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Biochemical mechanisms involved in pulmonary hypo-alveolarization induced by peroxides contaminating parenteral nutrition in newborn guinea pig

par

Wesam Elremaly

Département de Nutrition

Faculté de médecine

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Cette thèse intitulée:

Mécanismes biochimiques impliqués dans l'hypo alvéolarisation pulmonaire induite par des peroxydes générés dans la nutrition parentérale chez le cochon d'inde nouveau-né

Présentée par :
Wesam Elremaly

a été évaluée par un jury composé des personnes suivantes :

Dr Lise Coderre, président-rapporteur
Dr Jean Claude Lavoie, directeur de recherche
Dr Nathalie Grandvaux, membre du jury
Dr Jennifer Landry, examinateur externe
Dr Guy Lemay, représentant du doyen de la FESB

Résumé

La dysplasie broncho-pulmonaire (DBP), caractérisée par un défaut de l'alvéolarisation, est une complication pathologique associée à un stress oxydant chez le nouveau-né prématuré. La DBP est présente chez près de 50 % des nouveau-nés de moins de 29 semaines de gestation. La nutrition parentérale (NP) que ces nouveau-nés reçoivent pour cause d'immaturité gastro-intestinale est une source importante de stress oxydant. En effet, leur NP est contaminée par des peroxydes, dont l'ascorbylperoxyde qui est une forme peroxydée du déshydroascorbate. La génération des peroxydes est catalysée par la lumière ambiante. La photoprotection de la NP, quoique difficile d'application en clinique, est associée à une diminution de l'incidence de la DBP chez les enfants prématurés. Chez l'animal nouveau-né, la photoprotection de la NP est associée à un meilleur développement alvéolaire. Ainsi, nous émettons l'hypothèse que l'ascorbylperoxide infusé avec la NP cause la perte d'alvéoles suite à une apoptose exagérée induite par l'oxydation du potentiel redox du glutathion. Cette oxydation du potentiel redox serait occasionnée par l'inhibition de la transformation hépatique de la méthionine en cystéine, menant à une diminution de la synthèse de glutathion au foie et dans les tissus tels que les poumons. La confirmation de cette hypothèse suggérera qu'un ajout de glutathion dans la NP permettra une meilleure détoxification de l'ascorbylperoxide par l'action de la glutathion peroxydase, et préviendra l'oxydation du potentiel redox et ainsi, la perte d'alvéoles par apoptose.

Objectifs : Le but de mon projet de recherche est de comprendre les mécanismes biochimiques liant la NP et le développement de la DBP chez le nouveau-né prématuré et de proposer une alternative nutritionnelle prévenant le développement de cette complication fréquemment observée dans cette population. Les objectifs spécifiques sont : 1) d'évaluer l'impact, au poumon, de l'infusion de l'ascorbylperoxyde sur l'axe métabolique potentiel redox du glutathion - apoptose - le développement alvéolaire; 2) d'étudier l'impact de l'ascorbylperoxyde et du potentiel redox sur l'activité hépatique de la méthionine adénosyltransférase (MAT), première enzyme de la cascade métabolique transformant la méthionine en cystéine; et 3) de tenter de prévenir l'impact négatif de la NP ou de l'infusion d'ascorbylperoxyde sur le poumon en améliorant le statut en glutathion.

Méthodes: Par un cathéter fixé dans la jugulaire, des cochons d'Inde de trois jours de vie (n = 8 par groupe) ont reçu en continu durant 4 jours une NP ou une solution de base (dextrose + NaCl) enrichie des différentes molécules à l'essai. Le premier objectif a été atteint en enrichissant la solution de base en ascorbylperoxyde à 0, 20, 60 et 180 μM. Ces solutions contenaient ou non 350 μM H_2O_2 pour se rapprocher des conditions cliniques. Le second objectif a été atteint en investiguant les mécanismes d'inhibition de la MAT dans des animaux infusés ou non avec des solutions contenant la solution de base, des peroxydes, du glutathion et la NP (dextrose + acides aminés + multivitamines + lipides). Le troisième objectif a été atteint en ajoutant ou non à une solution d'ascorbylperoxide ou à la NP 10 μM de glutathion (GSSG), afin d'obtenir une concentration plasmatique normale de glutathion. Après 4 jours, les poumons étaient prélevés et traités pour la détermination de GSH et GSSG par électrophorèse capillaire, le potentiel redox était calculé selon l'équation de Nernst et le niveau de caspase-3 actif (marqueur d'apoptose) par Western blot et l'index d'alvéolarisation quantifié par le nombre d'interceptes entre des structures histologiques et une droite calibrée. Les données étaient comparées par ANOVA, les effets étaient considérés comme significatifs si le p était inférieur à 0,05.

Résultats: L'infusion de l'ascorbylperoxyde, indépendamment du H_2O_2, a induit une hypoalvéolarisation, une activation de la caspase-3 et une oxydation du potentiel redox de manière dose-dépendante. Ces effets ont été empêchés par l'ajout de GSSG à la NP ou à la solution d'ascorbylperoxyde (180 μM). L'ascorbylperoxyde et le H_2O_2 ont inhibé l'activité de MAT tandis qu'elle était linéairement modulée par la valeur du potentiel redox hépatique.

Conclusion : Nos résultats suggèrent que l'ascorbylperoxyde est l'agent actif de la NP conduisant au développement de la DBP. Ainsi la correction des bas niveaux de glutathion induits par les peroxydes de la NP favorise la détoxification des peroxydes et la correction du potentiel redox pulmonaire ; ce qui a protégé les poumons des effets délétères de la NP en outrepassant l'inhibition de la MAT hépatique. Nos résultats sont d'une grande importance car ils donnent de l'espoir pour une prévention possible de la DBP.

Mots-clés : dysplasie broncho-pulmonaire, nouveau-nés prématurés, stress oxydant, alvéolarisation, nutrition parentérale, ascorbylperoxyde.

