erk3) leads to IUGR, marked pulmonary hypoplasia, decreased serum IGF-2 levels, and early neonatal death during the first day of life [44]. Interestingly, in utero administration of glucocorticoids promoted fetal lung

maturity and rescued pneumocyte differentiation but failed to alter the neonatal lethality. These results suggest that Erk3 is a regulator of IGF-2 levels and plays a critical role in fetal growth and lung maturation.

## 7. SUMMARY

IUGR is a complex disorder with many phenotypic expressions that may rise from multiple etiologies including maternal/placental, fetal (genetic aberrations, epigenetic modifications), and environmental (fetal infections, teratogens) factors. Nutrition is the major intrauterine environmental factor that alters the expression of fetal genome and may have lifelong consequences. This phenomenon termed "fetal programming" has led to the recent theory of fetal origins of adult disease. Namely, alterations in fetal nutrition status may result in developmental adaptations that permanently change the structure, physiology, and metabolism of the offspring, thereby predisposing individuals to metabolic, endocrine, and cardiovascular diseases in adult life. Animal studies show that maternal undernutrition during late gestation decreased lung surfactant lipid levels in the immediate postnatal period and altered lung structural development during the postnatal period, but that surfactant protein gene expressions in postnatal rat lungs were not changed by maternal undernutrition. These results suggest that alteration of lung surfactant and structure may be important in the pathogenesis of impaired pulmonary function in IUGR infants and children. Since pulmonary function is a long-term predictor for overall survival rates in the general population, we contend that in prenatal care physicians should encourage the pregnant mother to gain body weight to reduce the risk potential of giving birth to an IUGR newborn.

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*Chapter 4*

# UNDERNUTRITION FROM FETAL LIFE TO PUBERTY, FETAL PROGRAMMING AND COMPENSATORY GROWTH IN RAT TESTES

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## ABSTRACT

24 adult Sprague-Dawley pregnant rats were divided into three groups: Control group (group C), n=8, fed *ad libitum* during gestation and lactation (until 25 days post-partum). Group underfed during gestation (group UG, n = 8), were offered only 40% of *ad libitum* rat chow intake of Control group pregnant dams. Group underfed during gestation and lactation (group UGL, n=8), were treated identically as group UG during gestation. After parturition, litters were adjusted to either 14 (group UGL) or 8 (Control and UG groups) pups. At 2 days of age, 11 group C and 12 group UG male pups were slaughtered. Their testes were processed for standard histology and morphometrical evaluation, and for proliferating cell nuclear antigen (PCNA) immunohistochemistry (IHC) of Leydig and myoid cells. At 25 days of age, 5 group C, 8 group UG and 10 group

UGL male pups were also slaughtered and their testes processed as in 2 days old pups. Semiquantitative results of PCNA IHC evaluation were converted to PCNA labeling index (LI). Body and testes weights, quantitative histological variables and PCNA LI were presented as means ± sd and analysed with anova. At 2 days of age, group UG pups had lower body weight (6.43 ± 1.07 vs. 7.63 ± 0.56 g), testes weight (0.0026 ± 0.0005 vs. 0.0035 ± 0.0005 g), gonadosomatic index (0.82119 ± 0.13162 vs. 0.92424 ± 0.08041) and total number of Sertoli cells per testis (0.50 ± 0.09 vs. 0.74 ± 0.15 x $10^3$) as compared to group C. At 25 days of age, group UGL had lower body weight (36.36 ± 3.27 vs. 61.39 ± 6,28 and 65.17 ± 5.00 g), testes weight (0.06 ± 0.02a vs. 0.12 ± 0.04b and 0.18 ± 0.03c g), seminiferous tubules diameter (196.74 ± 9.14 vs. 239.10 ± 7.68 and 244.87 ± 16.05 µm) and total number of Sertoli cells per testis (7.73 ± 2.11 vs. 12.22 ± 1.86 and 14.43 ± 2.55 x $10^4$) as compared to groups C and UG respectively. The only variable which was lower in group UGL than in group C at 25 days of age was testes weight. However, testicular weight and gonadosomatic index were higher in group UG than in both groups C and UGL, indicating compensatory growth. No differences were found in PCNA LI between groups. Present results indicate the effects of undernutrition during fetal life determine lower body and testes weight, and lower Sertoli cell numbers. Body and testicular weights, diameter of seminiferous tubules and Sertoli cell numbers in rats underfed during gestation and suckling period is lower than in their well fed controls but also lower than in rats which were underfed only during gestation. In summary, 1.- we confirmed the strong deleterious effect of undernutrition during fetal to postpubertal life on rat testicular development and final Sertoli cell numbers; 2.- most effects of undernutrition during gestation on testes structure, including Sertoli cell numbers, disappear at 25 days of age in rats fed *ad libitum* after birth and 3.- this kind of testicular compensatory growth in rats is accompanied by higher testicular weight than in control animals which never experienced undernutrition.

## INTRODUCTION

The nutritional status of females during pregnancy plays a critical role in the post-natal growth and development of the offspring, often leading to permanent changes (fetal programming, Lucas 1991). This concept was originally developed to explain variations in susceptibility of humans to disease, but has now been broadened and encompasses the effects of fetal malnutrition on pre- and post-natal development, including the development of the reproductive system before puberty (Engelbregt et al., 2000; Rhind et al.,

2001). Gonadotrophin secretion has been shown to be lower in underfed lamb foetuses (Deligeorgis et al. 1996). Furthermore, nutritional restrictions can influence the activity of the hypothalamo-pituitary axis and thus reduce gonadotrophin levels in ram lambs (review: Brown 1994). Recently it has been shown that dietary manipulation of *Bos indicus* heifers during gestation affects the prepubertal reproductive development of their bull calves (Sullivan et al., 2010). Thus, undernutrition during early life could affect the development of the testes and, in some circumstances, have permanent effect on their future capacity to produce spermatozoa.

Sertoli cells appear early in fetal life and divide until pre-pubertal age (rats: Orth 1982; sheep: Hochereau de Reviers et al., 1987). Sertoli cells are a particularly strong candidate for fetal programming of future performance, because the number of Sertoli cells is highly correlated with adult testicular size and the maximum rate of germ cell production (review: Sharpe et al., 2003). Furthermore, Sertoli cell numbers per testis is the most important factor that determines the ceiling of sperm production and output (Orth et al., 1988). Consequently, fetal nutrition, particularly during gonadal development, may be a determinant of maximum capacity for sperm output. In undernourished rat pups testes (Bansal-Rajbanshi and Mathur 1985) the cell generation cycle of spermatogonial germ cells and supporting cells (future Sertoli cells) on day 9 of age showed marked prolongation of DNA synthetic phase, and shortening of the pre-DNA synthetic phase indicating a depression in DNA synthesis in undernutrition. Male lambs undernourished *in utero* have reduced Sertoli cell numbers at 10 months of age (post puberty) (Kotsampasi et al., 2009). In a previous study (Genovese et al., 2010), we found that fetal to pubertal (until 25 days of age) undernourishment in rats is accompanied by changes in testicular structure and lower Sertoli cell numbers in adult life (100 days of age), strongly suggesting lower daily sperm production. However, the relative impact of undernutrition during the fetal and during the suckling periods is not clear. Thus, our objective for the present work was to determine the relative impact of undernutrition in male rats during fetal life and during the suckling period, until 25 days of age and to determine whether there is any degree of compensatory growth in body of testicular development from birth to 25 days of age.

## MATERIALS AND METHODS

Animals were bred and housed at the Faculty of Veterinary Medicine, Universidad de la República, Montevideo, Uruguay. Experimental procedures were approved by the Faculty of Veterinary Medicine's Ethical Committee. Eight adult young (age 5 month) virgin Sprague–Dawley rats (mean body weight 246.4 ± 4.0 g) were bred with only one male (body weight 257.5 g). Gestation was diagnosed by vaginal smears, taking as day 0 the day when sperm appeared in the smear. Pregnant rats were divided into three groups: Control group (C, n = 8) were kept in individual cages with water and standard rat chow (3 Cal / g, 21% protein, 10% fibre, 7% total minerals) *ad libitum*, during gestation and lactation (until 25 day post-partum). Litters were adjusted to eight pups until weaning. Pregnant dams in group Underfed during Gestation (UG, n=8) were offered only 40% of *ad libitum* rat chow intake of Control group pregnant dams. Later on, pups suckled subrogate mothers in litters adjusted to 8 pups, with water and standard rat chow *ad libitum*. Dams in group Underfed during Gestation and Lactation (UGL, n = 8) were treated as group UG dams during gestation, and were kept during lactation in specially prepared cages where only dams had access to food (food was located above a 'second floor' which was beyond reach of pups). After parturition, litters were adjusted to 14 pups until weaning. Water was always offered *ad libitum* to all animals. The amount of food offered to the UG and UGL pregnant dams was adjusted day by day according to a preliminary experiment where mean rat chow intake was measured along gestation in 10 sister rats of approximately same age and weight as the UGL mothers.

Pups from all three groups were weighed daily until 25 days of age. At 2 days of age, Group C pups (n=11) and Group UG pups (n=12) were weighed and euthanized with 25 mg sodium tiopenthal i / p. At 25 days of age, Group C pups (n=5), Group UG pups (n=8) and Group UGL pups (n=10) were also weighed and eutanized similarly. Testes from all euthanized pups were promptly dissected, weighed and immersion fixed in Bouin's solution for 24 h and stocked later in ethanol 70°C as described in detail in Bielli et al. (1999). Testes were sliced lengthwise in 3 mm thick sections, dehydrated in increasing concentrations of ethanol (70°C, 95°C and 100°C), immersed in chloroform and embedded in paraffin wax. Blocks were sectioned at 6 μm thickness and stained either with Haematoxylin–Eosin for morphometrical evaluation or with immunohistochemistry for PCNA.

## MORPHOMETRY

After qualitative evaluation of the slides, images of testicular parenchyma were retrieved from a light microscope (BX50, Olympus, Tokyo, Japan) using a video camera (SSC-C158P, Sony, Tokyo, Japan) and a personal computer with Infinity Capture software (Lumenera Corporation, Ottawa, Canada). Images were analyzed with Infinity Analyze software at a final magnification of 2500x on the computer monitor.

Testicular volume was calculated from testicular weight, assuming testicular density is 1 (Russell et al., 1990). Morphometrical procedures are described in further detail in Genovese et al., 2010. The volume density (Vv) of seminiferous tubules was measured by point counting (Weibel 1979). The diameter of the seminiferous tubules was determined by measuring two perpendicular diameters from 30 randomly chosen cross-sections of seminiferous tubules per testis. The total number of Sertoli cells per testis was estimated by measuring the mean number of Sertoli cells per seminiferous tubule cross section times seminiferous tubules length divided by section thickness. Only Sertoli cells with nuclei which were visible in the section were counted. Seminiferous tubules were assumed to be cylindrical and their lengths were estimated by calculating the seminiferous tubule absolute volume (seminiferous tubules Vv times testicular volume) and dividing seminiferous tubule absolute volume by seminiferous tubules cross section area. The testicular interstitium volume was calculated by subtracting the total volume of seminiferous tubules from absolute testicular volume. The gonadosomatic index of the pups at slaughter was calculated as paired testicular weight/body weight.

## PCNA LABELLING INDEX

Proliferating cell nuclear antigen (PCNA) immunohistochemistry (IHC) of Leydig and myoid cells was performed in order to evaluate their proliferation rate. Sections were deparaffinized, hydrated in decreasing concentrations of ethanol (100°C, 95°C and 70°) for 2 min each and rinsed in distilled water for 5 min.

Antigen retrieval was performed by microwaving the sections at 98°C in 0.2% trisodium citrate buffer (pH 6.02). The slides were then rinsed in PBS, and endogenous peroxidase activity was blocked with 10% hydrogen peroxide

during 5 min. Before incubation with a primary antibody against PCNA, the slides were rinsed in distilled water for 5 min.

The primary antibody against PCNA (clone PC 10, monoclonal, Dako Cytomation, Carpinteria, CA, USA) was diluted 1:100 in PBS, and processed with a developing kit LSAB+ (code K0679, Dako Cytomation, Carpinteria, CA, USA) as follows, according to the manufacturer's instructions. The slides were then rinsed and incubated with secondary biotinylated antibody (antimice and antirabbit) for 30 min. Sections were rinsed and incubated with streptavidin-labeled peroxidase complex for 20 min. After rinsing, the antibody was visualized as a brown staining with 0.6 mg/ml 4' diaminobenzidine tetrachloride (DAB). The sections were subsequently dehydrated and coverslipped with mounting balsam. Negative control sections were treated similarly, except the primary antibody was substituted with PBS.

## Semiquantitative Evaluation of PCNA Immunoreactivity

For the semiquantitative evaluation of PCNA contents, the staining intensity of

- the nuclei was scored at a final magnification of x 400 as being negative (O), weak (1),
- strong (2) or very strong (3), if nuclei exhibited no, light brown, brown or dark brown
- stain, respectively. Frequencies of different staining intensities (0 to 3) were assessed
- for Leydig and myoid cells and expressed in percent (%). PCNA immunoreactivity was expressed using a labelling index score (LI) which was calculated according to the following procedure (Boos et al., 1996):

  PCNA LI = 1 X *nCs,,)* + 2 X *n(,,*)* + 3 X *nCs,3j*

  *(n* = amount of cells exhibiting staining intensity 1, 2, or 3, expressed in %).

## STATISTICAL ANALYSIS

Variables were presented as means ± sd and analyzed with anova within each sampling date (2 and 25 days of age). Body weights from dams and pups were compared by repeated-measures ANOVA. The effects of group and dam were studied. The variation among dams within treatment was used as an error term when testing for differences between treatments. Individual means were compared by least significant differences.

## RESULTS

At qualitative histological evaluation, all testicular slides from all animals looked normal, regardless of the experimental treatment they received. Results for both sampling dates are depicted in table 1. At 2 days of age, pups which were underfed *in utero* (group UG) had lower body weight, testicular weight, gonadosomatic index, absolute volume of testicular cords and total number of Sertoli cells, as compared to their well-fed controls (group C) but there were no differences in testicular cords diameter and number of Sertoli cells/testicular cord cross-section. At 25 days of age, pups which were underfed *in utero* and during lactation (group UGL) had lower body weight, testicular weight, seminiferous tubules diameter, absolute volume of seminiferous tubules and total number of Sertoli cells than their well-fed controls. However, pups which had been underfed *in utero* but were fed *ad libitum* after birth (group UG), had higher testicular weight and gonadosomatic index, but no difference in body weight, seminiferous tubules diameter, absolute volume of seminiferous tubules, number of Sertoli cells/seminiferous tubule cross-section or total number of Sertoli cells. Furthermore, pups from group UGL, when compared to pups from group UG, had lower body weight, testicular weight, gonadosomatic index, diameter of seminiferous tubules, absolute volume of seminiferous tubules and total number of Sertoli cells, but were not different in the number of Sertoli cells/seminiferous tubule cross-section.

There were no differences in PCNA LI. At 2 days of age, Leydig cells PCNA LI were (group C vs group UG) 0.35 ± 0.08 vs. 0.42 ± 0.08, and myoid cells PCNA LI were 1.47 ± 0.10 vs. 1.54 ± 0.13. At 25 days of age, Leydig cells PCNA LI were (group C vs. group UGL vs. group UG) 0.46 ± 0.19 vs.

0.31 ± 0.13 vs. 0.50 ± 0.10, and myoid cells PCNA LI were 1.62 ± 0.09 vs. 1.55 ± 0.11 vs. 1.65 ± 0.24.

**Table 1. Body weight, testicular weight and testicular morphometrical variables in pups at 2 or 25 days of age which were underfed either *in utero* (group UG) or both *in utero* and during lactation (group UGL) and their well-fed controls (group C)**

| | Two days of age | | 25 days of age | | |
|---|---|---|---|---|---|
| | Group C | Group UG | Group C | Group UGL | Group UG |
| Body weight (g) | 7.63 ± 0.56 | 6.43 ± 1.07* | 61.39 ± 6.28 a | 36.36± 3.27b | 65.17± 5.00 a |
| Testicular weight (g) | 0.035 ± 0.0005 | 0.0026±0.0005** | 0.12 ± 0.04a | 0.06 ± 0.02b | 0.18 ± 0.03c |
| Gonadosomatic index ($x10^{-3}$) | 0.92424± 0.08041 | 0.82119± 0.13162* | 2.339303± 0.497133a | 2.031477± 0.484994a | 3.128588± 0.398964b |
| Diameter of testicular cords (2 days of age) or seminiferous tubules (25 days of age) (µm) | 96.07 ± 4.98 | 99.09 ± 4.19 | 239.10 ± 7.68 a | 196.74 ± 9.14b | 244.87 ± 16.05 a |
| Absolute volume of testicular cords (2 days of age) or seminiferous tubules (25 days of age) (mL) | 0.0016 ± 0.0003 | 0.0012 ± 0.0002* | 0.10 ± 0.02a | 0.05 ± 0.01b | 0.12 ± 0.03a |
| Nr. of Sertoli cells / cross-section of testicular cord (2 days of age) or seminiferous tubule (25 days of age) | 19.47 ± 0.82 | 19.09 ± 0.70 | 31.50 ± 0.77a | 30.64 ± 1.13a | 33.13 ± 1.01a |
| Total nr. of Sertoli cells / testis ($x 10^4$) | 0.74 ± 0.15 | 0.50 ± 0.09** | 12.22 ± 1.86a | 7.73 ± 2.11b | 14.43 ± 2.55a |

Values within the same row and sampling date which are not followed by the same letter are different. Asterisks *: P<0.05; **: 0.001<P<0.01; ***: P<0.001 as compared to Control group.