Abstract

Bronchopulmonary dysplasia (BPD) is a major complication of preterm newborns, affecting nearly 50% of infants born before 29 weeks of gestation. BPD is characterized by an arrest in alveolar development. The onset of BPD is related to oxidative stress. Research has shown that parenteral nutrition (PN), which is given to preterm newborns to bypass an immature gastrointestinal system, is a major source of oxidative stress. Indeed, PN is contaminated with peroxides, including ascorbylperoxide, an oxidized form of dehydroascorbic acid. Ambient light is a catalyst for the generation of peroxides. Photo-protection of PN, although difficult to apply in the clinical situation, is associated with a lower incidence of BPD in premature infants and with better alveolar outcomes in animal models of neonatal PN. We hypothesized that the ascorbylperoxide in PN disrupts alveolar development. The main mechanism of action is an inhibition of the transformation of methionine into cysteine in the liver, leading to a lower glutathione synthesis in the liver as well as in peripheral tissues such as lung. Lower glutathione (GSH) concentrations favour a shift of redox potential to a more oxidized state and consequently, to exaggerated apoptosis. If our hypothesis is correct, the addition of glutathione to PN would help detoxify ascorbylperoxide through the action of glutathione peroxidase and prevent the deleterious impact of PN.

Objectives: The aims of my research project were to investigate the biochemical mechanisms linking PN to the development of BPD in premature newborns and to propose a nutritional alternative that would prevent the occurrence of this frequently observed complication. Specific objectives were: 1) to assess the effect of intravenously infused ascorbylperoxide on the metabolic axis redox potential of glutathione in the lung; specifically, apoptosis and the alveolarization index; 2) to study the impact of ascorbylperoxide and the redox potential on the activity of methionine adenosyltransferase (MAT) in the liver; methionine adenosyltransferase is the first enzyme in the metabolic cascade from methionine to cysteine; and 3) to try to prevent the deleterious impact of PN or ascorbylperoxide infusions on the lung by improving glutathione status.

Methods: Through a catheter in the jugular vein, 3-day-old guinea pigs (n = 8 per group) received continuous infusions of PN or a simple solution (dextrose + NaCl) enriched with

different molecules for testing. The first objective was achieved by enriching the basic solution with ascorbylperoxide at concentrations of 0, 20, 60 and 180 μM. To mimic clinical conditions, these solutions contained, or not, 350 μM H_2O_2. The second objective was achieved by investigating the mechanisms of MAT inhibition in animals infused, or not, with solutions consisting of the basic solution, peroxides, glutathione, and PN (dextrose + amino acids + multivitamins + lipids). The third objective was achieved by adding, or not, 10 μM of glutathione (GSSG) to the ascorbylperoxide or PN solution until a normal plasma concentration of glutathione was obtained. After 4 days, the lungs were removed. GSH and GSSG levels in the lungs were determined by capillary electrophoresis. The redox potential was calculated using the Nernst equation. The activation and the concentration of active caspase-3 (marker of apoptosis) were determined by Western blot, and the alveolarization index quantified by the number of intercepts between histological structures and a calibrated straight line. Data were compared by ANOVA; effects were considered significant if *p* was less than 0.05.

Results: The infusion of ascorbylperoxide, independently of H_2O_2, induced hypoalveolarization, activation of caspase-3, and oxidation of the redox potential, in a dose-dependent manner. These effects were prevented by the addition of GSSG to the ascorbylperoxide (180 μM) or PN solutions. Ascorbylperoxide and H_2O_2 inhibited MAT activity in the liver. Hepatic MAT activity was linearly modulated by the value of the redox potential.

Conclusion: Our results suggest that ascorbylperoxide is the active ingredient in PN that leads to the development of BPD. Correcting the low glutathione levels induced by peroxides in PN solutions would promote the detoxification of peroxides and re-establish proper pulmonary redox potentials. Glutathione correction further protects the lungs from the deleterious effects of PN by bypassing hepatic MAT inhibition. This result is of great importance because it gives hope for the possible prevention of BPD.

Keywords: bronchopulmonary dysplasia, preterm newborns, oxidative stress, alveolarization, parenteral nutrition and ascorbylperoxide.

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APaf-1	Apoptotic protease – activating factor 1
ASCOOH	Ascorbylperoxide
BPD	Bronchopulmonary dysplasia
DHA	Dehydroascorbate
ELBW	Extremely low birth weight
FiO_2	Fractions of inspired oxygen
GA	Gestational age
γ GCL	Gamma glutamate cysteine ligase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione Reduced form
GSSG	Glutathione Disulfide form
H_2O_2	Hydrogen peroxide
Km	Kinetics constant
$LOO^{\cdot}$	Peroxide radical
LOOH	Hydroperoxyl radical
MAT	Methionine adenosyltransferase (EC 2.5.1.6)
MRP3	Multidrug resistance protein
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduce Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Erythroid 2- related factor 2

$O_2^{\bullet-}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
PaO_2	Partial pressure of oxygen
PMA	Postmenstrual age
PN	Parenteral nutrition
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
SAH	S- adenosyl homocysteine
SAM	S- adenosyl methionine
Se	Selenium
SOD	Superoxide dismutase
TGF-1	Transforming growth factor type 1
TNF1	Tumor necrosis factor receptor 1
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight

I dedicate this work to my family:

To my father's soul

To my mother

To my husband, my son and my daughter

To my brothers and my sister

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1. Introduction

In Canada, the number of extremely preterm babies (≤ 28 weeks' gestation) is increasing (1% of births). The incidence of several complications associated with prematurity is high; for example, bronchopulmonary dysplasia (BPD).[1] BPD is characterized by an arrest in alveolar development that makes the newborn dependent on oxygen supplementation until at least 36 weeks' postmenstrual age (PMA) . [2] In Canada, BPD affects one extremely premature infant (< 28 weeks' gestation) out of two. Consequences of BPD are many; for instance, lung function impairment during childhood, chronic lung disease, and impairment of neurodevelopment. For a long time, it was thought that oxygen supplementation was the root cause of the oxidative stress that underlies the etiology of BPD. More recently, it has been suggested that the administration of parenteral nutrition (PN) to bypass the immature gastrointestinal track of these infants might play an important role in BPD development. Indeed, light exposure to PN induces the production of peroxides such as ascorbylperoxide and hydrogen peroxide (H_2O_2) and increases the risk of oxidative stress. These peroxides are suspected to have a deleterious effect on the lung and to induce BPD. The aims of this research are to understand the mechanistic processes linking PN to BPD development, in order to optimize nutritional support of preterm infants without exposing these vulnerable children to any additional burden.

To better understand the issues raised by PN, I will review the main considerations in the following sections: 1.1 prematurity, 1.2 nutritional requirements for premature infants and parenteral nutrition, 1.3 lung development, 1.4 apoptosis and lung development, 1.5 bronchopulmonary dysplasia, 1.6 oxidative stress, and 1.7 premature antioxidant defense system.

1.1 Prematurity

According to the world health organization (WHO), preterm is defined as babies born alive before 37 weeks of pregnancy are completed. There are sub-categories of preterm birth, based on gestational age:

Moderate to late preterm (32 to <37 weeks): infants born between 32 and 37 weeks of gestation. This category is the most likely to survive with supportive care.

Very preterm (28 to <32 weeks): infants born between 28 and 32 weeks of gestation. These babies need supportive care.

Extremely preterm (<28 weeks): infants born before 28 weeks of gestation. This is the category with the highest mortality rate. These very small babies, if they survive, may require extensive and expensive intensive care [3, 4]

There is another definition for premature infants based on birth weight, as shown in Table 1. [5,6]

Table 1. Prematurity types based on birth weight.

Birth weight	Definition
1500 to 2500 g	Low birth weight
1000 to 1500 g	Very low birth weight
to1000 g	Extremely low birth weight

1.1.1 Incidence and cause of preterm birth

The preterm birth rate worldwide is about 11%, and about 15 million babies are born preterm each year. Of these preterm births, 84% occur between 32 - 36 weeks of gestational age (GA), 10% occur at 28 to < 32 weeks GA and 5% occur at < 28 weeks GA [7]. In 2010–2011, the preterm birth rate in Canada was 7.9%. This rate has remained stable since 2006–2007 (8.1%). Preterm births increased in developed countries in association with the number of older women having babies and multiple pregnancies.[8] However, in developing countries, preterm births increased due to high adolescent pregnancy rates as well as infections such as malaria. Preterm birth occurs primarily for three reasons: 1) through spontaneous delivery due to early labor or premature rupture of the placental membranes; 2) as an early delivery when the mother has health problems such as preeclampsia; or 3) through calculation error in due dates. [8]

Events leading to preterm birth are not well understood. The etiology is deemed to be multifactorial. The risk factors for preterm deliveries are medical conditions of the mother (obesity, diabetes, hypertension, smoking, infection, maternal age), genetics, environmental exposure, socioeconomic factors and multiple birth [9]. Prematurity has a great impact on the child's health, family and society. In fact, preterm birth is associated with highly costly intensive care services which would be challenging to deal with in poor countries.

The incidence of pathological complications associated with prematurity is inversely proportional to gestational age at birth; for example, BPD, a major complication of preterm birth, is characterized by reduced alveolar development requiring long-term respiratory support due to lung immaturity. [10, 11]

For this reason, BPD is considered a developmental disease with arrested lung development. In Section 1.4, I will describe the stages in lung development during

pregnancy and after birth. Further, I will show at which stages of lung development premature birth occurs. BPD is at the heart of my PhD work; see section 1.3.

1. 2 Nutritional requirements for premature infants

During intrauterine growth, the fetus gets its nutrients from the mother. Unfortunately, preterm birth perturbs the delivery of these nutrients [12]. The inadequate nutrition during the early postnatal period is associated with an increased incidence of complications of preterm birth, such as abnormal neurodevelopment and chronic lung disease [13]. Early adequate nutrition in premature infants is therefore essential.

1.2.1 Goals of nutrition in premature infants

In utero, the fetus grows at a minimum rate of 15 g/kg/day. Thus the goal of nutritional supplementation in premature infants is to reach a postnatal growth rate similar to that of the normal fetus at the same gestational age. [14, 15] However, this weight gain in preterm infants is often hard to achieve due to higher energy expenditures in extrauterine life as compared to intrauterine life. The reasons for higher expenditures are related to the intensive care environment, medical consequences, and inadequate nutrition [15].

Very preterm and extremely low birth weight (ELBW) infants will therefore need more intensive nutritional support [16]. Indeed, about 97% of very low birth weight (VLBW) infants had poor growth at 36 PMA. The reason is that preterm infants are often not fed enough protein and energy to meet the requirements for a growth rate similar to that of a normal fetus [17]. This observation is supported by several recent and older studies that have shown that adequate nutritional support can prevent the protein [18, 19, 20] and carbohydrate shortage and improve the growth of preterm infants. [21]

In order to achieve neonatal growth rates similar to those of the normal fetus, early PN is recommended soon after birth [22]. Infants born before 28 weeks' gestation, or VLBW

infants, required full PN. In premature infants, full enteral feedings are often delayed because of the medical problems associated with prematurity such as BPD, infections, necrotizing enterocolitis, and retinopathy of prematurity. In the first two weeks of birth, nutritional requirements of VLBW infants are frequently met by PN. PN provides balance in fluid homeostasis and electrolytes, avoids imbalance in macronutrients, and provides micronutrients and vitamins. [23]

Whereas previously this mode of nutrition for premature infants was often neglected, it is currently gaining in importance. The recommended PN includes: 1) sufficient calories for energy expenditure and infant growth; 2) carbohydrates to prevent hypoglycemia and proteins (essential amino acids) to meet nitrogen balance required for growth; 3) fatty acids to avoid essential fatty acid deficiency and prevent metabolic complications of glucose overfeeding; and 4) essential nutrients such as minerals, electrolytes, vitamins, and trace elements necessary for growth. [24]

Table 2. Protein, carbohydrates and lipid requirements in preterm infants on parenteral nutrition adapted from Velaphi et al.