## DISCUSSION

Recently (Genovese et al. 2010) we have shown that undernourishment during the intrauterine and suckling periods of life is accompanied by changes in testicular structure and lower numbers of Sertoli cells per testis in adult rats. In the present experiment, we have shown that the undernutrition treatment

that we had applied is accompanied, at birth, by lower body and testicular weight, lower volume of testicular cords and lower numbers of Sertoli cells per testis. Thus, the intrauterine life is a period which is very sensitive to undernutrition. This is no surprise, since it is well known that undernutrition tends to have a stronger effect in earlier periods of life (Mellor, 1983). Considering that testicular weight normally follows body weight until the pubertal accelerated growth period, the lower testicular weight in the UG group was also to be expected. However, the lower gonadosomatic index in the UG group as compared to Control group indicates a stronger effect in gonadal development than in general bodily development. This fact strongly suggests that severely undernourished fetuses assign a very low priority to nutritional investments in gonadal development. This tends to coincide with the well known partitioning pattern of nutritional energy prior to puberty, which assigns very few resources to reproductive growth, as compared to adult life, when reproductive activities are dedicated a higher proportion of nutritional intake and bodily energetic reserves. The diameter of the testicular cords is related to the number of cells in a given cross-section of testicular cord. It could also be expected that, consequently, the number of Sertoli cells/testicular cord cross-section did not vary as well, since most cells within the testicular cord are Sertoli cells. Thus, both the higher absolute volume of testicular cords and the higher testicular weight can be abscribed to longer testicular cords. The existence of longer testicular cords can also be explained by the higher number of Sertoli cells (Hochereau-de Reviers et al., 1987). It should be born in mind that Sertoli cells stop proliferating when puberty begins. Consequently, lower Sertoli cells numbers at birth (2 days of age) does not necessarily mean lower Sertoli cell numbers in adult life.

At 25 days of age, the situation was quite different. When comparing group UGL with their well-fed controls, a big difference in body weight and testicular weight was seen. Interestingly, the gonadosomatic indexes in UGL and C groups were not different, indicating that the history of undernutrition during fetal life provoked lower gonadosomatic index at birth, but such consequence was short lived, and had disappeared at postpuberty. Probably, the experimental treatment that we had applied after birth was not as strong as the treatment applied *in utero*, at least since the former had comparable effects on body and testes weight. Moreover, the absolute volume of seminiferous tubules (which is the histological variable most closely correlated with daily sperm output, Hochereau-de Reviers et al., 1987) was also lower in group UGL. Furthermore, both the diameter of the seminiferous tubules and the total number of Sertoli cells per testis were also lower in group UGL than in group

C. The diameter of seminiferous tubules also is highly correlated with daily sperm output, since most of the diameter length can be ascribed to the number of germ cells present in a given cross-section. The total number of Sertoli cells, on the other hand, is highly correlated with maximal potential sperm output (Sharpe et al., 2003). Thus, as in Genovese et al. (2010), we confirmed that undernutrition during intrauterine and suckling life determines histological changes indicating lower sperm output and lower maximal spermatogenetic capacity in adult life.

When comparing the group UG with groups C and UGL (at 25 days of age), it is interesting to note that there were no differences between groups UG and C, exception made of the testicular weight and the gonadosomatic index, which were higher in group UG. Thus, we found compensatory growth in body weight and similarly, compensatory mechanisms determined that most morphometrical variables studied were not different between rats which had been underfed during intrauterine life and their well-fed controls. Surprisingly, testicular weight was 50% higher in group UG than in group C, demonstrating that strong compensatory mechanisms of yet unknown nature had been acting, and provoked group UG developing bigger testes than normal at 25 days of age, i.e., at the postpubertal period. The compensatory growth mechanisms which were acting accelerated both body (since body weight was lower in group UG at 2 days of age than in group C) and testicular growth, but the testes were stimulated at a higher rate. The compensatory growth that we have found indicates that, at least in rats and under the conditions described in the present paper, the fetal programming provoked by undernutrition during fetal to pubertal life does not occur when only fetal undernutrition is present. Moreover, undernutrition during fetal life followed by *ad libitum* nutrition from birth to postpuberty is accompanied by bigger than normal testes, although there is no difference in Sertoli cell numbers per testis and consequently, in maximal potential daily sperm output. Surprisingly enough, our results indicate that undernutrition during fetal life, if followed by *ad libitum* feeding, can have beneficial effects on testes size in rats. This is not the first report on compensatory growth of testicular weight and Sertoli cell numbers. Recently, it has been shown that fetal exposure to phthalate and/or flutamide reduce Sertoli cell numbers in the rat at birth (Auharek et al., 2010) but Sertoli cell numbers recover to normal by day 25. This effect is dependent on androgen levels, and the same thing might have happened with the effect we found in our present experiment, since androgen levels can be affected by nutrition in lamb fetuses (Rae et al., 2002). However, as far as we know, our present results are the first report of compensatory growth of Sertoli cell

numbers and testicular weight after undernourishment, in any mammalian species.

It is tempting to speculate to what degree the present results can be extrapolated to bigger and longer-lived mammals (i.e., human beings and farm animals). We think much caution should be taken. In general, the rat is very sensitive to nutritional influences on reproduction, and rodents are born at a much earlier developmental stage than farm animals and humans. Consequently, part of the reproductive developmental processes which occurred with *ad libitum* nutrition in the group UG newborn rats of the present experiment would have occurred during fetal life in farm animals and humans being underfed *in utero*. On the other hand, primate Sertoli cells can multiply much later in life than rat Sertoli cells, and the general proliferation dymamics of testicular cells is different in rodents and primates (Mitchell et al., 2008).

In summary, 1.- we confirmed the strong deleterious effect of undernutrition during fetal to postpubertal life on rat testicular development and final Sertoli cell numbers; 2.- most effects of undernutrition during gestation on testes structure, including Sertoli cell numbers, disappear at 25 days of age in rats fed *ad libitum* after birth and 3.- this kind of testicular compensatory growth in rats is accompanied by higher testicular weight than in control animals which never experienced undernutrition.



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*Chapter 5*

# DOES EDUCATIONAL ATTAINMENT AFFECT NUTRITIONAL STATUS? A STUDY AMONG UNDERPRIVILEGED ADULTS OF SIX DISTRICTS OF WEST BENGAL, INDIA

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## ABSTRACT

*Aim*: To investigate the prevalence of undernutrition and the impact of level of education on undernutrition among ***SAHAI*** (living below poverty line) adults from six selected districts of West Bengal, India.

*Methods:* This cross-sectional study was conducted among 600 randomly selected SAHAI families from six selected districts of West Bengal. A total of 1068 adults (> 18 years of age) were studied of whom 499 and 569 were men and women, respectively. Mid-upper arm circumference (MUAC) of each subject was recorded to the nearest 0.1 cm. Evaluation of nutritional status was based on internationally accepted

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MUAC cut-off values. Information on educational status of the subjects was also obtained.

*Results:* The mean (sd) ages in men and women were 40.4 years (15.0) and 38.4 years (14.6), respectively. The population had a very high rate (62.0%) of illiteracy (men = 54.5%; women = 68.5%). There was significant sex-difference in educational status (chi-square = 24.36; $p < 0.001$). There was a significant sex difference ($t = 9.71$; $p < 0.001$) in mean MUAC (men: mean = 24.2 cm, sd = 2.5 and women: mean = 22.6 cm, sd = 2.7). Results revealed that the overall sex-combined prevalence of undernutrition was 34.6%. Significantly (chi-square = 16.41; $p < 0.001$) more females (40.1%) were undernourished compared to males (28.3%). Overall, there was a very strong significant (both sexes combined: chi-square = 21.51; $p < 0.001$) association of educational status with undernutrition. In men, there was a significantly (chi-square = 8.45; $p < 0.01$) decreasing prevalence of undernutrition with increasing level of education. The highest rate of undernutrition was observed among illiterates (33.5%) while the lowest rate was observed among individuals with secondary or above level of education (18.8%). Subjects with primary level of education had intermediate level of undernutrition (23.3%). A similar trend (chi-square = 8.60; $p < 0.01$) existed among females. Among them, the highest rate was observed among illiterates (44.10%) while the lowest rate was observed among individuals with secondary or above level of education (28.21%). Subjects with primary level of education had intermediate level of undernutrition (32.14%). Logistic regression analyses revealed that educational status (independent variable) had significant impact on nutritional status (dependant outcome variable) among the SAHAI people (Wald = 20.01, $p < 0.001$). This impact was similarly strong in both sexes (men: Wald = 7.95; $p < 0.005$; women: Wald = 7.96; $p < 0.005$). These results implied that education is a significant predictor of undernutrition in this population.

*Conclusion:* Our study provided strong evidence that the level of undernutrition among adults belonging to SAHAI families was high, more so among women. These adults were experiencing severe nutritional stress which was indicative of acute nutritional deprivation. Moreover, educational attainment was strongly related to nutritional status. Urgent appropriate nutritional supplementation/intervention programs are required to reduce this high prevalence of undernutrition. There is also a need to increase the level of educational attainment.

**Keywords:** India; Undernutrition; Educational status; Mid-upper arm circumference

## INTRODUCTION

Although there has been a recent spurt in economic growth at the national level in India, concern has been raised over the regional disparity of poverty levels as well as the slow rate of poverty reduction in recent years (Jha and Gaiha, 2003; Kijima 2006; Himanshu 2007). The disparity could be associated with geographical locations (among different states or between urban and rural areas) or among social groups or castes (Kijima, 2006, Gang et al., 2008). A recent study has shown that undernutrition is more prevalent in rural than urban areas, the recent report also reported that 38% of males and 41% females of rural areas were suffering from undernutrition. In contrast, 27% of males and 25% females of urban areas were suffering from undernutrition (NFHS III 2006). According to the National Sample Survey Organization (NSSO, 2006) around 9% of the Indian population do not get adequate food. In the last Rural Household Survey (RHS, 2007), 3.5% of the population have reported that even one meal a day is not certain for them. Another 16.5% face difficulty in arranging two square meals a day for all the months in a year. Based on these assessments, the State Government of West Bengal in the Panchayat and Rural Development Department introduced the concept of Anti-poverty Sub Plan at the level of Panchayats. Therefore *Panchayati Raj Institutions* have implemented various poverty alleviation programmes as well as some of the important social security programmes with an objective to ushering economic development and social justice of rural peoples known as *State Action against Hunger and Inequality (SAHAI).* One of the major expectations from these interventions is that every individual living within the respective areas of a Gram Panchayat may remain totally free from hunger and is generally above the level of destitution (Govt. W.B, 2007).

Hitherto, data have been scanty on the anthropometric and nutritional status of various underprivileged populations of India (Arlappa et al. 2005; Bose and Chakraborty 2005; Bose et al. 2006 a, b). It has been suggested (Bose and Chakraborty 2005) that there is urgent need to evaluate the nutritional status of various rural populations of India. To the best of our knowledge, hitherto, there are no investigations on nutritional status among rural adult population from SAHAI families in India.

In view of this, the objective of the present study was to evaluate mid-upper arm circumference (MUAC) based prevalence of undernutrition as well as explore the association between nutritional status and educational status of adult members of SAHAI families from six selected district of West Bengal, an Eastern Province of India.

According to World Health Organization (1995), anthropometry can be used to assess the nutritional and health status of adults. There are many anthropometric tools or measurements to evaluate nutritional status among adults. The Body Mass Index (BMI) and MUAC are well established and easy to perform methods to evaluate nutritional status among adults. The MUAC is particularly effective in the evaluation of undernutrition among adults in developing countries (James et al., 1994). It has been found that MUAC is a simpler measure than BMI requiring a minimum of equipment and in practice has now been found to predict morbidity and mortality as accurately as deficits in weight (Breind et al, 1989). James et al. (1994) had suggested that MUAC could be used for simple screening of nutritional state after an extensive study of 8 countries (Mali, India, Senegal, Zimbabwe, Somalia, Ethiopia, Papua New Guinea and China). It has also been stated that since MUAC is a simpler measure than BMI, it could be used not only in emergencies but also when semi-skilled monitors are available. It can be used as a substitute for BMI when rapid screening of an adult population is required as a prelude to targeting help for the undernourished (James et al., 1994). Several recent investigations have studied the relationships of socio-economic status with undernutrition among different populations (Delpeuch et al., 1994; Ahmed et al., 1998; Reddy, 1998; Khongsdier, 2002; Pryer et al., 2003; Monteiro et al., 2004; Clausen et al., 2006; Mahmud et al., 2006).



## MATERIALS AND METHODS

### Sample and Study Area

Our cross- sectional study was conducted during May - July, 2010, in 600 randomly selected SAHAI (living below poverty line, BPL) families from different Gram Panchayats (GP) of six selected districts of West Bengal, India. The districts were: Purulia, Mushidabad, Birbhum, Uttar Dinajpur, Jalpaiguri and Cooch Behar. A total of 1068 adults (≥ 18 years of age) were studied of whom 499 and 569 were men and women, respectively. The mean (sd) ages in men and women were 40.4 years (15.0 years) and 38.4 years (14.6 years), respectively. This study was conducted based on family, and the families were selected from BPL list at the mentioned districts under selected Gram Panchayats. These families are generally termed as *SAHAI families.* A total of 100 SAHAI families were studied from each district. The final sample consisted of 600 SAHAI families from six selected districts.

## Anthropometric Measurements and Information on Education

Ethical permission was obtained from relevant authorities. Informed consent was also obtained from all subjects. Anthropometric measurements and information on educational status were collected by a research team of Vidyasagar University following the standard techniques of Lohman et al. (1988). The MUAC were recorded to the nearest 0.1 cm. Technical errors of measurements (TEM) were computed, and they were found to be within acceptable limits (Ulijaszek and Kerr 1999).

Subjects were asked about their educational status. Thereafter, educational status of the subjects was grouped in to three categories, i.e., illiterate, primary and secondary and above.

## Evaluation of Nutritional Status

Nutritional status of the subjects was evaluated using the internationally accepted guide line (WHO 1995) of MUAC cut-off.

The following cut-off points were used.

MUAC < 23.0 cm = Undernutrition among men

MUAC < 22.0 cm = Undernutrition among women.

## Statistical Analyses

Student's t-test was performed to test for sex differences in mean MUAC and chi-square test was undertaken to find out significance sex difference in educational status as well prevalence of undernutrition based on sex and educational status. Logistics regression analyses (dependent = undernutrition = yes/no) were performed with educational status as an independent variable. Significance level was set at $p < 0.05$.

# RESULTS

*Table 1* presents the characteristics of the subjects. There were significant sex differences ($t = 9.71$; $p < 0.01$) in mean MUAC (men: mean = 24.2 cm, sd = 2.5 and women: mean = 22.6 cm, sd = 2.7) as well as mean age (men: mean = 40.44 years, sd = 14.97 and women: mean = 38.39 years, sd = 14.65, t =

2.26, $p < 0.05$). Educational status of the subjects is presented in *table 2*. The population had a very high rate (61.0%) of illiteracy (men = 54.5%; women = 68.5%). Overall sex-combined attainment of primary and secondary or above level of education were 28.4% (men = 32.7% and women = 24.6%) and 9.64% (men = 12.8% and women = 6.8%), respectively. There was significant sex-difference in educational status (chi-square = 24.36; $p < 0.001$). Results *(table 3)* revealed that the overall sex-combined prevalence of undernutrition was 34.6%. Significantly (chi-square = 16.41; $p < 0.001$) more females (40.1%) were undernourished compared to males (28.3%). Overall, there was a very strong significant (both sexes combined: chi-square = 21.51; $p < 0.001$) association of educational status with undernutrition. In men, there was a significantly (chi-square = 8.45; $p < 0.01$) decreasing prevalence of undernutrition with increasing level of education. The highest rate of undernutrition was observed among illiterates (33.5%) while the lowest rate was observed among individuals with secondary level of education (18.8%). Subjects with primary level of education had intermediate level of undernutrition (23.3%).