Energy	110-120 kcal/kg/day
Protein	
< 30 weeks	3.5-4.0 mg/kg/day
30-36 weeks	2.5-3.5 mg/kg/day
> 36 weeks	1.5-2.5 mg/kg/day
Carbohydrates (glucose)	6-8 mg/kg/day
Lipids	2.5-3.5 g/kg/day

1.2.2 Energy requirements

Preterm infants have low energy reserves because they were born before they could accumulate fat and glycogen reserves [15]. Energy requirements depend on the stage of development and gestational age; for instance, the energy needs for infants born at 24 weeks' GA are higher than for those born at 38 weeks' GA. The recommended daily energy intake of healthy premature infants is 110-120 kcal/kg/day to allow for growth rates similar to those in utero [15].

1.2.3 Carbohydrates

Carbohydrates are the main energy source for premature infants receiving PN in the form of glucose. The rate of glucose synthesis is different for preterm and term infants: 6-8 mg/kg/minute and 3-5 mg/kg/minute, respectively [15]. Because an adequate amount of

glucose is critical for growth and neurodevelopment, glucose levels are monitored at initiation of and during PN in order to maintain serum glucose concentrations and avoid hypo- and hyperglycemia. [25, 15]

1.2.4 Lipid requirements

Lipids are considered an important source of energy in PN for premature infants. Additionally, lipids provide essential fatty acids which are required, among others, for brain and visual development [26]. Parenteral lipid emulsions also facilitate the delivery of lipid-soluble vitamins. A lipid infusion rate of 3 g/kg/day provides the necessary essential fatty acids and energy levels [27].

1.2.5 Protein requirements

Proteins are essential for premature growth and development. Several clinical studies have reported that higher protein and energy intakes have markedly increased the growth, head circumference, brain size, and cognitive function of premature infants [28]. The protein recommendation for infants depends on GA at birth. As shown in Table 2, intravenous amino acids must be administered to provide protein of 3.5–4.0 g/kg/day for infants < 30 weeks' GA at birth; 2.5-3.5 g/kg/day for infants 30 to 36 weeks and 1.5-2.5 g/kg/day for infants > 36 weeks of GA at birth. [29]

1.2.6 Electrolytes, minerals, and vitamins

During the first postnatal week, electrolyte needs are relatively low because of free water diuresis. [15] However, during the growing phase, sodium requirements are increased. Calcium, magnesium and phosphorus are necessary for tissue structure and function. During the last trimester of pregnancy, calcium is actively transferred from mother to fetus at the rate of 120-150 mg/kg of fetal weight per day [15]. Thus, preterm infants born before

the last trimester are at risk of low bone mass and bone disease. Calcium and phosphorus in PN is a challenge because of their limited solubility. [30]

Water-soluble and lipid-soluble vitamins are low in the premature infant population. They should be infused as soon as PN is started. There are different types of multivitamin preparations in the world. However, in North America, only one multivitamin preparation is available that contains both water-soluble and lipid-soluble vitamins (Multi-12 from Sandoz, Boucherville, Qc, Canada). Elsewhere, e.g. in Europe, there are several multivitamin preparations with these two types of vitamins. In my doctoral work, we used the Multi-12 pediatric preparation. [15]

Parenteral multivitamin preparations are very important for the nutritional needs of the infant; however, this mode of vitamin delivery has undesirable effects. See Section (1.7.6) on PN (or parenteral multivitamin preparation) as a major source of oxidative stress.

1.3 Lung

1.3.1 Normal structure of lung

The lungs are the primary organs responsible of respiration in humans and animals. The lungs are located in the central area of the thoracic cavity. The lungs are paired on either side of this central area. The right lung has three lobes, and the left lung has two lobes allowing a space for the heart. A lobe is further divided into lobules, and each lobule has a bronchiole serving many alveoli. The first structure allowing the air to entering the lung is the trachea. It is divided subsequently into two bronchi. The cartilaginous component of their wall maintains these ways open. Inside the bronchi, the epithelium is composed of pseudostratified columnar cells, which include basal cells, ciliated cells and goblets cells. These last one are responsible for mucus production.

Bronchi are subsequently divided in bronchioles that are further divided into smaller bronchioles. This last one, also named terminal bronchiole, leads air to acini (primary lobule), a group of alveoli that constitutes the first component of gas exchange. At the junction of bronchioles and alveoli, the Clara cells, similarly to alveolar type II cells, are secretory cells contributing the lining layer of the lungs. In addition, they also act as stem cell for epithelial renewal. [31, 32]

Alveoli, at number about 300 million in lungs, are the main point of gas exchange. It is a hollow cavity that is surrounded by an epithelium. The gas exchange occurs, by diffusion, with the numerous blood capillaries surrounding the alveoli. [34]

The epithelium of alveoli contains three types of cell. Type I cell forms the structure of the alveoli. Type II cell produces the surfactant and acts as stem cell for alveolar epithelium. The type III cell (pulmonary alveolar macrophage) derived from blood monocyte, acts as scavenger (dust cell). [31, 33]

1.3.2 Lung development in infancy

Lung development is a process that involves interactions between epithelial and mesenchymal tissue starting at the fourth week of gestation and continuing after birth. [31] The embryonic stage (4-7 weeks of gestation) includes the first appearance of the lung. It is a critical period of cellular differentiation and branching morphogenesis, when gross changes in lung structure can occur. The pseudoglandular stage (7-16 weeks' gestation) includes branching of the airways and blood vessels. Next, in the canalicular stage (16-26 weeks' gestation) there is further development of the blood supply to the peripheral mesenchyme. [32] The saccular phase includes differentiation of the respiratory airways and differentiation of future respiratory gas exchange units; it is thought to begin by 24 to 26 weeks' gestation and is completed by 40 weeks' gestation. [33] The alveolarization

stage includes the differentiation and multiplication of alveolar cells. The alveolarization stage extends from birth until 3 years of age; lung volume increases mainly because of an increasing number of alveoli. Alveoli may still develop up to 8 years of age.[34, 35] In contrast, the number of conducting airways is complete at birth; thereafter, the airways increase only in size. After alveolar multiplication is completed, lung growth continues in size only.[36, 37] Interference with developmental programming of the lung by undesirable effects of an environmental factor during any of these phases may affect lung development, leading to increased risks of lung disease or decreased ability of gas exchange later in life [38] see Figure.1 In the following section, I will describe the factors affecting lung development.

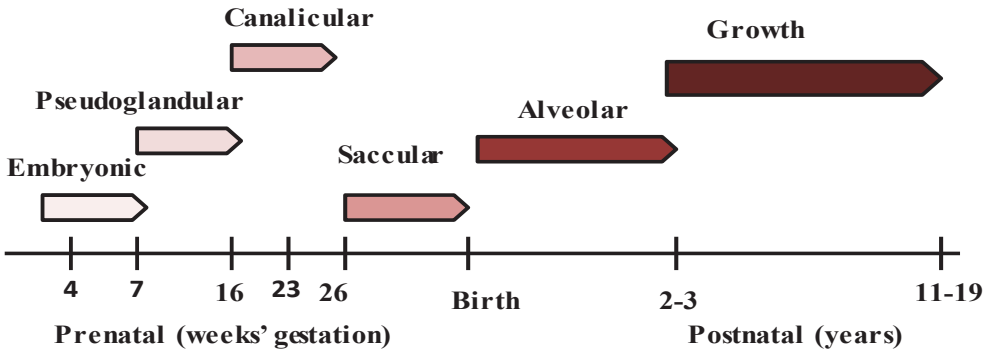


Figure 1. Stages of normal lung development

1.3.3 Antenatal-prenatal environmental influences on lung development

1.3.3.1 Fetal growth restriction

Intrauterine growth restriction is associated with an increased risk of compromised lung development leading to BPD [39] and impaired lung function in children [40] and adults. [41] In animals, studies have established that intrauterine growth restriction causes a reduction in the number of alveoli, with consequently impaired blood gas exchange which persists into adulthood. [42, 43]

1.3.3.2Maternal smoking and nicotine

Infants of mothers who smoke have reduced respiratory function and are more likely to develop wheezing than others. Experimental studies in various animal models have demonstrated that exposure to nicotine leads to smaller lungs with a reduced number of alveoli. [44] There is evidence from animal models that prenatal exposure to nicotine causes abnormalities in airway branching, leading to increased airway smooth muscle and collagen deposition, reductions in airflow, and airway hyperreactivity.[45] Several epidemiological and physiological studies [46, 47, 48, 49] have confirmed the postnatal effects of intrauterine smoke exposure in humans, which include disrupted lung function and increased respiratory morbidity. Prenatal nicotine exposure is associated with poor lung function at birth, which persists into early adulthood. [47, 50]

Taken together, the results of these studies indicate that nicotine exposure leads to persistent reduction of lung capacity for gas exchange. This may contribute to reduced exercise capacity as well as poor response to respiratory infections. [51]

1.3.3.3 Prenatal infection

Chorioamnionitis is a prenatal inflammatory response associated with bacterial infection of the uterus and infiltration of neutrophils into the placenta. Chorioamnionitis causes increased concentrations of proinflammatory cytokines such as interleukin (IL) -6, tumor necrosis factor alpha, interleukin-1 beta, and IL -8 in the amniotic fluid. This process is reported to increase the infant's risk of developing BPD. [52]

1.3.4 Postnatal environmental influences on lung development

1.3.4.1 Prematurity

When birth occurs at 28-32 weeks' gestation, the lung is in the saccular stage of development. During the saccular stage, the lungs start producing surfactant. Surfactant is essential after birth as it serves to lower the surface tension at the alveolar air-liquid interface and to stabilize the alveoli during expiration. [33] Before 29 weeks of gestation, the pulmonary surfactant system is immature and infants often require administration of exogenous surfactant to prevent alveolar collapse. An immature pulmonary surfactant system in combination with structurally immature alveoli is the major cause of respiratory distress syndrome (RDS). RDS is common in extremely preterm and very preterm infants, leading to a requirement for prolonged respiratory support. [33] Respiratory support in the presence of severe lung immaturity is the major cause of lung injury and is associated with BPD. [53]

Moreover, infants born prematurely or with various clinical syndromes may suffer delayed development of the lungs and airways, with consequences in later life. [54, 55] Several studies have shown that extremely premature children have reduced lung function with respiratory disease in later childhood [56, 57, 58] or early adulthood. [55]

1.3.4.2 Early exposure to hyperoxic gas

Preterm infants born before 30 weeks of gestation are exposed to elevated fractions of inspired oxygen (FiO_2) for extended periods of time, owing to respiratory insufficiency. Exposing the immature lung to hyperoxic gas can initiate the generation of reactive oxygen species (ROS). ROS can directly damage DNA and proteins, cause lipid peroxidation, and contribute to inflammation [59] and decreased alveolarization. [60]