**Table 1. Characteristics of the subjects**

| Variables | Sex | n | Mean | SD | t- value |
|---|---|---|---|---|---|
| Age (Years) | Male | 499 | 40.44 | 14.97 | |
| | Female | 569 | 38.39 | 14.65 | 2.26* |
| MUAC (cm) | Male | 499 | 24.20 | 2.52 | |
| | Female | 569 | 22.63 | 2.73 | 9.71** |

* $p < 0.05$; ** $p < 0.001$.

**Table 2. Educational status of the subjects**

| Educational Status | Male | Female | Sex-combined | Chi-square |
|---|---|---|---|---|
| | | | | |
| Illiterate | 54.51 | 68.54 | 61.99 | |
| | | | | $\chi^2 = 24.36^{***}$, df = 2 |
| Primary | 32.67 | 24.60 | 28.37 | |
| | | | | |
| Secondary and above | 12.83 | 6.85 | 9.64 | |

All figures refer to percentages.

*** $p < 0.001$.

**Table 3. The association of educational status and prevalence (%) of MUAC-based undernutrition among the subjects**

| Sex | Nutritional status | Educational status | | | Total | Chi-squares |
|---|---|---|---|---|---|---|
| | | Illiterate | Primary | Secondary and above | | |
| Male | Undernutrition | 33.46 | 23.31 | 18.75 | 28.26 | $\chi^2 = 8.45^{**}$, df = 2 |
| | Normal | 66.54 | 76.69 | 81.25 | 71.74 | |
| Female | Undernutrition | 44.10 | 32.14 | 28.21 | 40.07 | $\chi^2 = 8.60^{**}$, df = 2 ^$\chi^2 = 16.41^{***}$, df = 2 |
| | Normal | 55.90 | 67.86 | 71.79 | 59.93 | |
| Sex combined | Undernutrition | 39.73 | 27.39 | 22.33 | 34.55 | $\chi^2 = 21.51^{***}$, df = 2 |
| | Normal | 60.27 | 72.61 | 77.67 | 65.45 | |

^ sex difference.
** $p < 0.01$; *** $p < 0.001$.

**Table 4. Logistic regression of undernutrition (dependent variable) and educational status (independent variable)**

| Independent variable | Sex | B | seB | Beta | 95% C.I. for Beta | | Wald | Sig. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | |
| Educational status | Male | 0.429 | 0.152 | 1.535 | 1.140 | 2.068 | 7.945 | 0.005 |
| | Female | 0.421 | 0.149 | 1.523 | 1.137 | 2.040 | 7.961 | 0.005 |
| | Sex combined | 0.472 | 0.105 | 1.603 | 1.304 | 1.971 | 20.082 | 0.001 |

B = Regression Coefficient,
seB = Standard Error of Regression Coefficient,
Beta = Population Estimate,
CI = Confidence Interval.

A similar trend (chi-square = 8.60; $p < 0.01$) existed among females. Among women, the highest rate of undernutrition was observed among illiterates (44.10%) while the lowest rate was observed among individuals with secondary level of education (28.21%). Subjects with primary level of education had intermediate level of undernutrition (32.14%). Logistic regression analyses revealed *(table 4)* that educational status (independent variable) had significant impact on nutritional status (dependant outcome variable) among the SAHAI people (sex combined: Wald = 20.01, $p < 0.001$). This impact was similarly strong in men (Wald = 7.95; $p < 0.001$) as well as women (Wald = 7.96; $p < 0.005$). These results implied that education is a significant predictor of undernutrition in this population.

## DISCUSSION

Malnutrition still remains a significant public health problem in developing world including South Asian countries (Wickramasinghe et al. 2004). After over 60 years of independence, India is still a country in developmental transition and continues to battle with infectious diseases and conditions related to undernutrition. Over 30% of adults are undernourished as judged by anthropometric indices and over 70% of women and children suffer from anaemia (NFHS-III).

Worldwide studies have clearly established that low socio-economic status is associated with undernutrition among adults (Shetty and James, 1994; Ene-Obong et al., 2001; Pryer et al., 2003; Clausen et al., 2006; Pryer and Rogers, 2006) in different populations. Although, a major section of the Indian population live in poverty (World Bank, 2000), extensive efforts have not been made to study the nature and extent of association of educational status with MUAC-based undernutrition among them. The present study has investigated the impact of educational status on MUAC-based undernutrition among adult members of SAHAI families of six selected districts in West Bengal, India.

Results of our study clearly demonstrated that nutritional status of this population was unsatisfactory. Furthermore, significantly (chi-square = 16.41; $p < 0.001$) more females were undernourished compared to males. There was a very strong significant (chi-square = 21.51; $p < 0.001$) association of educational status with undernutrition. Educational status had significant impact (sex combined: Wald = 20.01, $p < 0.001$; male: Wald = 7.95, $p < 0.005$ and female: Wald = 7.96, $p < 0.005$) on nutritional status. These results thus implied that education is a significant predictor of undernutrition in this population. Similar findings were found in a very recent study conducted by Bose et al. (2009) among adult rural Bengalee population in West Bengal, India. Several investigations have also been reported in similar rates of undernutrition among other populations who are economically disadvantaged (Bose and Chakraborty, 2005; Bose et al., 2006). These high rates are strongly influenced by poor socio-economic condition including poor educational attainment. The high rate of undernutrition is indicative of nutritional stress being experience by these individuals.

It has already been emphasized (Topal and Samal, 2001) that variation exists in social and economic conditions among rural populations of India. Nutritional status is intricately linked with dietary habits as well as the ecology of the population. One of the major limitations of our study was the absence of detailed information on dietary intake and other socio-economic parameters

including house type, source of drinking water, access to sanitation, etc. Therefore, we recommend that further research be undertaken to investigate, in detail, these factors. Moreover, there are distinct inter-ethnical differences in the environment in which they reside, i.e., the ecology of the population (Mandal et al. 2002). The present report did not deal with these factors as they were beyond the scope of study. This is another clear limitation of the present study. However, it is imperative that future studies on rural underprivileged populations include these parameters when investigating their nutritional status.

In conclusion, our study provided robust evidence that the level of undernutrition among adults belonging to SAHAI families was high, more so among women. These adults were experiencing severe nutritional stress which was indicative of acute nutritional deprivation. Moreover, educational attainment was very strongly related to nutritional status. Urgent appropriate nutritional supplementation/intervention programs are required to reduce this high prevalence of undernutrition. There is also a need to increase their level of educational attainment.

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## CONFLICT OF INTEREST

None.

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*Chapter 6*

# PREVALENCE OF THINNESS AMONG RURAL BENGALEE MUSLIM SCHOOL CHILDREN FROM EASTERN INDIA

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## ABSTRACT

Child undernutrition is the most serious health problem in developing countries. It is a serious public health challenge that continues to be a primary cause of ill-health and premature mortality among children in these countries. However, there exists scanty information of the prevalence of undernutrition among Muslim school children in India. The present cross-sectional study investigated the prevalence of undernutrition among 6-16 years old Bengalee Muslim school children from a rural area of eastern India. The children (304 boys and 334 girls) were randomly selected from different schools and Madrashas

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(government schools) of Chapra Block Nadia District, West Bengal, India. Significant ($p < 0.001$) age difference existed in mean height and weight in boys (height: $F = 202.66$; weight: $F = 129.00$) as well as girls (height: $F = 40.12$; weight: $F = 45.03$). Overall (age and sex combined) prevalence of thinness was 56.70%. Overall prevalence of thinness (age combined) were 58.77% and 56.70%, among boys and girls, respectively. Significant sex (age combined) difference in different grades of thinness were observed among the subjects (chi-square = 77.12, $p < 0.001$). In conclusion, our study provided strong evidence that these children were under acute and chronic nutritional stress in form of thinness. There is a requirement for the implementation of immediate appropriate public health nutritional intervention programmes.

**Keywords**: India; Bengalee Muslim; School; Madrasah; School; Thinness

## INTRODUCTION

The incidence of child undernutrition is high in developing countries like India. It is a serious public health problem that continues to be a primary cause of ill-health and premature mortality among children in these countries (Nandy et al., 2005). It is well established that half of the world's malnourished children are to be found in only three south Asian countries including India, Bangladesh and Pakistan (Rattan, 1997). Out of the global total, India accounts for about 40 percent of undernourished children, which is significantly associated with high rates of morbidity and mortality in the country (Levinson, 1998). Generally, childhood undernutrition is assessed by conventional anthropometric indices of stunting (low height for age), underweight (low weight for age) or wasting (low weight for height) following different internationally and regionally recommended standards (WHO, 1995). The body mass index (BMI) is an inexpensive and non-invasive measure that has been extensively utilized to assess the nutritional status of adults and thinness in adolescents (WHO, 1995). This index (BMI) provides a simple measure of a person's "fatness" or "thinness", allowing health professionals to discuss over- and under-weight problems more objectively with their patients. Therefore it has been widely used for assessing nutritional status of adults (WHO, 1995; WHO, 2000) and more recently in children aged 0-5 years (WHO, 2006). Very recently, new international cut offs of child overweight and obesity for the age range of 2-18 (Cole et al., 2000) and for underweight or thinness (Cole et al.,

2007) have been developed. In the latter study, undernutrition has been termed as thinness (as in adults) defined as low BMI for age and it has been graded as III, II, I (severe, moderate and mild, respectively) similar to adult chronic energy deficiency (CED) grades of CED III, II and II. The BMI is measured by weight in kilogram (kg) divided by height in meter squared.

According to the Indian Census 2001, the ratio of Muslims in total population is 13.43%, which implies that total Muslim population in India is second only to Indonesia. The states with large number of Muslim population are Uttar Pradesh (31 million), West Bengal (20 million), Bihar (14 million), Maharashtra (10 million), Kerala (8 million), Andhra Pradesh (7 million) and Jammu and Kashmir (7 million). In J and K and Lakshadweep, Muslims are in the majority. There are 43 districts in UP, West Bengal, Bihar, Assam, J and K, Jharkhand, Haryana and Uttaranchal where the Muslim population is substantial (over 30 percent of total population in most of these districts). Within these districts, 10 are in UP, 5 in West Bengal, 4 in Bihar, 10 each in Assam and J and K, 2 in Jharkhand and 1 each in Haryana and Uttaranchal. It has been observed that the literacy rate is low (49%) among Muslims in comparison with Hindu (53%), Christians (81%) and other minorities (54%) However, it is higher than the literacy rate of Scheduled castes (42%) and Scheduled tribes (39%) (Human Development Report, 1999).

Undernutrition among school children is an important health problem in developing countries (Pryer et al., 2004) including rural India (Roy, 2005). In West Bengal, half of children of this age group suffered from different types of under nutrition (Bose et al., 2008). However, there is scanty information of the prevalence of undernutrition among Muslims school children in India (Bishnoi et al 2004, George et al 2000, Kumari 2005, Biswas et al., 2009) and West Bengal (Shaikh et al., 2003, Mustaphi and Dobe 2005, Biswas et al., 2009). Till date, there are only a few investigations on thinness in children in India. (Mustaphi and Dobe, 2005, Kumari, 2005, George et al., 2000, Shaikh et al., 2003, Biswas et al., 2009). Therefore, the objective of the present study was to evaluate the prevalence of different grades of thinness using the recently recommended age and sex specific cut off values of BMI (Cole et al., 2007). There exists no published literature that has utilized these cut-off points to evaluate the rates of thinness among Muslims school children. Thus, the results of the present study will be useful for national and international comparisons of rates of thinness among school children.

# MATERIALS AND METHODS

## Location and Subjects

This cross sectional study was undertaken at Chapra Block, Nadia District, West Bengal, India. The study area is situated at the India–Bangladesh international border, 140 km from Kolkata, the provincial capital of West Bengal**.** The area is remote and dominated by Bengalee Muslims. All children (6–16 years old) living in Chapra Block are enrolled at different primary schools and Madrashas (Govt. of West Bengal aided).

The subjects were selected from different primary schools and Madrashas of the Chapra Block. A total of 642 primary and Madrasha children (304 boys and 334 girls) aged 6-16 years were measured. Age and ethnicity of the subjects were verified from official records. Formal ethical approval was obtained from Vidyasagar University and school and Madrasha authorities prior to the commencement of the study.

## Anthropometry and Assessment of Thinness

Height and weight measurements were measured to the nearest 0.1cm and 0.5 kg, respectively, by one author (SB) on each subject following the standard techniques (Lohman et al., 1988). The BMI was computed following internationally accepted standard equation as weight in kg divided by square in height in meter. Nutritional status was evaluated using the age and sex specific cut-off points *(Table 1)* of BMI as described by Cole et al. (2007). Thinness (as in adults) was defined as low BMI for age and were graded as III, II, I (severe, moderate and mild, respectively) defined to pass through BMI values of 16.0, 17.0, and 18.5 $kg/m^2$, respectively, at age 18 (Cole et al 2007). Technical errors of measurements (TEM) were found to be within reference values (Ulijaszek and Kerr, 1999) and thus not incorporated in statistical analyses. Student's t-tests were used to see the significance of differences in means between the sexes at each age group as well as between sexes on an overall basis. One-way ANOVA tests were done to evaluate the significance of differences in means for each sex as well as for the sex-combined mean values across the age groups. Chi-square tests were undertaken to test for sex differences in overall thinness in each age group and age combined, respectively. Statistical significance was set at $p < 0.05$.

**Table 1. Age and sex specific international cut-off values for assessing thinness among children (based on Cole et al., 2007)**

| Age (years) | Grades of thinness | | | | Grades of thinness | | | |
|---|---|---|---|---|---|---|---|---|
| | Grade III | Grade II | Grade I | Normal | Grade III | Grade II | Grade I | Normal |
| 6 | 12.45 | 13.10 | 14.04 | >14.04 | 12.28 | 12.90 | 13.82 | >13.82 |
| 7 | 12.41 | 13.09 | 14.08 | >14.08 | 12.27 | 12.95 | 13.93 | >13.93 |
| 8 | 12.45 | 13.17 | 14.24 | >14.24 | 12.37 | 13.08 | 14.14 | >14.14 |
| 9 | 12.57 | 13.34 | 14.49 | >14.49 | 12.53 | 13.29 | 14.43 | >14.43 |
| 10 | 12.77 | 13.58 | 14.80 | >14.80 | 12.78 | 13.59 | 14.81 | >14.81 |
| 11 | 13.03 | 13.87 | 15.16 | >15.16 | 13.15 | 14.01 | 15.32 | >15.32 |
| 12 | 13.37 | 14.25 | 15.58 | >15.58 | 13.65 | 14.56 | 15.93 | >15.93 |
| 13 | 13.83 | 14.74 | 16.12 | >16.12 | 14.20 | 15.14 | 16.57 | >16.57 |
| 14 | 14.35 | 15.28 | 16.69 | >16.69 | 14.75 | 15.72 | 17.18 | >17.18 |
| 15 | 14.86 | 15.82 | 17.26 | >17.26 | 15.25 | 16.22 | 17.69 | >17.69 |
| 16 | 15.36 | 16.34 | 17.80 | >17.80 | 15.63 | 16.62 | 18.09 | >18.09 |

# RESULTS

## Anthropometric Characteristics

*Table 2* shows the mean, height (cm) weight (kg.) and BMI ($kg/m^2$) of the subjects. Significant sex differences ($p < 0.05$) were observed in height and weight at ages 14, 15, 16 years and 6, 11, 16 years, respectively. There were increasing trends in height and weight with increasing ages, in both sexes. Mean BMI increased with increasing of ages from 8 year up to age 16 year among boys. Similarly increasing trend of mean BMI was observed from 6 to 15 years among girls. Significant ($p < 0.001$) age difference existed in mean height and weight in boys (height: $F = 202.66$; weight: $F = 129.00$) as well as girls (height: $F = 40.12$; weight: $F = 45.03$).

## Nutritional Status

*Table 3* presents the prevalence of thinness by age and sex among the subjects. Overall (age and sex combined) prevalence of thinness was 56.70%. Overall prevalence of thinness (age combined) were 58.77% and 56.70% among boys and girls, respectively.