Studies in rodents have found that neonates exposed to higher levels of FiO_2 during the saccular and alveolar stages of lung development had fewer and larger alveoli, with altered vascular development. Oxygen-dependent loss of vascular endothelial growth factor (VEGF) contributed to these changes [61, 62]. The blocking of VEGF signaling disrupted postnatal alveolar development. [63,64] Further, the number of circulating endothelial cell precursors found in the blood and lung was reduced in neonatal mice exposed to hyperoxia. [65, 66] Today, advances in medical practice and the use of exogenous surfactant and antenatal steroids have led to reduced mortality in BPD. However, infants born prematurely have an increased risk of asthma and are often re-hospitalized when infected with respiratory syncytial virus. [67, 68]

1.3.4.3 Ventilatory support

Most very preterm infants will need respiratory support due to respiratory distress syndrome. This ventilatory support increases the risk of BPD [69]. It has been shown that mechanical ventilation alone leads to impaired alveolarization in the very immature lung. [70, 71] Several studies have shown an early inflammatory reaction and inflammatory cytokines such as IL-8, IL-6 in bronchoalveolar lavage fluid from premature infants who

develop BPD. [72, 73, 74] Moreover, ventilation in preterm sheep increases inflammation and airway injury [75].

1.3.4.4 Postnatal growth restriction

After birth, the lung continues to develop, with increasing numbers of new alveoli. Postnatal nutrition and growth may affect the architecture of the adult lung. An animal study in adult sheep showed that postnatal growth correlated with the final number of alveoli. [76] Poor nutrition and growth in preterm infants could therefore contribute to reduced lung function and increased risks of pulmonary illness during childhood and adolescence. [77]

1.3.4.5 Early lung infections

Recurrent infections in early life such as respiratory syncytial virus may affect lung development, especially when sustained within the critical period of immune system development.[78] Several studies [79, 80, 81, 82] have shown that respiratory syncytial virus infection was associated with wheezing and bronchiolitis in infants and contributed to the development of asthma. The recurrent wheezing may result from losses of pulmonary function, lung growth and development due to impaired repair mechanisms. Children are most susceptible to respiratory tract infections in their first year of life because of an immature immune defense system. [83] This first year also corresponds with the period of lung alveolarization; there is therefore an increased risk of altering the development of the gas exchange region of the lung. Lower respiratory tract infections have been associated with reduced forced expiratory flow at school age. [84, 85] A cohort study has addressed the developmental aspect of lung function from childhood to adulthood in relation to early lower respiratory tract infections. This study reported an association between early infections in the first 2 years of life and reduced lung function in adulthood. [86]

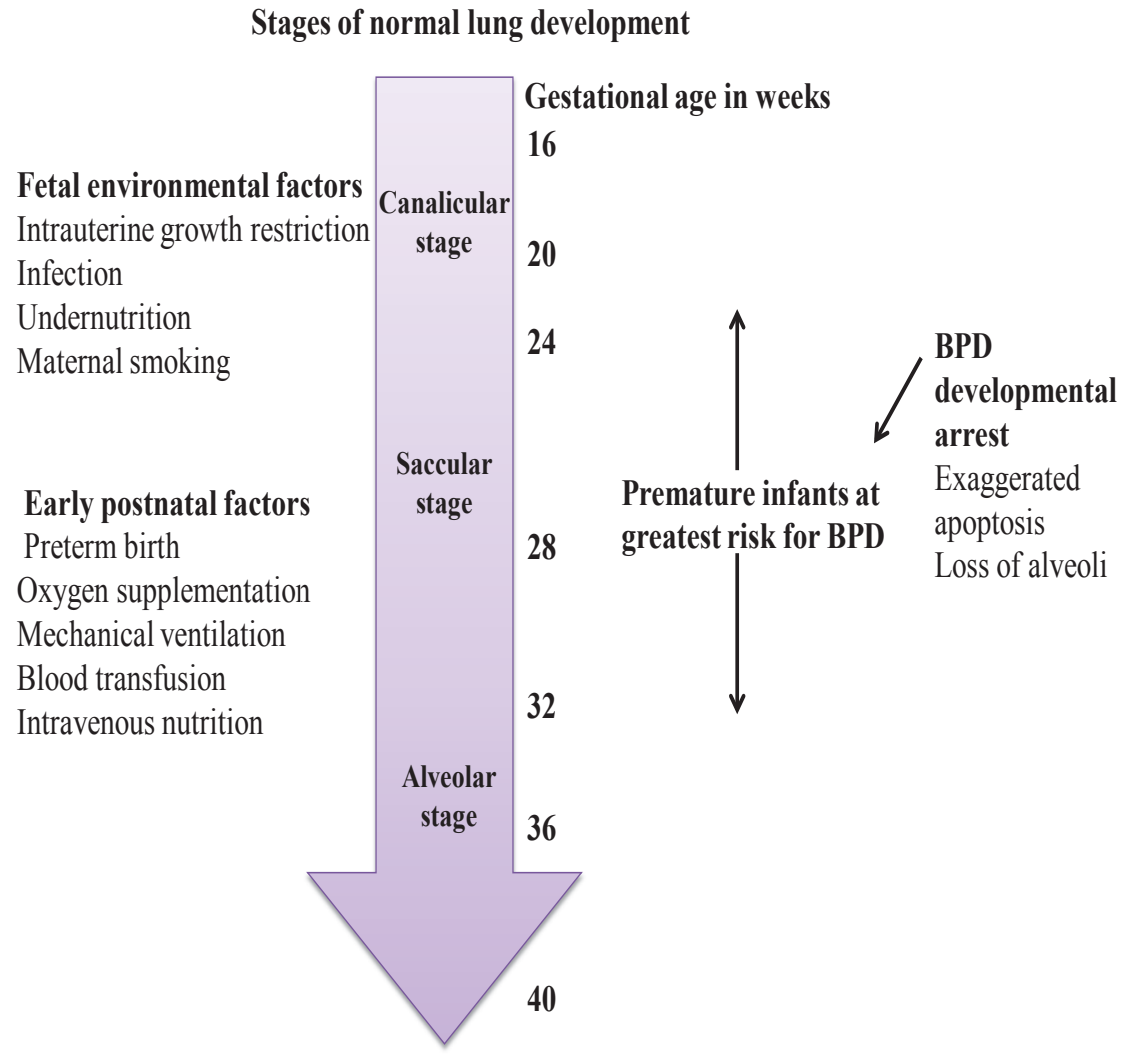


Figure 2. Stages of normal lung development and factors affecting lung development

Lung maturation must pass through the cell cycle stages of proliferation, differentiation, and apoptosis in order to continue lung remodeling. These cycle stages are dependent on the redox potential.