## Table 2. Anthropometric Characteristics of the subjects

| Age (years) | Boys | | | | | | | Girls | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Height (cm) | | Weight (Kg) | | BMI (Kg/m$^2$) | | n | Height (cm) | | Weight (Kg) | | BMI (Kg/m$^2$) | |
| | | Mean | SD | Mean | SD | Mean | SD | | Mean | SD | Mean | SD | Mean | SD |
| 6 | 36 | 104.34 | 7.18 | 14.89^ | 2.11 | 13.63 | 0.82 | 34 | 102.87 | 4.52 | 13.98 | 1.29 | 13.22 | 0.95 |
| 7 | 27 | 112.96 | 6.93 | 17.36 | 2.96 | 13.50 | 1.10 | 26 | 114.16 | 3.52 | 17.26 | 2.06 | 13.20 | 0.99 |
| 8 | 24 | 119.84 | 7.14 | 19.30 | 3.04 | 13.38 | 0.79 | 24 | 117.64 | 5.68 | 18.92 | 2.12 | 13.67 | 1.35 |
| 9 | 24 | 122.13 | 5.25 | 20.93 | 2.09 | 14.02 | 0.80 | 23 | 123.83 | 4.01 | 21.34 | 2.29 | 13.89 | 1.02 |
| 10 | 21 | 128.84 | 4.56 | 24.05 | 1.75 | 14.50 | 1.02 | 32 | 130.68 | 8.14 | 24.35 | 3.89 | 14.18 | 1.05 |
| 11 | 28 | 134.15 | 7.90 | 26.11^ | 3.43 | 14.48 | 1.21 | 29 | 137.58 | 7.61 | 29.04 | 6.49 | 15.19 | 2.20 |
| 12 | 30 | 144.68 | 11.82 | 32.31 | 8.12 | 15.17 | 1.60 | 30 | 145.00 | 6.16 | 33.42 | 4.87 | 15.85 | 1.72 |
| 13 | 30 | 150.29 | 8.78 | 36.19 | 7.95 | 15.83^^ | 1.80 | 32 | 149.36 | 3.76 | 38.01 | 3.57 | 17.05 | 1.62 |
| 14 | 28 | 154.66^^ | 5.82 | 42.36 | 8.54 | 17.64 | 3.18 | 32 | 150.75 | 3.99 | 42.23 | 6.79 | 18.50 | 2.41 |
| 15 | 32 | 159.46^^ | 6.62 | 46.05 | 7.39 | 17.99 | 1.80 | 35 | 151.09 | 5.68 | 42.57 | 8.20 | 18.56 | 2.91 |
| 16 | 28 | 161.20^^ | 7.40 | 47.95^^ | 6.15 | 18.39 | 1.50 | 37 | 152.53 | 3.41 | 42.32 | 4.77 | 18.17 | 1.77 |
| ANOVA | | F = 202.66** | | F = 129.00** | | F = 40.12** | | | F = 330.27** | | F = 156.12** | | F = 45.03** | |

** $p < 0.001$.

^ = significant sex differences ($p < 0.05$).

^^ = significant sex differences ($p < 0.001$).

**Table 3. Prevalence (%) of thinness of the subjects based on Cole et al., 2007**

| Age (years) | Thinness | | | | | | Overall thinness | | |
|---|---|---|---|---|---|---|---|---|---|
| | Grade-III | | Grade-II | | Grade-I | | | | |
| | Boys | Girls | Boys | Girls | Boys | Girls | Boys | Girls | Sex-combined |
| 6 | 5.56 | 20.59 | 22.22 | 17.65 | 41.67 | 41.18 | 69.44 | 79.41 | 74.29 |
| 7 | 14.81 | 15.38 | 22.22 | 30.77 | 33.33 | 34.62 | 70.37 | 80.77 | 75.47 |
| 8 | 12.50 | 8.33 | 20.83 | 16.67 | 45.83 | 54.17 | 79.17 | 79.17 | 79.17 |
| 9 | 4.17 | 4.35 | 16.67 | 34.78 | 50.00 | 30.43 | 70.83 | 69.57 | 70.21 |
| 10 | 4.76 | 12.50 | 14.29 | 15.63 | 52.38 | 40.63 | 71.43 | 68.75 | 69.81 |
| 11 | 10.71 | 3.45 | 21.43 | 6.90 | 39.29 | 27.59 | 71.43 | 37.93 | 54.39 |
| 12 | 13.33 | 0.00 | 20.00 | 23.33 | 30.00 | 20.00 | 63.33 | 43.33 | 53.33 |
| 13 | 3.33 | 3.13 | 23.33 | 6.25 | 40.00 | 34.38 | 66.67 | 43.75 | 54.84 |
| 14 | 10.71 | 9.38 | 25.00 | 6.25 | 0.00 | 9.38 | 35.71 | 25.00 | 30.00 |
| 15 | 6.25 | 11.43 | 0.00 | 8.57 | 28.13 | 22.86 | 34.38 | 42.86 | 38.81 |
| 16 | 0.00 | 16.22 | 10.71 | 0.00 | 10.71 | 29.73 | 21.43 | 45.95 | 35.38* |
| Overall | 7.79* | 9.88* | 17.86* | 14.07* | 33.12* | 30.84* | 58.77* | 54.79* | 56.70* |
| | | | | | | | | | |

* Significant sex in different grades of thinness ($p < 0.05$).

The rates of Grade-II and Grade-I thinness were higher among boys (Grade-II = 17.86%, Grade-I = 33.12%) compared with girls (Grade-II = 14.07%, Grade-I = 30.84%). In contrast, the frequency of thinness Grade-III was higher among girls (9.88%) than boys (7.79%). Prevalence of thinness was higher among 8 year old children (79.17%) and vise-versa lower prevalence of thinness was observed among 14 year old children (30.00%). Significant sex (age combined) difference in different grades of thinness were observed among the subjects (chi-square = 77.12, $p < 0.001$).

## DISCUSSION

Undernutrition among children and adolescents is a serious public health problem globally, especially in developing countries in South Asia (Pelletier, 2003, El-Ghannam, 2003, Staton and Harding, 2003). The very recent study of Cole et al. (2007) has demonstrated that undernutrition is better assessed as thinness (low BMI for age) than as wasting (low weight for height). Prior to this study of Cole et al. (2007) there were no suitable thinness cut-offs for this age group. These new cut-off points are suggested to encourage direct comparison of trends in child and adolescent thinness worldwide. These cut-offs provide a classification of thinness for public health purposes. In spite of having some limitations such as low sample size in some age groups and

inability to employ any strict sampling strategy, which makes the state level of the sample questionable, the results of the present study clearly indicated that the nutritional status of these children was unsatisfactory with very high rates of thinness of 56.70%, 58.77% and 56.70% in sex-combined, boys and girls, respectively. A noteworthy point was that both sexes had more or less similar rates of thinness. We propose that future investigations in India should utilize the cut-off points proposed by Cole et al. (2007) to determine the rates of thinness in children and adolescents among different caste and religious groups. Such studies should provide data on prevalence of thinness which can be used for the formulation of effective public health policies. Moreover, they would also provide useful datasets for comparisons, both nationally and internationally.

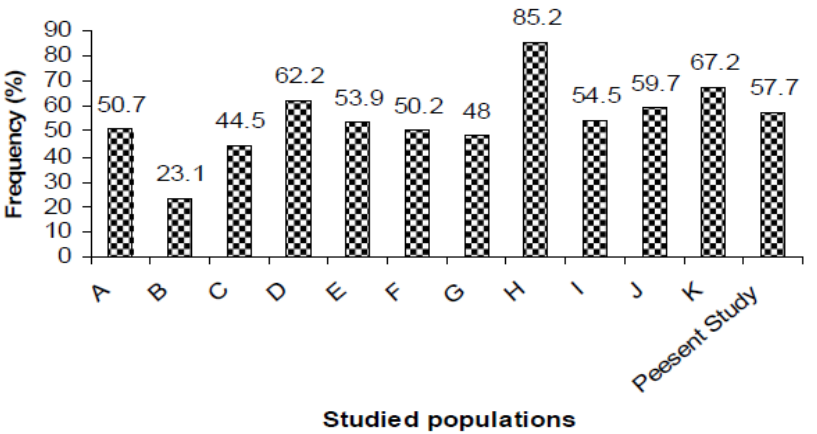


Figure 1. A = Preschool children, Nadia district, West Bengal. (Biswas et al., 2009). B = School children, Bankura District, West Bengal. (Bose et al., 2008). C = School children, Paschim Medinipur and Puruliya district, West Bengal. (Bose and Bisai, 2008). D = School children, Purba Medinipur, West Bengal. (Chakraborty and Bose, 2009). E = School children, Dibrugarh district, Assam. (Medhi et al., 2006). F = Children, Dibrugarh district, Assam. (Medhi et al., 2007). G = Children of Central Orissa. India. (Mishra and Mishra, 2007). H = ICDS children, Hooghly district, West Bengal. (Mandal et al., 2009). I = Tribal children, India. (IIPS, 2007). J = Tribal children, West Bengal. (IIPS, 2008). K = Kora-Mudi tribal children, Paschim Mediniapur, West Bengal. (Bisai et al. 2010).

*Figure 1* shows the comparison of prevalence of thinness among different Indian children and adolescents. Most of the studies showed lower prevalence of thinness than the present study (Biswas et al., 2009, Bose et al., 2008, Bose and Bisai, 2008, Medhi et al., 2006, Medhi et al., 2007, Mishra and Mishra,

2007). Some other studies (Chakraborty and Bose, 2009, Mandal et al., 2009, Bisai et al 2010) had reported higher prevalence of thinness compared to present study. Similar rates of thinness have been reported from tribal populations of different parts of India (IIPS, 2008) including West Bengal (IIPS, 2007).

A noteworthy point was that all studies have reported that both sexes had similar rates of thinness. It is well documented that thinness is an indicator of acute undernutrition, the results of more recent food deprivation (WHO, 1995). Malnutrition (under-nutrition and over-nutrition) continues to be a problem of considerable magnitude in most developing countries of the world (Som et al., 2006). Different worldwide studies have shown that dietary and environmental constraints are the major determinants of differences in growth patterns between children of both developed and developing countries (Serenius, 1988, Dugdale et al., 1994, Quinn et al., 1995, Mei and Trowbridge, 1995). A previous report has showed that in recent years, undernutrition among children has increased (Chatterjee, 2007). Better nutrition during school age period leads to better growth and development of children and has been regarded as one of the universal humanitarian goals (Rajaram, 2003). Under the above scenario, we recommend that further investigations in India may utilize these cut-off points proposed by Cole et al. (2007)] to determine the rates of thinness among children and adolescents of different caste and religious groups. These studies may provide valuable information towards the formulation of effective public health policies to combat child malnutrition in India and adjoining countries where high prevalence of child and adolescent thinness exist.

In conclusion, our study provided evidence that these children were under acute and chronic nutritional stress. There is a need for immediate appropriate public health nutritional intervention programmes.

## ACKNOWLEDGMENTS

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*Chapter 7*

# LONG-TERM EFFECTS OF EARLY UNDERNUTRITION ON WOUND HEALING

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## ABSTRACT

Epidemiological studies have revealed that protein-calorie malnutrition is a widespread problem among infants and young children of developing countries, and in poverty areas of the industrialized nations, and a moderate degree of undernutrition is more common among children, generally associated with negligence and carelessness from the parents. Malnutrition is still a problem in the Brazil, and studies have pointed out that 31% of younger children (up to 5 year old) may present moderate or severe undernutrition. Considering these facts, we think it is very important to understand the effects of undernutrition on wound healing, particularly after recovering from the undernutritional state.

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## WOUND HEALING AND UNDERNUTRITION

Malnutrition in early life has been associated with a number of acute and chronic sequelae. Early malnourished children may present anatomical, neurological, neurochemical and hehavioral alterations, some of them persisting even after recovery from undernutrition. Malnourished children also presented lymphoid tissues atrophy and their cell-mediated immunity is significantly altered (Chandra, 1991; Keller et al., 1992; Manjarrez et al., 1996).

Clinical reports have found poor wound healing in malnourished patients, and experimental studies indicated that wound healing is impaired after prolonged dietary restriction. Clinical studies consider a weight loss of 10% as a significant evidence of undernutrition (Emery and Sanderson, 1995; Ayello et al., 1999).

The wound healing process is basically divided into three phases: i) an initial inflammatory phase; ii) a proliferative phase; and iii) a remodeling phase. The inflammatory phase is characterized by infiltration of neutrophils, which are rapidly replaced by mononuclear cells. The proliferative phase is characterized by the presence of mononuclear inflammatory cells, followed by the formation of granulation tissue, and the proliferation of fibroblasts and keratinocytes. The final remodeling phase is that of maturation of the neoformed tissue (Moore et al., 1997).

It is well known that during the wound healing, a sequence of inflammation, synthesis and reorganization of tissue is necessary, and alterations or impairment of wound healing are recognized in a number of conditions, including undernutrition. These changes in wound healing appear to be mostly related to underlying immune functions.

Malnutrition, caused by a low protein-calorie diet may significantly alter the processes of tissue regeneration, inflammatory reaction, and immune functions, and studies have demonstrated that deffects during the inflammatory phase of wound healing may impair subsequent fibroblast growth and collagen synthesis (Goodson III and Hunt, 1979).

Epidemiological studies have revealed that protein-calorie malnutrition is a widespread problem among infants and young children of developing countries, and in poverty areas of the industrialized nations, and a moderate degree of undernutrition is more common among children, generally associated with negligence and carelessness from the parents. Malnutrition is still a problem in the Brazil, and studies have pointed out that 31% of younger children (up to 5 year old) may present moderate or severe undernutrition.

(Reichenhein and Hasselmann, 1977). Considering these facts, we think it is very important to understand the effects of undernutrition on wound healing, particularly after recovering from the undernutritional state.

## EXPERIMENTAL STUDIES ON WOUND HEALING

There are several methods for causing experimental undernutrition (Shakir, 1979). Our experimental method to produce undernutrition was to separate the pups from the mother for a portion of the day (10 hours) during the suckling period (21 days after birth) (Wolff et al., 2003). Undernourished rats were kept on normal food for recovering, and as control were used rats at same age not submitted to suckling restriction. Undernutrition and recovered states were assessed by body weight according to Barret and Frank (1987) considering: *normal* those with at least 90% of the expected body weight; *slight undernutrition* 76% - 89%; *moderate undernutrition* 61% to 75%; and *severe undernutrition* ≤ 60% of the expected body weight. Recovered animals were considered when reaching at least 90% of the control group body weight.

After undernutrition (21 days), it was possible to identify a moderate state of undernutrition in our animal population (71.44% of the expected body weight). Recovery times was 3 months, when those undernourished animals submitted to nutritional rescue showed recovery of physical growth (at least 90% of the body weight of controls).

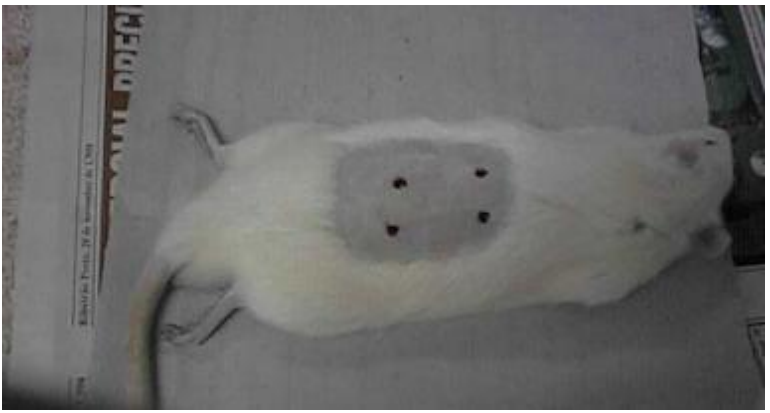

Figure 1. Young adult male rat 200-250g, anesthetized, shaved and presenting 4cm diamether skin wounds at each side of the median line, aproximatelly 2cm far from each other.

The animals were submitted to surgical procedures as follows: under tribromoethanol (Aldrich, 25mg/100g body weight) the back of all the rats (control and experimental groups) were shaved and skin wounds were prepared (4cm diameter wounds each side of the median line, aproximatelly 2cm from each other) [as a modification of Mustoe et al., 1987; Whitby and Ferguson, 1991; Most et al., 1996] (Figure 1).

## HISTOPATHOLOGY OF WOUND HEALING

The skin structure of the rat is, in many aspects, similar to human skin. Both are formed by the corium or dermis (connective tissue) and epidermis (epithelium), and the tissues have the same general characteristics, however in the animal, the epithelial appendages are mostly hair follicles and sebaceous glands. Sweat glands are not observed in rats. The epidermis of the dorsum of a normal rat is formed by four or five cells thick with basal, spinous, granular and cornified (keratin) layers.

In a way to follow the healing process, animals were sacrificed 1 and 6 hours, 1, 3 and 7 days after surgery (Figures 2 to 5). The specimens were fixed in 10% formalin (24 hours) or Carnoy (4 hours) and embedded in paraffin (Gaffney, 1983). Hematoxilin and eosin (HE), or Giemsa stained sections (6μm thickness) were examined under a light microscope. Wound healing area were accessed.

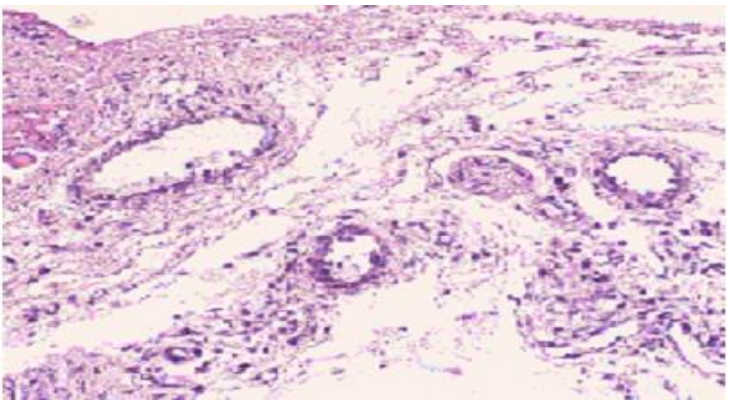

Figure 2. Wounded area on X25 magnification. Observe the initial inflammation characteristics such as the presence of edema, vasodilatation and vascular exsudative processes (HE).

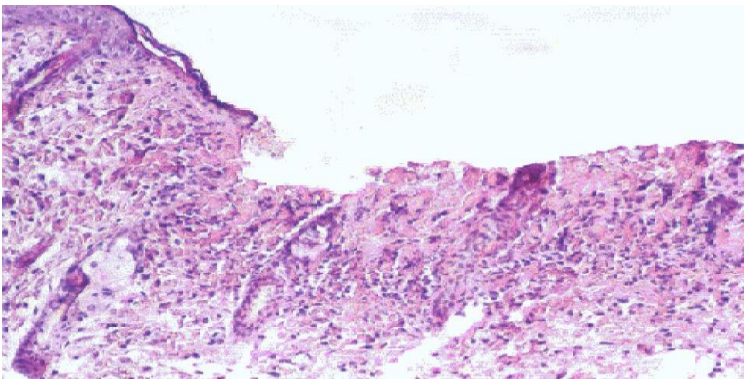

Figure 3. HE stain 6 hours after surgery. Magnification x10. Within 6 hours after injury, defense cells start to appear in the area. A band of polimorphonuclear and round cells begin to be noticeable at the edges limit between viable and unviable tissues.

It was possible to observe that tissue repair process occurs in an ordered sequence of events, beginning with an inflammatory response of the wounded area (that lasts about 3 ys), followed by the formation of granulation tissue and epithelial neoformation (3 to 7 days after lesion); then followed by the remodeling phase (which may take some months to be completed).

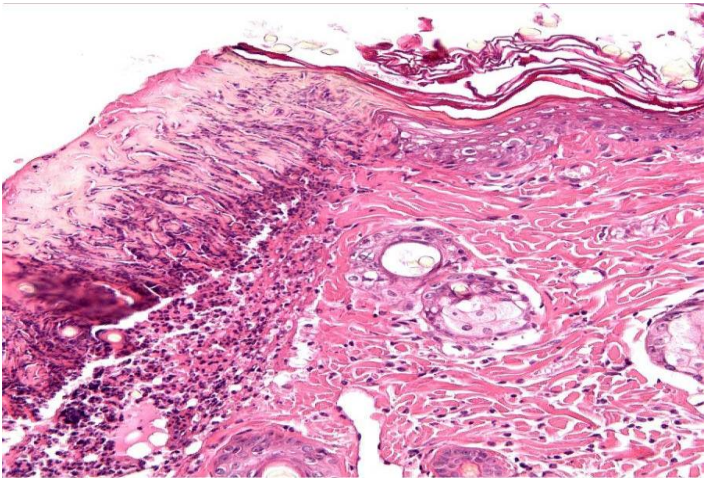

Figure 4. HE stain or rat wounded skin, 1 day after surgery.The defect was covered by a pseudomembrane. Fibrin exsudate and defense cells are observed. At 3 days after surgery, the pseudomembrane is still present and it will be possible to observe the epithelial neoformation begining to migrate accross the incisional gap moving from along wound edge. Magnification x10.

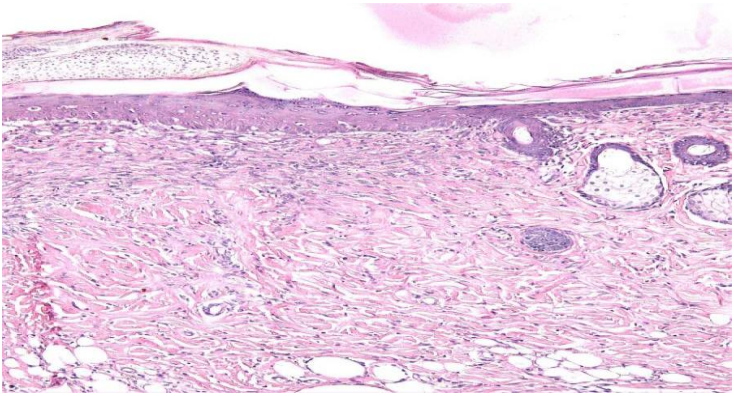

Figure 5. HE stain 7 days after surgery. Observe the collagen fibrils organization on the wounded. The arrangement and orientation of the cells is different from the non wounded area, and most of the healing area is covered by the new epithelium. Inflammatory cell infiltration is less remarkable. Magnification 10x.

Some interesting studies (Tarlton et al., 1997; Loots et al., 1998; Salmon-Ehr et al., 2000) have shown that the inflammatory response is a necessary and important stage of wound healing, and it is also assumed that an abnormal inflammatory response may have profound effects on the resolution of the repair process. Iinhibition of the repair process might also facilitate wound infection.

## CONTROL OF WOUND HEALING

After injury with tissue disruption, wheter surgical or traumatic, the major priorities of any organism are cessation of hemorrhage, prevention of infection and restoration of tissue integrity and function. These objectives represents the wound healing process.

The control of the wound healing process is not completely known. Tissue repair proceeds as an ordered sequence of events controlled by interactions between different cell types (blood and inflammatory cells, endothelial cells, fibroblasts, and keratinocytes), their interactions with the extracellular matrix, and it is also dependent on the expression of many different soluble mediators that either stimulate or inhibit the process (Salmon-Ehr et al., 2000).

Tissue injury leads to platelet degranulation. The liberation of biochemical signals lead not only to the clot formation but also the accumulation of a number of chemoattractants to the site of wounding, and inflammatory

reaction. Whiting 6 hours after injury, circulating immune cells start to be visible in the wounded area. Polymorphonuclear leukocytes are the first blood leukocytes to enter the wound site. They appear shortly after injury, and their numbers increase reaching the peak at around 24-48 hours, then tend to decrease rapidly after the third day after injury if bacterial contamination has not ocurred. Their main function seems to be phagocytosis of bacteria and other foreingn materials introduced into the wound during injury, and it was observed that healing proceeds normaly even in the absence of neutrophils, in the absence of infection.

The next cellular immune element to enter the wound area are macrophages. They are derived from circulating monocytes, and seems to appear within 48-96 hours after injury, reaching peak numbers around the third day and persist in the wound area until bealing is completed. Like neutrophils, macrophages phagocytosis and digest pathological organisms and tissue debris, but also secrete and release many different biologically active substances with different functions in the healing process. Activated macrophages are capable of influencing many aspects of wound healing, and cytokines and growth factors released by macrophages acts on proliferative and synthetic activities of fibroblasts, induction of neovascularization and to epitelial neoformation (Diegelman et al., 1981; Barbul and Regan, 1995).

T lymphocytes, the third immune cell to enter the wound area seems to be present in significant numbers around the day 5 after injury, reach peak numbers around the day 7, and as well as macrophages, seems to have an important regulatory paper in the control of the healing process. Lymphocytes are responsible for secretion of many important cytokines and growth factors.

Fibroblasts within the wound healing are the major synthetic element, responsible for the production and reorganization of the structural proteins required for the repair. It seems like these cells first appear in significant numbers in the wound area on the third day after injury and reach peak numbers around the seventh day. Fibroblast are attracted into the wound area, migrate and proliferate induced by cytokines and growth factors present in the area. These cells are mostly responsible for the production of proteins, mostly extracellular matrix constituents such as fibronectin, hialuronic acid, glycosaminoglycans and collagen, but they are also closely related to the other cells and molecules in the wounded area, and also express cytokines and growth factors.

Fibroblasts present changing in phenotypes during the wound healing, and the more remarkable change is the presence of myofibroblasts. Myofibroblasts are present in certain normal tissues, in healing wounds and in fibrocontractive

disease. Wound contrations take place when granulation tissue is populated with myofibroblasts, characterized by the expression of α-smooth muscla actin (α-SMA), the actin isoform typical of vascular smooth muscle cells. The factors regulating the transition between one fibroblast phenotyping to another is not completely identified, but TGF-β is accept as the major growth factor involved in α-SMA expression and myofibroblast differentiation (Hinz et al., 2001).

About the second day after injury, endothelial cells from the side of the wound begin to migrate in response to angiogenic stimuli, and revascularization proceeds in parallel with fibroplasia. Also, while these events are proceeding deep in the wound, restoration of epithelial integrity is taking place at the wound surface. Reepithelialization of the wound begins within hours after the injury, but may be clearly observed only after the second day from injury.

The last and longest phase of wound healing is the maturation period, when the new tissue is reorganized, wound contraction occur, and the normal anatomical and functional characteristics of the new tissue restored.

Many studies showed that cytokines and growth factors produced by inflammatory and structural cells are capable of regulating the healing process. Extracellular fluid and wound cells recovered from normal wounds at different times after wounding may present expressive differences in phenotype and action. In the literature, it is possible to observe hundreds of cytokines and growth factors already identified, characterized and classified into families and superfamilies on the basis of structural homologies. These soluble factors are numerous in the wound healing, but their role in the process are not completely known. The more frequently studied are: the platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), basic- and acidic fibroblast growth factors (FGFs), vascular-endothelial growth factor (VEGF), transforming growth factors –α and β (TGFs), and epidermal growth factor (EGF). Some other proteins have may also be important in the healing process, such as the hepatocyte growth factor (HGF), and conective tissue growth factor (CTGF) (Barbul and Regan, 1995).

## CYTOKINES AND GROWTH FACTORS

Cytokines and growth factors are a group of polypeptides that affect a wide variety of biological systems through binding to specific high-affinity

cell membrane receptor, generally transmembrane glycoproteins, belonging to either the receptor tyrosine kinase (RTK) or G-Protein-coupled receptor (GPCR) family. They are different from hormones, which are basically carried in the circulation, and from pheromones, which are dispersed in the air, and their action may be through intracrine (growth factor/receptor complex is internalized), autocrine (when affect the own cell), juxtacrine (when affect adjoining cells), paracrine (when affect nearby cells), or endocrine (target cells is distant) mechanisms (Lee, 2000).

The described scenario for growth factors action seems to be: a) growth factor binds to its cell surface receptor; b) becouse of the binding, a redistribution or an association with other membrane proteins may occur; c) activated growth factor receptor activates intracellular substrats; and d) as a result, there is an activation exist a reorganization of the cytoskeleton. The growth factor receptors in cellular membrane present an extracellular and an intracellular domains, and their unities are responsible for ligand binding and signal transduction. The multiplicity of growth factors in various tissues, the varying cell type specificity, or target, for the growth factors, and the requirement for multiple growth factors for stimulation of specific cell types provide the fine tuning of the control in different processes (Goustin et al., 1986; Falanga, 1993; Klenkler and Sheardown, 2004).

Healing process include the migration and proliferation of inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes, synthesis of extracellular matrix components and a sophisticated interaction between these elements (Matsuda et al., 1998). Growth factors expression and suppresion in the course of wound healing may reflects the presence of a well controlled mechanism that regulates tissue regeneration and remodeling. Studies suggested that adjacent cells, near the site of injury, and the new proliferating cells are the major source of growth factors during wound healing (Antoniades et al., 1993).

## PLATELET-DERIVED GROWTH FACTOR (PDGF)

Platelet-derived growth factors (PDGFs) are a family of glycoprotein dimers (27-35 kDa) that exert potent mitogenic and chemotactic activities toward cells of mesenchymal origin. PDGF was first identified as the major mitogen released from the α-granules of activated platelets. Four PDGF polypeptide subunities, designated PDGF-A, PDGF-B, PDGF-C and PDGF-D have been identified. They share considerable amino acid identity, but are

encoded by separate genes, located on different chromosomes. These subunities dimerize via disulfide linkages to form the homodimers PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and the heterodimer PDGF-AB (Kaetzel, 2003).

PDGF, and PDGF-like peptides are released not only from platelets, but also from other connective tissue and epithelial cells, however diferently. For example, it seems like smooth muscle cells and fibroblast synthesize only the PDGF-A chain, whereas endothelial cells and macrophages synthesize PDGF-A and PDGF-B chains.

PDGFs are major mitogens for connective tissue cells, and are involved in different biological processes, such as embryonic development, cellular differentiation, response to tissue damage, tumorigenese and atherosclerosis. Constitutive expressison of PDGFs is rarely observed, except during embrionic development, but its release occur under stimulation, such as during inflammation, and mostly associated to other cytokines and growth factors. PDGF-B chain products seems to be helpful wound repair for diabetic ulcers, and studies are been conducted to use them for the treatment of impaired wound healing. Topically applied, recombinant human PDGF is a new pharmacologically active therapy for chronic neuropathic, lower extremity diabetic ulcers (Falanga, 1993; Lee, 2000; Takehara, 2000; Kaetzel, 2003).

Dermal fibroblasts were considered the major target cells of PDGFs, but these growth factors are now also very clearly associated to angiogenesis. PDGF-AA and PDGF-BB seems to act as promoters of angiogenesis when associated to other angiogenic factors, such as bFGF and VEGF, and the angiogenic mechanism and therapeutic potential of PDGF-CC was only recently characterized. It seems like PDGF-CC mobilize progenitor cell in ischemic conditions, induce diferentiation of bone marrow cells into endothelial cells, and smooth muscle cells, stimulate the growth of these smooth muscle cells during vessel sprouting and stimulate migration of the neoformed endothelial cells promoting neovasculatization in ischemic sites (Li et al., 2005).

## EPIDERMAL GROWTH FACTOR (EGF)

Epidermal growth factor (EGF) is a compact 6kDa, ≅53 amino acid polypeptide, potent mitogen for epithelial cells. It binds to both low affinity and high affinity sites on cells expressing the EGF-receptor (EFGR), which is also a receptor for EGF-related cytokines such as transforming growth factor-

α (TNF-α, a ≅50 amino acids polypeptide). Ligand binding EGFR activates RTK activity, leading to DNA synthesis, production of extracellular matrix molecules, cellular proliferation, cytoskeletal rearrangement and cell motility. In normal adult epidermis, EGFR is predominantly expressed in basal keratinocytes, and the signalling events related to this receptors are known to affect epithelial cells proliferation, differentiation and migration. In healing skin wound, EGFR expression is upregulated producing migrating and proliferating keratinocytes adjacent to the wound (Haase et al., 2003).

EGF seems to upregulates expression and activation of different integrins, enhances degradation and neoformation of hemidesmossomos, acting in epithelial cell migration as well as epithelial cells proliferation after injury. It is one of the number growth factors investigated for their potential to improve wound healing (Lee, 2000; Klenkler and Sheardown, 2004).

## INSULIN-LIKE GROWTH FACTOR-I (IGF-I)

Insulin-like growth factor-I (IGF-I) is a peptide with structural homology to proinsulin. The major source of circulating IGF-I in postnatal life is the liver, bur IGF-I can also be produced by many other tissues, where it is thought to act in a paracrine fashion. In skin, IGF-I is produced by dermal fibroblasts and macrophages, is thought to signal to basal keratinocytes that express IGF-I receptors as well as regulates keratinocytes shape by stimulating plasma membrane protrusion and spreading, and also stimulate proliferation and to contribute to hair follicle morphogenesis. The action of IGF-I seems to result in acceletarion of wound neoepithelialization. (Haase et al., 2003).

Two known receptors, present on most cell types, recognize the IGFs. IGFR1 a hetertetremeric tyrosine kinase, homologous to the insulin receptor, nas been shown to mediate the effects of both IGF-I and IGF-II; and the IGFR-2, structurally distict from IGFR-1 which role is not completely elucidate. However, the bioactivity of IGFs is not only dependent on their interactions wth their receptors. It has been shown that they requeres the presence of another protein to have any effect. IGFs actions are modulated bu proteins by proteins found in pericellular space, called IGF-binding proteins (IGFBPs). The six members of the IGFBP family have become increasingly appreciated as powerful determinants of IFG-I bioactivity (Galiano et al., 1996; Firth and Baxter, 2002).

## TRANSFORMING GROWTH FACTOR BETA (TGF-β)

The term transforming growth factor (TGF) has been used for peptides with capacity to induce untransformed cells in transformed cell phenotypes. That means, these cells loss the density-dependent inhibition of growth in monolayer, they overgrowth in monolayer, acquire characteristic changes in cellular morphology, and also an anchorage indenpendence with make them capable to growth in soft agar. However, although the TGFs were discovered in conditioned medium of virally transformed neoplastic cells, they were later found in almost all tissues, neoplastic and nonneoplastic, from all animal species. It is now observed that transformed phenotype is a physiological state associated with functional normal functions, such as development and tissue repair, and transforming (or *onc*) genes have been found in normal cells of vertebrates (Roberts et al., 1983).