Since apoptosis is needed for proper lung development and is important to the hypothesis proposed in this thesis, the next section will be devoted to the role of apoptosis in lung development and to how apoptosis occurs.

1.4 Apoptosis and lung development

Several studies have observed apoptotic activity in the various stages of lung development, suggesting a role for apoptosis in these processes. [87, 88, 89, 90, 91] During the embryonic stage of lung development, apoptosis was found in the peripheral mesenchyme. Studies have shown that apoptosis in the canalicular stage at 18 days of gestation leads to a progressive increase in epithelial and interstitial apoptosis. [92,88] During late gestational development (canalicular, saccular stages), there is lung remodeling with an increase in alveolar type II epithelial cell apoptosis and a decrease in cell proliferation. [93, 89] After birth (alveolar stage), apoptosis serves as an important process to remove excess cells and transform saccules into functional alveoli. [91, 90] Alveolar type II epithelial cell proliferation is important because these cells serve as precursors to alveolar type I epithelial cells, which cover over 95% of the alveolar surface [94]. This proliferation plays a role in the maturation of the air-blood barrier during microvascularization [94]. Also, alveolar type II epithelial cells produce surfactant, which decreases surface tension and prevents alveolar collapse during exhalation after birth. After alveolarization, excess type II epithelial cells decrease in number by differentiating into type I epithelial cells and by apoptosis. [90]

On the other hand, apoptosis may participate in the development of lung disease. Increased apoptosis of epithelial cells would lead to inefficient re-epithelialisation or ineffective removal of apoptotic cells. [95]

1.4.1 Mechanisms of apoptosis

During apoptotic events, cells undergo various morphological changes, including cell shrinkage, fragmentation of chromosomal DNA, and the release of apoptotic bodies. The mechanisms of apoptosis involve an initiation phase, whereby a stimulus activates caspase

activities; these enzymes then induce cell death. [95] Two signaling pathways can activate a caspase cascade in mammalian cells: the extrinsic pathway and the intrinsic pathway.

1.4.2 Apoptosis signaling pathways

The extrinsic or death receptor pathway involves the activation of death receptors present in the cell membrane, such as Fas and tumor necrosis factor receptor 1 (TNF-1). Connection of the death ligand to its death receptor leads to activation of an adaptor protein called activated death domain and the subsequent activation of procaspase-8 or -10; this then activates the caspase -3 leading to apoptosis. [96]

The intrinsic or mitochondrial pathway is induced in response to stress stimuli, such as ROS, DNA damage, irradiation, or reduced anti-apoptotic mitochondrial factors (e.g. Bcl2). The intrinsic pathway is triggered by alterations in mitochondrial membrane permeability and the consequent release of cytochrome c and other apoptogenic factors (e.g. Bak, Bax, Bim) from the inner mitochondrial membrane. Cytochrome c binds with apoptotic protease-activating factor 1 (Apaf-1) to form an apoptosome. Subsequently, activated caspase-9 and caspase-3 induce apoptosis. [97]

There is now increasing evidence demonstrating that the endoplasmic reticulum also executes apoptosis. Various stressors can impair protein folding and induce endoplasmic reticulum stress, thus further exacerbating the endoplasmic reticulum stress which transduces apoptotic signals. [98]

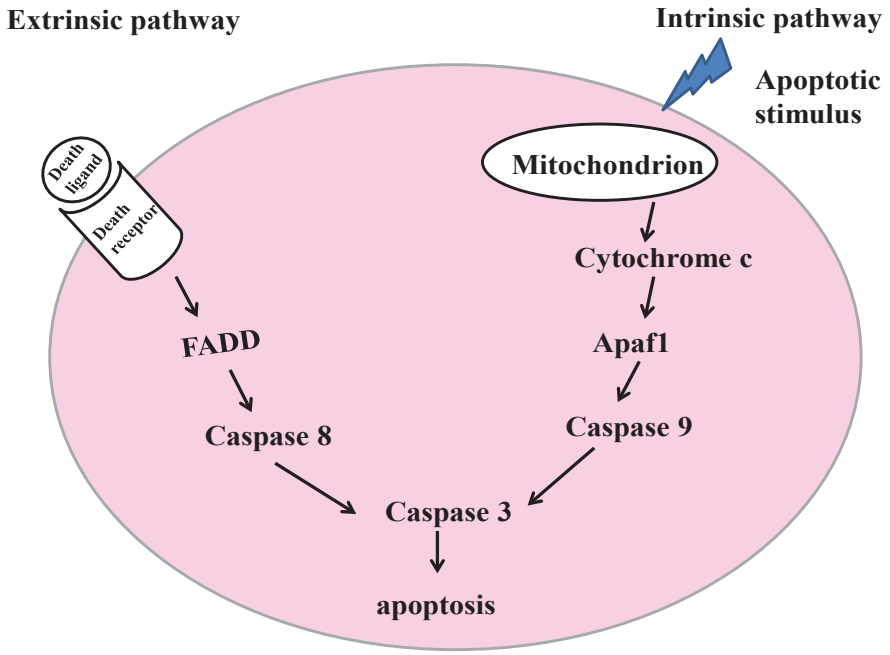


Figure 3. Apoptosis pathways: Activation of caspase-3 is caused by the initiator caspases, caspase-8 and/or caspase-9, as part of the extrinsic and intrinsic apoptotic signaling pathways.