TGF-βs exist in a number of structurally related but functionally distinct isoforms. The initial described form of TGF-β was as a 25 kDa (25.000 molecular weight) dissulfide-linked homodimer (composed of two identical polypeptide chains) of 112 amino acids each, and it was called sarcoma growth factor. It apparently can stimulate or arrest differentiated cellular functions, and the nature of its action on a particular target cell is dependent on many parameters, including cell type, state of differentiation, growth conditions and the presence or the absence of other peptid growth factors. It was observed their capacity for, mostly, to inhibit epithelial, endothelial and leukocyte cell growth, and to stimulate fibroblast proliferation. Becouse of these characteristics, TGF-β may be considered, rather than a growth promoter, a multifunctional growth regulator (Roberts et al., 1983; Goustin et al., 1986; Sporn and Roberts, 1991; Yang et al., 1999).

In mammals, three isoforms, TGF-β1, -2, and -3 have been identified, each one synthesized as a large precursor that has to be lost for activation. TGFs-ß are secreted associated to LAP (TGF-ß latency-associated protein). LAP makes the protein unable to bind to its receptors until dissociated. Proteases are thought to be the most common mechanism of TGF-β activation in vivo, but the activation of the latent TGF-β can happened by a variety of situations such as extreme pH, high temperature, and presence of enzymes other proteases (Blanchette et al., 1997; Clarck et al., 1997; Ehrhart et al., 1997; Taipale and Keski-Oja, 1997; Yang et al., 1999).

TGF-β1 is one of the most abundant TGF-βs mammalian isoforms and has been detected by immunohistichemistry both intra- and extra-cellularly but the

intensity of satining varies between tissues and cell types. TGF-β1 expression seems to be an important part of the wound healing process (Pittelkow et al., 1988; Stavenezer, 1995). TGF-β1 helps the formation of granulation tissue, stimulates chemotaxis, proliferation and activation of fibroblast. TGF-β1 also stimulates the synthesis of extra cellular matrix components (Pittelkow et al., 1988; Fukamizu and Grinnell, 1990; Locci et al., 1995; Clarck et al., 1997; Diegelmen, 1997; Loots et al., 1998; Okuda et al., 1998; Tipton and Dabbous, 1998; Yang et al., 1999).

TGF-β as a protein seems to be produced by a number of cell types involved in wound repair, including platelets, macrophages, fibroblasts, endothelial cells, and keratinocytes. Essentially all cells have funtional receptors for TGF-β (Pittelkow et al., 1988; Clarck et al., 1997; Taipale and Keski-Oja, 1997; Gao et al., 1998; Yang et al., 1999).

During the process of repair, even since 1 hour after injury, has been described a remarkably intense expression of TGF-β1 in the subepithelial area at the injuried site, and in later phases of wound healing TGF-β1 expression seems to decrease significantly. In general, TGF-β shows great promise for use in the therapy of poorly healing wound, however, has been observed some deleterius effects of TGF-β when administered in excess, and it requires the development of a very well controlled delivery system when trying to use this growth factor as a therapy (Pittelkow et al., 1988; Bennet and Schultz, 1993; Yang et al., 1999; Lee, 2000).

## FIBROBLAST GROWTH FACTORS (FGFS)

Fibroblast growth factors (FGFs), proteins of approximately ≅18 kDa, are a major growth factor family involved in angiogenesis, directing endothelial cell migration and proliferation. They are classified also as heparin-binding growth factors (HBGFs) or heparin-binding angiogenic factors (HBAFs) (Baird et al., 1987; Folkman et al., 1988).

Initially, two forms of FGF were identified, one with an acidic isoeletric point (aFGF, 16 kDa) and the other with a basic isoeletric point (bFGF, 17 kDa), with 53% total homology in their primary structure. After this classification, seven additional FGFs were identified and the family has gained a new nomenclature. Acidic FDF (aFGF) is also FGF1, basic FGF is FGF2, and the new forms are FGF-3 through FGF9. bFGF, is the protopypic member of the FGF family, and studies indicate a sequence of 146 animo acids

molecule (Baird et al., 1987; Werner et al., 1992; El-Hariry et al., 1997; Mutsaers et al., 1997; Nguyen and D'Amore, 2001).

Studies have shown that FGFs are expressed in normal skin, and induced upon injury. Have also been established that the growth factor is distributed in many different cells and tissues, however, the immunoreactivity of the FGFs is aways 80-95% cell associated. The demonstration that FGFs are bound to heparin-related glycosaminoglycans in the extracellular matrix can explain how these proteins did not show a clear signal when outside the cells (Baird and Ling, 1987; Folkman et al., 1988; Werner et al., 1992; Qu et al., 1995; Murata et al., 1997).

This protein can be stored in the extracellular matrix in a stable, and probably inactive form, due to its affinity for heparin-like molecules, being inaccessible to the target cells under physiologic conditions. This storage also provides a mechanism for the regulation of capillaries, so platelets and macrophages may release the growth factor content upon activation and degrranulation, and endothelial cells may release FGF upon blood vessel damage (Mutsaers et al., 1997; Bashkin et al., 1989; Cronauer et al., 1999).

The heparan sulfate degradation seems to be a major enzymatic step in the breakdown of the extracellular matrix (ECM), and possibly an important way for the release of stored FGFs. The importance of matrix solubilization and degradation preceding the release of FGFs, and the growth factor availability for biological funtions has been already established. bFGF has also been detected in saliva (Baird and Ling, 1987; Cronauer et al., 1999; Oda et al., 2004).

It is believed that FGFs effect cell adhesion and induce proliferative response on cells (vascular endothelial cells, smooth muscle cells, adrenocortical cells, condrocytes and neuroectodermal cells), also act as mitogens for keratinocytes and fibroblasts. They may have also important functions on differentiation of keratinocytes, and promote endothelial tubule formation, significantly enhancing vascularization and re-epithelialization in wound healing. Recent studies have shown that bFGF induces the expression of another growth factor, the vascular endothelial cell growth factor (VEGF). Different systems, and various carriers have been investigated, including fibrin scaffold for the delivey of FGF-1, and for *in vivo* release of bFGF from biodegradagle gelatin hydrogel carrier, trying to use this growth factor in the treatment of impaired wound healing (Baird et al., 1987; Baird and Ling, 1987; Broadley et al., 1989; Gonzalez et al., 1990; Mutsaers et al., 1997; Cronauer et al., 1999; Lee, 2000).

## KERATINOCYTE GROWTH FACTOR (KGF)

Keratinocyte growth factor (KGF) a member of the FGF family, consists of a single polypeptide chain of approximately 28 kDa not physiologically expressed in keratinocytes, but produced during epidermal repair. It is widely expressed in stromal cell types and it is mitogenic for keratinocytes in particular and for epithelial cells in general. Epithelial cells express the KGF receptor and are the main target for this growth factor.

In addition to its direct effect on tpiehtlial cells, KGF induces TGF-α expression and EGFR signaling in exposed tissues. It was also demonstred that KGF signal transduction in keratinocytes increases cell proliferation and differentiation. The characteristic to bind heparin leads to its deposition in the extracellular matrix where storage and release to target cells is regulated. Initial clinical studies on topical application of growth factor preparations to accelerate wound healing processes presented controversial results (Atabai et al., 2002; Kopp et al., 2004).

## VASCULAR-ENDOTHELIAL GROWTH FACTOR (VEGF)

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) is a potent multifunctional growth factor that exerts several important actions on vascular endothelium. It is a highly conserved, disulfide-bonded dimeric glycoprotein, 35-45 kDa, which, upon reduction, loses all of its biological activities. VEGF shares low but significant sequence homology with platelet-derived growth factor (PDGF) and close homology with placenta growth factor (Jakeman et al., 1989; Dvorak et al., 1995).

Four different VEGFs transcripts encoding polypeptides of 206, 189, 165 and 121 amino acids may be expressed. It appears that smaller isoforms (121 and 165 amino acids isoforms) are secreted in soluble form, whereas the larger (189 and 206 amino acids isoforms) remain cell-associated (Jakeman et al., 1989; Dvorak et al., 1995).

VEGF has been aassociated to angiogenesis in numerous situations, mostly pathological, such as tumor growth, proliferative retinopaty and rheumatoid arthritis, but also in tissue repair. It seems like to be secreted by fibroblasts, macrophages, and keratinocytes, and act directly and selectively on vascular endothelial cells, inducing transient accumulation of cytoplamic calcium, shape changes, cell division, cell migration, and degradation of

extracellular matrix components. It acts mostly by two Class III receptors tyrosine kinases, flt-1, and KDR, that are expressed predominantly, if not exclusively, on vascular endothelium (Ballaun et al., 1995; Dvorak et al., 1995; Nissen et al., 1998; Bouloumié et al., 1999).

VEGF was originally discovered becouse its ability to increase the permeability of microvessels, and actually acts as one of the most potent vascular permeabilizing agents, even more potent than histamine, helping the transendothelial transport of macromolecules (Dvorak et al., 1995).

## HEPATOCYTE GROWTH FACTOR (HGF)

Hepatocyte growth factor (HGF) is a glycoprotein of approximately 90 kDa with sequence similar to plasminogen. It is tipically synthesized by cells of mesenchimal origin as an inactive precursor, which is activated to a heterodimer by injury, inflammation, or by proteases of the coagulation cascade. Similar to KGF, it binds to various extracellular matrix cmoponents. The high-affinity HGF receptor, c-Met, is a tyrosine kinase, proto-oncogene product, found mainly on epithelial cells.

In addition to its mitogenic effect on hepatocytes, HGF binding leads to proliferation, motility and shape change in a range of epithelial, endothelial and melanocytic cells, and causes individual epithelial cells to adoppt a fibroblast-like phenotype. While the roles of HGF and KGF are a little different in epidermal physiology, both similarly stimulate proliferation and migration of human keratinocytes; HGF stimulates plasminogen activator activity of normal humam keratinocytes and are greatly related to the regeneration of the epithelium. HGF also stimulates the fibroblasts to produce matrix metalloprotease-1 (MMP-1) and inhibit the expression of TGF-β, preventing the hypertrophic scar formation (Okazaki et al., 2002; Klenkler and Sheardown, 2004).

## CONNECTIVE TISSUE GROWTH FACTOR (CTGF)

The connective tissue growth factor (CTGF) was identified originally from cultured human umbilical vein endoothelial cells. This factor seems to be also produced by skin fibroblasts, but only when they are stimulaed with TGF-β. CTGF is recognized by anti-PDGF polyclonal antibody and CTGF-cDNA

was cloned using anti-PDGF antibody. This factor exhibits PDGF-like chemotactic and mitogenic activities, and binds to PDGF receptors (Takehara, 2000).

Another way to look at the wound healing process is to observe specific activation of intracellular signaling pathway. The classical mitogen activated protein kinase cascade (MAPK or ERK) is a modulo of three protein kinases acting in a hierarchical order. The MAPKs (ERKs) are activated by a KAPK kinase (MEK-1) through phophorylation of conserved threonines and tyrosines within a TXY motif; the MAPKK kinase (Raf) reculate MEK-1 activity, and the signals stimulating the activity of the MAPK cascade are mediated by many different growth factors, cytokines and cell-matrix and cell-cell adhesion molecules. Another important kinase involved in growth factor mediated signal transduction is phosphatidylinositol-3-kinase (PI-3K). As a lipid kinase, this enzyme phosphorylates the inositol ring, which is thought to organize the interactions of signaling molecules spatially, thereby facilitating the formation of signalling complexes. PI-3K has been implicated in the regulation of cell proliferation, transformation, protein trafficking, actin cytoskeletal organization and apoptosis.

Although the effects of individual growth factors are been studied in wound healing process, the signaling mechanisms by which these factors exert their functions or the interactions between the signaling pathways are not completely understood yet.

Wound healing is a complex event, regulated by an interplay of mechanisms involving different cells, cytokines, growth factors and signaling molecules. The biological significance of each factor has to be very well studied and understood.

Studies in our laboratories, so far have shown that:

1. Recovering in body weight is not aways followed by recovering in cellular or nuclear levels, showing long-term sequelae, mostly in early undernutrition on epithelial cells (Cavenaghi et al., 2000);
2. Inflammatory cells such as neutrophils, lymphocytes and activated macrophages were observed in small numbers on wounded areas of undernourished/recovered animals (Cavenaghi et al., 2001);
3. In spite of recovering the body weight, undernourished/recovered animals did not showed recovered capacity for producing growth factors in the wounded areas – such as bFGF (Basic Fibroblast Growth Factor) which leads to the supposition that cells are not recovered at a molecular level (Komesu et al., 2003).

Epidemiological studies revealed that protein-calorie malnutrition is a widespread problem among infants and young children of developing countries, and in poverty areas of the industrialized nations. Severe forms of malnutrition can be easily detected, but milder degrees of malnutrition can be misinterpreted. However, a moderate degree of undernutrition is more common among children, generally associated with negligence and carelessness from the parents (Reichnhein and Hasselmann, 1977). In these studies we had a moderate degree of undernutrition.

It is generally accepted that individuals have variable cellular predisposition to respond to injury. When the reasons for this variability are not completely understood, it can be, many times, explained as "background" or the "gene expressions". Nevertheless, the specifical mechanisms of failure of the wound healing process are still unknown.

Wound healing is a complex process involving multiple cellular and extracellular components, all of them necessarily present in a normal healing process, such as inflammatory cells, fibroblasts, keratinocytes and their products (cytokines, growth factors and other proteins), and these cells seems to modulate the reconstruction of the injured area. Alterations in inflammatory cell infiltration and activation might also mean altered expression of growth factors in the wounded area, as demonstrated in experimental studies (Punch and Ivinski, 1977).

There are indications that undernutrition at early life stages produces lymphoid tissue atrophy (Frank et al., 1996), and the results obtained in our studies provided further support for the hypothesis that cellular deficiencies are associated with impaired wound healing. This subject deserves further research, but so far, our results support the hypothesis that early malnutrition has late effects on the wound healing process, even after physical recovering from undernutrition.



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*Chapter 8*

# EFFECTS OF PROTEIN DEPRIVATION ON THE TEMPOROMANDIBULAR JOINT

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## ABSTRACT

The impact of malnutrition on oral cavity, especially of protein deprivation, is considered an important morbidity that significantly decreases the quality of life. Poor protein intake predisposes the oral mucosa to various diseases and affects the dental structure. Recently, the effects of protein deprivation on jaw bones, including the temporomandibular joint (TMJ), have been discussed mainly due the high frequency of protein malnutrition in hospitalized and immunosuppressed patients, as well as in patients with chronic diseases. In the case of bone fracture, these patients frequently exhibit poor bone repair, and this situation may be present in fractured TMJ. In this chapter, we discuss the effects of protein deprivation on fracture repair of TMJ, including the role of low concentrations of proteins and vitamins in the delay of repair in TMJ structures. Some mechanisms of malnutrition on TMJ bone and cartilage cells are emphasized, as well as on hematopoietic marrow of mandibular condyle and temporal bone, and on the TMJ masticatory muscles. Additionally we comment the effects of malnutrition on the TMJ growth, including the problems of malnutrition on infancy that involves the jaw bones. The themes discussed in this chapter have been analyzed by our research group using experimental models in animals with TMJ fracture and protein deprivation. We observe that an ingestion

of hypoprotein and isocaloric diet provokes delay on TMJ repair, impairment on bone cells in some periods of repair process, serum biochemical alterations, mainly in albumin and calcium, and muscle atrophy after the consolidation of TMJ fracture. We also detect poor formation of cartilage tissue and, in some cases, important anatomic alteration of TMJ, such as disappearance of articular disc, malformation of head condyle, and pseudoarthrosis. These alterations are extensively described in this chapter, focusing the most susceptible structures of TMJ when they are exposed to malnutrition. As conclusion, we alert the surgeons in general and the dental professionals in particular with respect to the importance of malnutrition diagnosis during the treatment of TMJ trauma.

## 1. INTRODUCTION

The impact of undernutrition on the stomatognathic system is poorly understood. There are few studies focused on the effects of low-protein diet and hypo caloric diet on the physiology of the oral structures. It is known that the oral mucosa may exhibit the systemic conditions of undernutrition at an early stage [1], and that the deficiencies of some vitamins and proteins can predispose the teeth, salivary glands, jaw bones, and oral mucosa to pathological conditions. Table 1 presents the most important of these conditions in the oral cavity caused by vitamin and mineral deficiencies.

Recently, the effects of protein deprivation on jaw bones, including the temporomandibular joint (TMJ), have been discussed mainly due the high frequency of protein malnutrition in hospitalized and immunosuppressed patients and those with chronic diseases. These patients frequently exhibit poor bone repair probably as a consequence of nutritional imbalance. Additionally there is a global concern as regards the risk of bone fractures, not only in the elderly, but also among children of poor Western countries due dietary deficiencies.

The TMJ is one of the structures of the stomatognathic system responsible for masticatory movements. Our studies have evidenced that the TMJ is affected by nutritional deficiencies that compromise the individual at a high level and result in permanent sequelae [2].

In this chapter, the effects of protein deprivation on TMJ fracture repair are discussed, including the role of low concentrations of proteins and vitamins in the delay of this repair. Some of the effects of malnutrition on TMJ bone and cartilage cells are emphasized, as well as those on the hematopoietic

marrow of mandibular condyle and temporal bone, TMJ masticatory muscles, body mass and blood constituents during TMJ repair. The effects of malnutrition on the TMJ growth are also mentioned.

**Table 1. Main alterations in the oral cavity caused by vitamin and mineral deficiencies**

| Oral structure | Clinical sign | Nutritional deficiency (↓) | |
|---|---|---|---|
| | | Vitamins | Minerals |
| Teeth | Dental caries | $B_3$ | Fluoride, iron |
| | Enamel pigmentation and pitting | | Fluoride (↑) |
| | Tooth loss | C | Calcium |
| | Periodontal disease | D | Calcium |
| | Impairment of tooth development | A | |
| | Disturbances in odontogenesis | D | Calcium, magnesium |
| Oral mucosa | | | |
| | Mucous membrane edema | $B_2$ | |
| | Angular cheilitis | $B_2$, $B_6$, $B_9$, A (C) | Iron |
| | Stomatitis | $B_2$, $B_9$+$B_{12}$+iron | Iron |
| | Glossitis, atrophic burning tongue | $B_2$, $B_3$, $B_6$, $B_9$, $B_{12}$ | Iron, zinc |
| | Decrease in ability to taste | | Zinc |
| | Burning mouth | $B_3$, $B_9$ | |
| | Gingivitis (bleeding, swallowing, erythema) | $B_3$, $B_6$, C, A (↑) | |
| | Xerostomia | A | Zinc |
| | Impaired healing | A(↑), C | Zinc |
| | Pathological pigmentation | A (↑) (orange pigmentation) | |
| | Oral cancer | E | |
| | Predisposition to oral infection | | Iron, Zinc |
| Jaw bone | Resorption and decrease in bone mineral density | D, K | Calcium, phosphorus |
| | Low organic matrix deposition | Vitamin C | Zinc, magnesium |
| | Inhibition of craniofacial growth | Moderate to intense undernutrition | |

↓ Deficiencies due to lower ingestion. ↑ Deficiencies due to higher ingestion.

## 2. General Effects of Undernutrition on the Repair Process

Undernutrition, especially the type derived from protein deprivation, can cause severe alterations in the repair process [3]. Practically all the repair phases are affected by undernutrition [4], mainly the inflammatory and proliferation phases. The inflammatory phase consists of inflammatory cell recruitment and macrophage differentiation in the tissue, as well as vasodilatation and plasmatic exudation. The proliferation phase includes granulation tissue formation and tissue stroma reorganization, facilitating cell migration and differentiation. In this phase the original tissue architecture is reconstructed by well-differentiated cells. Both phases are linked to cytokine and growth factor release mainly by fibroblasts, macrophages, and endothelial cells.

There is consensus that undernutrition causes immunosuppression in various degrees and in different segments of the immunological system [5,6,7,8]. Table 2 shows some of the effects that interfere in the repair process. One of the significant alterations is the impairment of hematopoiesis [8], which causes hematological disturbances and lower lymphocyte and neutrophil differentiation. Cell-mediated immune responses are also affected by undernutrition. Studies have demonstrated that undernutrition interferes with macrophages, diminishing their capacity for phagocytosis and inducing apoptosis in these cells [9]. This is a crucial alteration for the immune system since a large part of cell signaling is dependent on macrophage activities. Especially in the repair process, macrophage inhibition implies severe reduction in the production of cytokines and growth factors that are essential to the tissue regeneration [10]. Humoral immunity is also reduced due to lower immunoglobulin secretion, which exposes the tissue to secondary infection [11,12]. As a result of this scenario of immunosuppression, the inflammatory phase of the repair process is more prolonged; the proliferation phase is delayed, and the remodeling phase, which is responsible for the recovery of tissue mechanical properties, is compromised [3].

As regards growth factor and cytokine levels, protein undernutrition has a complex effect. The levels of some growth factors and cytokines can be reduced or increased according to amino acid, lipid, antioxidants, and vitamin imbalance [13]. For instance, vitamin D is essential for production of fibroblast growth factor 23 (FGF-23), keratinocyte growth factor (KGF), nerve growth factor (NGF), and transforming growth factor b (TFG-b), but inhibits

the insulin-like growth factor I-II (IGF-I-II). Regarding pro and anti inflammatory cytokines, the vitamin D inhibits the expression of IL-2, IL-4, and IL-6. Therefore, the imbalance of this vitamin can predispose to impairment in growth factor production and to increase on interleukins expression [14].

**Table 2. Influence of nutritional disturbances on different segments of repair process**

| Immune system | Effects |
|---|---|
| Hematopoiesis | Impairment of cell proliferation |
| | Low number of pluripotent cells |
| | Pancytopenia |
| | Alteration in the microenvironment of bone marrow, with degeneration of extracellular matrix |
| | Atrophy of medullar space |
| | Presence of anemia and leukopenia |
| Cell-mediated immunity | Atrophy of the thymus |
| | Low number of T and B lymphocytes |
| | Impairment of action of Natural Killer cells (NK cells) |
| | Reduction in phagocytosis by macrophages |
| | Increase of apoptosis in macrophages |
| | Susceptibility to infection |
| | Susceptibility to autoimmune diseases |
| Humoral immunity | Low production of immunoglobulins |
| | Susceptibility to recurrent and chronic infections |
| Innate immunity | High epithelial cell apoptopic indexes in superficial tissues (mucosa and skin) |
| | Reduction of the corneum layer |
| Cytokines and growth factors | Increase or decrease depending upon the nutrient imbalance |
| Collagenous secretion, deposition and maturation | Low collagenous synthesis |
| | Low fibroblast proliferation |
| Angiogenesis | Reduction in microvascular branches |
| | Impairment of vascular endothelial growth factor (VEGF) |
| | Induction of endothelial cell apoptosis |

A low protein diet may also inhibit angiogenesis. Studies on rat maternal food restriction during pregnancy have demonstrated that neonatal offspring exhibit marked structural alterations in blood vessels, such as extensive vessel

remodeling due high concentrations of matrix metalloproteinases, reduction in microvascular branches, impairment of vascular endothelial growth factor (VEGF), and induction of endothelial cell apoptosis [15]. As the repair process covers the embryological steps of tissue formation, angiogenesis may probably also be reduced in malnourished adult individuals.

## 2.1. Particularities for Bone, Muscle, and Cartilage in Joint Fracture

There are profound effects of protein and/or caloric undernutrition on the bone, muscle, and cartilage. Experimental studies using partial or total diet restriction in adult animals have indicated that acute diet restriction (for about 4 days) reduces aerobic metabolism in skeletal muscles [16]. Chronic protein restriction causes reduction in the size of skeletal muscle fibers and focal degeneration in myofibrils, morphological signs that indicate muscle atrophy [17]. In young animals, the nutritional deficiencies cause reduction in size and number of muscle cells, causing permanent or prolonged block in animal growth [18]. To compensate the low levels of amino acids during protein deprivation, muscle proteins are hydrolyzed for production of free amino acids [19]. Under protein deprivation, it was shown that some skeletal muscles distant to wound healing sites exhibit a deficit in protein mass, but the site with repair process maintain high protein levels [20]. As the muscles are the main source of proteins in the case of deficient diet, healing in muscles may be negatively affected by protein undernutrition.

Poor protein diets exert great influence on the quality of bone fracture healing. Clinical and experimental studies have demonstrated that there is a reduction in bone diameter after fracture consolidation under low protein intake, as well as lower BMC (bone mineral content) [21]. In addition, protein restriction inhibits osteoblast activity and osteoid deposition, as well as increasing calcium loss from bone. The latter effect may due to a decrease in total-body calcium retention and intestinal calcium absorption. Bone remodeling is also harmed by osteoclast activity inhibition. The absence of essential amino acids contributes directly to impairment of bone matrix deposition in fracture healing. For example, lysine is essential for the formation of hydroxyapatite in bone matrix during endochondral ossification. Hydrolysine plus lysine are fundamental to enabling collagen to form intermolecular links. During fracture healing, these amino acids are recruited from other sites of body. It is unknown how long this process continues and if

these amino acids are incorporated into bone callus, but there is consensus that the diet must supply this necessity and its absence leads to important consequences [21]. In addition to the effects on the quantity of bone formation, the poor protein diet causes a decrease in the biomechanical properties of new-formed bone. Torsional strength of bone callus seems significantly more reduced under low levels of proteins than under a mineral-deficient diet, indicating an important dependence on adequate protein levels for good bone healing [22].

Joint cartilage is affected by undernutrition mainly with respect to its growth. Growth inhibition during malnourished infancy is reflected by an atrophy of skeletal growth centers, which frequently contain hyaline cartilage that may be significantly affected by protein deprivation. Articular cartilages present an extracellular matrix enriched by soluble proteins that are substituted by bone matrix and mineralization by endochondral ossification during growth. The maintenance of cartilaginous matrix is dependent upon protein synthesis by chondrocytes and chondroblasts. Chondrocytes produce vesicles containing metalloproteinases (collagenases) that dissolve proteoglycans located in the extracellular matrix. This event is crucial for bone tissue mineralization and formation. The enzymes production by chondrocytes is vitamin D-dependent. Rackitic rats exhibit hyperplasia of growth cartilage zones, indicating inhibition of extracellular matrix dissolution [23]. Other nutritional deficiencies in addition to protein deprivation can interfere in joint function. For example, vitamin C deficiency causes athralgia and arthritis in the knees, ankles, and wrists. Bleeding and loss of joint space are also present, indicating persistent malfunction secondary to vitamin imbalance [24].

The majority of studies observing the impact of undernutrition on fracture healing are focused on femoral fracture. A large portion of these studies includes protein or caloric restriction or both as models of undernutrition. The major indicators of undernutrition in these models are loss of body weight and low levels of serum albumin. This induced undernutrition causes a decrease in the injured muscle mass and muscle protein content, and a reduction in bone callus mineral density. As an overall result, studies have affirmed that there is delay in fracture healing and harm to bone and muscle physiology [25]. On the other hand, it seems that there is a compensation of protein loss evidenced by a high production of growth factors during fracture healing, mainly of insulin-like growth factor (IGF) and endothelial growth factor (EGF). This increase in the production of growth factors suggests that there is an increase in gene transcription during protein deprivation [26].

Supplementation with an anabolic diet has been studied, especially in older animal models with bone fracture. Glutamine, arginine, cysteine, histidine, proline, taurine, and tyrosine are considered essential aminoacids and are very important for tissue healing. In these models it has been observed that there is better recovery of muscle mass and protein content in the fractured bone and formation of bone callus with a high concentration of minerals [27]. Therefore clinical studies have recommended diet supplementation for patients with bone fracture and malnutrition, but this has not been applied to TMJ fractures (see item 6).

## 3. ANATOMY OF THE TEMPOROMANDIBULAR JOINT (TMJ)

The TMJ is a sinovial joint that plays an important role in masticatory movements. It is responsible for the articulation between the head of the mandible and the articular fossa of the temporal bone. The joint is covered with a thick fibrous capsule and has an articular disc interposed between the articular surface of the mandibular condyle and the articular fossa. The articular disc is composed of an upper and lower collagenous lamina and has an exuberant nervous and venous plexus in its posterior portion. This disc is directly attached to the temporal fossa, mandibular condyle, and to the masseter and lateral pterigoid muscle fibers, in the posterior, anterior, and lateral portions respectively [28]. The articular capsule contains an outer fibrous layer and inner synovial membrane that produces the synovial fluid. This fluid is essential for mandibular condyle movement with a high degree of freedom and smoothness. It invades the cartilage of the mandibular condyle surface, also promoting growth of the condyle during craniofacial development [29]. The condyle cartilage is considered a growth center due to endochondral ossification that is present until the human adolescent phase. Various muscles are connected to the TMJ bones and disc, such as the lateral and medial pterigoid muscle and masseter muscle fibers. Contraction of these muscles is responsible for mandibular condyle displacement into the articular fossa during masticatory movements. There is rich vascular irrigation in the TMJ, mainly in the muscle stroma, lateral and posterior border of the articular disc, and in the articular capsule [28]. Under normal conditions, the tissues of the TMJ are renewed periodically, mainly in the mandibular bone, which exhibits constant remodeling. Because of this, fracture healing in the TMJ is

dependent upon the maintenance of mandibular bone vitality and proper vascular irrigation.

## 4. THE PHYSIOLOGY OF FRACTURE HEALING IN THE TMJ UNDER NORMAL NUTRITIONAL CONDITIONS

During TMJ bone healing, some phases of TMJ embryology are recruited, such as endochondral ossification of the condyle and intramembranous ossification of the mandibular process [2,30,31]. Table 3 summarizes this process through the description of microscopic findings observed during TMJ repair in fractures induced in animals. The first phenomenon present in fractured condyle healing is the invasion of inflammatory cells surrounding the bone fracture and an expressive vascular hyperemia and edema. In general, fractures in the TMJ lead to medial dislocation of the mandibular condyle, which in turn causes muscle compression with posterior muscle fiber necrosis. Therefore, the inflammatory cells present in the first hours of healing are signaled for both bone and muscle structures. In eutrophic patients, the necrosis indexes are moderated in this phase. The proliferation phase of TMJ healing includes the formation of an exuberant granulation tissue in the fracture region and muscle insertions, with a mixture of mesenchymal cells, osteoprogenitor cells, satellite cells in the muscle, and chondroblast cells. Under normal conditions, these cells already have an anatomical distribution coherent with the correct formation of different tissues (connective tissue in the muscle stroma, muscle fibers, bone tissue in the condyle and mandibular ramus, and cartilaginous tissue in the condyle head). The next step is bone callus formation that begins at about 7 days. In this period, it is possible to observe a mixture of bone and cartilaginous tissue in the middle of the callus. There is constant bone matrix deposition and remodeling up to 30 days, when the two bone fragments are consolidated. The peak of remodeling is at about 90 days, and may be continuous for several months. Clinically a good fracture healing result in the TMJ is the correct position of the mandibular condyle in the articular fossa, replacement of the articular disc, and the re-establishment of jaw movements without symptoms.

**Table 3. Microscopic findings of the different healing phases of TMJ fracture and alterations in these processes due nutritional deficiencies resulting from protein deprivation.**

| Healing phase | Normal nutritional conditions | Abnormal nutritional conditions (protein deprivation) |
|---|---|---|
| 24h | Inflammatory phase: presence of neutrophils and lymphocytes, plasmatic exudation, necrosis, and hyperemia surrounding the bone fracture; mandibular condyle dislocation, and low frequency of bone necrosis | Inflammatory phase: minor inflammatory cell invasion, high indexes of necrosis both in bone and muscle, low intensity of hyperemia, mandibular condyle dislocation |
| 7 days | Initial of proliferation phase: granulation tissue formation surrounding the bone fracture, mesenchymal cell proliferation in bone, muscle, and connective tissue, high angiogenesis, new bone deposition | Mixture of inflammatory and proliferation phase: persistent inflammatory exudation, high indexes of muscle necrosis, presence of bone sequestrum, immature granulation tissue with low angiogenesis |
| 15 days | Peak of proliferation phase: visible bone callus in the fracture region, initial bone union, presence of cartilaginous tissue in the callus, initial muscle insertion, initial reposition of articular disc and condyle in the articular fossa | Mixture of inflammatory and proliferation phase: lymphocyte infiltrate, muscle necrosis, beginning of bone deposition interposed with cartilaginous tissue, presence of granulation tissue in the fracture region |
| 30 days | Remodeling phase: Total bone callus formation, complete bone union, initial remodeling in bone and muscle | Proliferation phase: Initial muscle regeneration, initial bone callus formation, absence or few dislocation of disc and mandibular condyle into articular fossa, high deposition of collagenous fibers in the necrosis region |
| 90 days | Remodeling phase: Bone callus resorption, total repositioning of mandibular condyle and articular disc in the temporal fossa, complete muscle insertion | Mixture of proliferation and remodeling phase: signs of bone callus in the fracture region, absence of correct repositioning of the articular disc and the mandibular condyle, partial muscle reinsertion, high deposition of fibrous tissue in the articular space (pseudoarthrosis) |

Microscopic characteristics derived from the animal models [2,30,31].

# 5. Effects of Protein Deprivation in the Healing of Fractured TMJ Observed in Experimental Studies

## 5.1. General Aspect

Healing in the fractured temporomandibular joint involves the repair of multiple tissues and depends upon a sequence of orchestrated events. Not only the bone, but cartilaginous tissue, muscles, nerves, and vascular network suffer rupture and degeneration which must be repaired.

As regards the muscles, in the majority of condylar fractures there is massive rupture of muscle fibers, mainly of the masseter, temporal, and lateral pterigoid. This injury may contribute to the long duration of the repair process at the site, since muscle regeneration is dependent upon stable satellite cells with low differentiation in comparison with osteoprogenitor cells. In addition to the muscles, the condylar cartilage may also be compromised by several factors, many of them related to rapid condylar dislocation secondary to the fracture. This dislocation may determine poor irrigation in the condylar head which may affect the chondroblast and chondrocyte cells due low nutrient diffusion. Articular cartilage nutrition depends on the synovial fluid and vascular irrigation in the bone. Condylar dislocation may cause disruption of the articular capsule affecting the release of synovial fluid to the cartilage cells. The blood flow is also interrupted due to rupture of the vascular network creating an ischemic environment that allows profound effects on bone and cartilage cells.

In our experimental studies, we have demonstrated that bone, cartilage, muscles, and even hematopoietic bone marrow are profoundly affected by undernutrition [2]. Poor healing observed in this situation resulted from innumerous and concomitant faults in these tissues. As was described above, Table 3 summarizes the tissue reactions during temporomandibular joint fracture repair both in well-nourished and malnourished individuals submitted to low protein intake.

At 24h, the main difference between the well- and malnourished individual is the degree of inflammatory cell recruitment to the fracture site. In protein deprivation, there is discrete invasion of inflammatory cells and prominence of necrosis in the tissues. The necrosis becomes massive at 7 days and involves mainly the muscle tissue. This fact may explain the delay in repair events that occurs under abnormal nutritional conditions. The necrosis

must be phagocyted by macrophages through direct and indirect signaling by lymphocytes. Protein deprivation causes impairment of the immune system and increase in macrophage apoptosis (Table 2), which results in a delay in necrosis absorption. Moreover, at 7 days, complete formation and initial maturation of granulation tissue is important. In malnourished individuals, the granulation tissue is scarce and shows low angiogenesis. The impact of these alterations is evident in the following phases. At 15 days, in well-nourished individuals, there is a peak of the proliferation phase. By contrast, malnourished individuals exhibit significant indexes of tissue necrosis interposed by immature granulation tissue and new-formed bone and cartilaginous matrix. The proliferation phase reaches its peak only at 30 days in malnourished individuals, and inadequate replacement of the condyle and articular disc is evident in the temporal fossa. Furthermore, in this phase the necrotic tissue is replaced by a fibrous tissue and the muscle fibers exhibit partial regeneration without reinsertion. Finally, after 3 months, there may be incomplete bone union, muscle reinsertion may be insufficient to support the mandibular movements, and pseudoarthrosis may be present. In this case, joint function is fully compromised.

The causes of these asynchronous events culminating in a delay in the repair process are poorly understood. We have attempted to explain the effects of protein deprivation in fracture healing by describing the hematological and serum alterations observed in malnourished individuals with TMJ fracture, as well as its effects on hematopoietic, bone and cartilaginous cells, and on muscle fibers.

## 5.2. Body Mass Index and Level of Blood Constituents as Markers of Undernutrition

The body mass and food ingestion, as well as hematological and some serum indexes have been observed in our experimental studies in order to monitor nutritional conditions during fracture healing. We have observed that in these models, the main indicators of nutritional status are body mass and serum albumin levels. We also have monitored the serum alkaline phosphatase and serum calcium levels as basic serum markers of bone physiology.

During TMJ fracture healing in eutrophic individuals, there is a significant decrease in body mass and serum albumin levels due low food intake, particularly at 15 days after fracture. The animals are in a state of mild malnutrition in this period, but exhibit a rapid recovery at 30 days, when there

is expressive gain in body mass and serum albumin. On the other hand, the fractured animals under protein deprivation exhibit negative body mass gain throughout the entire experiment and show the lowest total serum proteins and serum albumin values. In addition, these animals had different oscillations in serum alkaline phosphatase. In eutrophic animals, this protein peak is at 30 days, which is coincident with the peak of bone callus formation [32]. The malnourished animals presented high levels of alkaline phosphatase at 7 days, which may be related to poor callus formation in these animals. Serum calcium is also fully altered by fracture and malnutrition. We have observed significantly low serum calcium levels in malnourished individuals during fracture healing, indicating the profound influence of protein deprivation on calcium metabolism. Studies have shown that low protein intake decreases the levels of vitamin D transport and/ or calcium binding proteins [33] and reduces the total-body calcium retention. Additionally, there is an increase in calcium loss from bone, as described above (item 2.1), setting up a cycle of bone resorption and low calcemia that culminates in poor bone healing.

## 5.3. Effects on Hematopoietic Bone Marrow

Hypocaloric nutrition may interfere directly in hematopoietic bone marrow function since causes hypocellularity, necrosis, and extracellular matrix modifications [34,35]. These alterations may reflect the blood cell impairment observed in malnourished individuals, such as decreased erythrocyte and leukocyte counts [36]. A large part of the immunosuppression present in undernutrition may originate from a reduction in hematopoietic cell differentiation in bone marrow. Experimental studies in adult and young animals under caloric restriction have shown morphological alterations in femur and sternum bone marrow, such as impairment of myeloid cell differentiation [37], presence of basophilic and gelatinous extracellular matrix [38], loss of local micro architecture, and ineffective erythropoiesis [39].

In TMJ embryogenesis, the mandibular and temporal bones, as well as the majority of craniofacial bones present hematopoietic bone marrow throughout their entire extension [40]. In animals, this tissue is active until the young adult age and slowly suffers atrophy and bone replacement. During the course of TMJ fracture, hematopoietic tissue participates actively in bone regeneration, particularly with regard to osteoblast and osteoclast differentiation. Osteoclast precursors, i.e., monocytic cells, are present in this tissue and are stimulated to osteoclastic differentiation mainly through growth factor secretion by

osteoblasts. This stimulation depends on the proximity of precursor cells to osteoprogenitor cells, which is related to harmonious bone marrow microarchictecture. Therefore the maintenance of cellularity and extracellular matrix in hematopoietic tissue is crucial for bone regeneration. In a fractured condyle, hematopoietic bone marrow is present in all the phases of bone regeneration. Within the first hours after fracture, there is ischemic necrosis in the hematopoietic tissue close to the fracture line in the mandibular ramus and throughout the entire extension of the condylar fragment. At the peak of callus formation, the hematopoietic tissue is exuberant and exhibits high cellularity occupying the majority of the new-formed bone marrow spaces. Angiogenesis, extracellular fibril production, and initial extracellular matrix network organization are evident. In the remodeling phase, the number of hematopoietic cells is reduced to normal proportions of various cell types, which are organized in clusters and niches typically seen in hematopoietic bone marrow present in the mandibles of young adults [41].

In animals under protein restriction, there is no hematopoietic tissue recovery in both the mandibular condyle and temporal bone. Permanence of basophilic degeneration in the extracellular matrix, low number of hematopoietic cells, and loss of ratio between cell types indicate poor regeneration of this tissue. These morphological signs are seen with high intensity in the temporal bone, although this bone does not suffer fracture in the described animal models. The basic causes of this result may be undernutrition in addition to the absence of joint function due to poor bone consolidation and erroneous repositioning of articular disc and condylar head in the temporal fossa. As there is an intrinsic relationship between bone cell and hematopoietic cell function, the absence of joint function mainly at articular surface level probably reflects negatively in the hematopoietic tissue [41].

The function of hematopoietic tissue present in the TMJ may be restricted to local homeostasis, since blood cell production is dependant on the hematopoietic cells of the long bones and sternum. However these morphological alterations observed in the craniofacial complex indicate the strong susceptibility of hematopoietic structures to nutritional oscillations and constitutes an important factor that must be considered in patients with nutritional disorders.

## 5.4. Effects on Masticatory Muscles

The effects of protein deprivation on masticatory muscles have not yet been elucidated. It is well known that during undernutrition, skeletal muscle proteins are responsible for maintaining the protein ratio in the individual, supplying the body protein demand [19]. Studies have shown that there is no reduction in protein synthesis in the muscles in undernutrition, indicating an evident participation of this tissue in protein homeostasis. During healing, a large portion of the proteins recruited for tissue reconstruction originates from the muscles. As muscles have to supply the body with proteins under abnormal nutritional conditions, healing is this tissue may be harmed. Indeed, a profound effect of protein deprivation on the masticatory muscles of the TMJ has been observed, similar to that described above in Topic 2.1. In addition to the massive necrosis normally present in cases of condylar fracture, malnourished individuals exhibit a significant delay in muscle regeneration, with frequent cicatrization and atrophy in this tissue. As muscle insertion is crucial for bone remodeling and even matrix bone deposition, this has great impact both on bone fracture consolidation and TMJ function [2].

## 5.5. Orthognathic Alterations

In order to investigate the facial symmetry after experimental mandibular condyle fracture and with protein undernutrition (8% of protein), experimental studies have been carried out by our group using cephalometric measurements. The main results indicate an important deviation of the median line of the mandible relative to the median line of the maxilla in malnourished individuals, as well as asymmetry of the maxilla and mandible, in special at the final period of experiment. This asymmetry is characterized mainly by small length both of the maxilla and the mandible on the same side of the fracture. It may be caused by a compensation of the masticatory function through a neuromuscular mechanism [31] as the protein deprivation decreases the bone callus formation and leads unsatisfactory mandibular condyle repositioning. There is also prejudice on the dental occlusal intercuspation, which promotes serious masticatory dysfunctions [42].

## 6. NUTRITIONAL CONDITIONS OF PATIENTS WITH TMJ FRACTURE

Trauma and surgical stress produce a catabolic state that can result in a net loss of body protein with excessive nitrogen excretion [25]. These conditions are followed by a period of low food consumption, whereas the body demands more metabolic fuels. Hospitalized patients with severe bone fractures frequently exhibited undernutrition. Other studies have demonstrated that patients with open fractures of the lower limbs showed some nutrient deficiency symptoms, such as low serum albumin levels and low serum transferrin levels [43]. These indicators are important for the clinical detection of nutritional deficiencies, but there is no consensus that they indicate a worse prognosis for wound healing.

Temporomandibular joint fracture is considered a severe trauma with high catabolism during the first 15 days. Jaw movements are frequently compromised during the first week of bone healing, which determine changes in diet and/or adoption of a soft or liquid diet [44]. Various complications may occur during and after fracture consolidation, such as hypomobility, ankylosis, pain syndrome, malocclusion, and chronic dislocation of the condyle [45]. In addition, TMJ or other mandibular fractures frequently demand surgical intervention, with internal fixation or immobilization. All these conditions interfere in mandibular movements and mastication. Therefore nutritional deficiencies may be present and may interfere in fracture consolidation.

Apart from the nutritional deficiencies derived directly from TMJ fracture, some may be inherent to patients at risk for this fracture. Epidemiological studies have indicated that the main cause of mandibular fractures is automobile and motorcycle accidents, followed by physical aggression. Frequently patients were under the influence of alcohol and exhibited other facial fractures, which incremented the catabolic state. Persons of adult age and men are the most affected, but a significant percentage of patients are older, with high chances of nutritional disorders. Children are particularly affected, especially those from low socioeconomic levels in under-developed countries. In this age group, malnutrition is frequently present [45].

There is no confirmation that alcoholism, being older or at the age of childhood, accompanied by undernutrition, or multiple fractures in the face can predispose the fractured patient to poor healing. Clinical studies involving mandibular fractures have not been focused on the nutritional status. However some concern has been shown in studies with regard to maintaining the

patient's nutritional status by prescribing a balanced soft or liquid diet up to the time of surgical wound healing. There are some past reports that have described the adoption of enteral feeding by nasogastric tube in hospitalized patients with mandibular fractures [46]. The adverse effects of this artificial feeding, such as esophageal and gastric irritation and vomiting, are so intense that its indication is not recommended at present.

## 7. CRANIOFACIAL GROWTH AND UNDERNUTRITION

Actually there is consensus that the bone mass of adult individual depends upon the peak obtained during skeletal growth. Epidemiological studies have indicated that poor growth during fetal life, infancy, and childhood is associated with decreased bone mass in adulthood and an increased risk of fracture [21].

Bone growth in the postnatal period depends upon mesenchymal stem cell differentiation into cells of the osteogenic, adipogenic, fibroblastic, and reticular lineages. Recent studies have indicated that maternal protein deprivation causes delay in bone cell differentiation that occurs in her offspring. There is a reduction in colony-forming unit-fibroblasts (CFU-F) and alkaline phosphastase-positive CFU-F, as well as chronological alteration in osteoblast proliferation indexes. Moreover, the CFU-F responsiveness to the growth hormone (GH) and insulin-like growth hormone (IGF-1) is impaired under low protein circumstances [47]. This may indicate that maternal nutritional imbalance affects the intrauterine programming of growth, which may arise from endocrine system alterations.

Some structures of the temporomandibular joint are responsible for the growth of the inferior region of the cranium during craniofacial development. Mandible genesis and growth depends upon the ossification of mesenchymal tissue formed in the neighborhood of Meckel's cartilage. This intramembranous ossification gives origin to a large part of the mandibular body. Mandibular processes are derived from a secondary cartilage present in the posterior region of the mandibular body. This is a mass of cartilage that is rapidly converted to bone by endochondral ossification. Only a thin layer of this cartilage is maintained in the condylar head forming the articular surface of this bone in the temporomandibular joint. This region constitutes a growth center for future long growth of the mandibular processes, in a manner similar to that of the epiphysis cartilage in long bones.

The craniofacial skeleton is very susceptible to nutritional variations and to epigenetic factors. There is a lack of detailed knowledge as regards the individual outcomes in the craniofacial skeleton, derived from undernutrition. A number of studies have shown that there is inhibition of craniofacial growth, with more intensity in the viscerocranium than the neurocranium. The viscerocranium contains bones that recruit more amino acids during growth, and this recruitment is preserved even under protein deprivation for a certain time [48]. In addition, it seems that craniofacial growth alterations are perpetuated in the next generations and contribute to descendants being small-sized adults [49].

An experimental study on a hard and soft calcium- and vitamin D-deficient diet for growing rats showed that the animals exhibited less bone mass in premaxilla and nasal bones, and premature obliteration of nasal sutures [50]. In addition, animals submitted to hard and soft-deficient diets exhibited upwards rotation of the viscerocranium (orthocranialization), causing severe masticatory disturbances. Low masticatory function also contributed to craniofacial abnormalities [51].

In jaw bones, maternal protein deprivation causes specific alterations in endochondral and intramembranous ossification in the mandibular condyle. Experimental studies have shown that there is atrophy of proliferative and hypertrophic zones of the mandibular condyle in rat fetuses during maternal low protein intake. In general, low prechrondoblast and chrondoblast densities in the mandibular condyle are observed in 21 days postnatal pups, as well as a drastic reduction in the amount of proteoglycans in the extracellular matrix. As an overall result, rat pups with a history of maternal undernutrition, exhibited atrophy in the mandibular growth center, which probably compromises craniofacial growth [52]. Another study showed that rachitic rats had calcium-free unmodified proteoglycans in the mandibular condylar cartilage, which prevents matrix ossification [23].

The effect of protein deprivation on mandibular functional units is not uniform, and some deformation may occur as a result of proportional alterations. In an experimental study with neonatal rats nursed by dams put on a protein-free diet to depress milk production and thus create a state of protein-energy malnutrition in the offspring, the mandibles exhibited impairment of growth, which persisted throughout the entire experiment. These findings suggest that catch-up growth was incomplete in rats malnourished during the suckling period and that mandibular bone architecture adaptation to body growth was apparently insufficient to attain normal values [53]. An ascorbic acid-deficient diet also promotes impairment of craniofacial growth in

growing rats and reduces the periodontal ligament space width, but the jaw bone density is not affected [54]. In the comparison of mandibular growth with femur growth in growing rats under protein deprivation, the femur was more strongly affected than the mandible. Severe impairment of femur length was observed under suboptimal nutritional conditions, differently from mandibular size alterations, which were observed only under hard-deficient diet during a specific period of time [55].

## CONCLUSION

Undernutrition derived from protein deficiency exerts important effects on the TMJ, both in its repair and growth processes. A constellation of malnutrition effects can be present in TMJ repair and growth due the diversity of TMJ structures with regard to tissue origin and function. These differences must be considered during clinical management of TMJ disorders, in order to avoid or attenuate the consequences of nutritional imbalance on these tissues. Finally, more comprehensive studies involving nutritional status and TMJ dysfunction must be conducted.

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