In the next section, I will demonstrate what we know concerning the apoptosis signaling pathway in relation to neonatal lung injury and how excessive apoptosis can contribute to BPD.

1.4.3 Apoptosis in neonatal lung injury or BPD

Although oxygen and mechanical ventilation are important to premature survival, excess oxygen and pressure overload from mechanical ventilation are injurious to a newborn's lungs, leading to BPD. [59, 99,100]

The administration of supplemental oxygen to preterm infants is standard therapy. However, exposure to high concentrations of oxygen is a major contributor to lung injury and BPD [101]. Exposing the immature lung to hyperoxic gas can initiate the generation of

ROS. ROS can directly damage DNA and proteins, cause lipid peroxidation, and contribute to inflammation [59] and decreased alveolarization. [60] Several studies have shown an early inflammatory reaction and the presence of inflammatory cytokines such as IL-8, IL-6 [72, 73], and transforming growth factor type 1(TGF-1) [74] in bronchoalveolar lavage fluid from premature infants who develop BPD. Hyperoxia-induced lung injury is considered a major risk factor for the development of BPD. In the developing lung, hyperoxia results in epithelial and endothelial cell loss, disordered proliferation, and other changes that may hinder alveolar and microvascular development [102]. Thus, ventilatory support in very preterm infants also increases the risk of BPD [69]. Indeed, it has been shown that mechanical ventilation alone leads to impaired alveolarization in the very immature lung. [70, 71]

An elevated apoptotic ratio was found on autopsy in alveolar and bronchiolar cells of 24 patients with BPD. [103] These data highlight the importance of apoptosis in the development of BPD in the premature infant. In fact, growing evidence from animal and human studies supports an increase in apoptotic signals.

Lukkarinen et al. [104] showed that preterm infants who had RDS and received mechanical ventilation had higher levels of apoptosis, particularly in epithelial cells.

Similarly, increased levels of epithelial apoptosis have been observed in neonatal mice exposed to high concentrations of oxygen [105] and mechanical forces. [106]

Exposure to high concentrations of oxygen in the neonatal period may impair lung growth and is a major contributing factor to the development of BPD. [107,108] Cell death from hyperoxic injury may occur through either an apoptotic or non-apoptotic pathway. Sharon et al. found increased levels of Bax messenger RNA, a gene transcript associated with apoptosis, in the lungs of neonatal mice born and raised in 92% oxygen levels. In neonatal

mice exposed to hyperoxia, apoptotic cells in the lungs increased with longer exposure times. [105] The increase in apoptosis from hyperoxic exposure during a critical period of lung development may be an important factor in the impaired lung growth and remodeling of BPD.

Li et al. found increased expression of P53, a gene associated with apoptosis, in the lungs of neonatal rats born and raised in 95% hyperoxia. These results suggest that 95% hyperoxia could upregulate the gene expression of P53, which induces the transcription of P21 [109]. Furthermore, P21could lead to cell cycle arrest and inhibit proliferation of lung cells. At the same time, P53 could also promote apoptosis of lung cells. Similarly, Das et al. showed increased apoptosis and expression of P21 and P53 in the premature infant baboon model of BPD [110]. Therefore, exposure to high concentrations of oxygen in the neonatal period may impair lung growth and be a major contributing factor to the development of BPD.

The Fas ligand (FasL) pathway is a system of signaling- and receptor-molecules leading to apoptosis. Fas antigen is expressed in various cells and tissues including the lung [111]. FasL can be released as a biologically active, death-inducing mediator capable of inducing apoptosis of epithelial cells during acute lung injury [112]. In FasL transgenic mice, upregulation of Fas-L was associated with dramatically increased apoptosis of alveolar type II cells and Clara cells, disrupted alveolar development, decreased vascular density, and increased postnatal lethality. [113] These results suggest that inhibiting this pathway may lead to novel targets against BPD.

TGF-1 is the most potent promoter of extracellular matrix production. In addition, TGF-1 can induce apoptosis directly in various cells. [114] TGF-1 can mediate apoptosis through activation of caspase-3, upregulation of P21, and downregulation of Bcl-2 expression [115,

116]. However, TGF-1 is also an enhancer of Fas-mediated apoptosis of lung epithelial cells [117]. Increased levels of TGF-1 are found in airway lavage samples of infants in the early stages of BPD [74]. Moreover, high levels of TGF-1 are associated with more severe cases of BPD [74]. In BPD during tissue remodeling, dysregulation of the TGF-1 pathway may interact with Fas-mediated epithelial cell apoptosis during the pathogenesis of pulmonary fibrosis. Repair after BPD requires the elimination of proliferating mesenchymal and inflammatory cells. Failure to clear unwanted cells by apoptosis will increase the inflammation [118]. Therefore, apoptosis may have both beneficial and detrimental effects during BPD.

Finally, in animal studies, the infusion of PN without adequate photo protection was associated with a lower level of alveolarization (i.e. hypoplasia), the principal characteristic of BPD [119]. Moreover, a higher level of apoptosis was observed in lungs with alveolar hypoplasia [119]. However, little is known about the biochemical mechanisms involved and the present thesis will hopefully lead to a better understanding of this mechanism.

In the following section, I will review the definition and characteristics of BPD, who is at risk for developing BPD, differences between old and new BPD, the clinical definition of BPD, and finally, the long-term outcomes of BPD.

1.5 Bronchopulmonary dysplasia

BPD is a lung disease of premature infants, characterized by abnormal alveolarization and pulmonary vascularization. BPD was first described by Northway in 1967 [120]. He and his colleges defined BPD on the basis of clinical, radiographic and pathological findings following clinical practices such as aggressive mechanical ventilation and high levels of oxygen in RDS [121]. Today this lung disease is called classic or old BPD, which was characterized by lung inflammation, airway injury, lung fibrosis, and oxidative stress.

This definition has become less relevant in current practice, with improved care of RDS and the survival of very tiny babies. In 2001, the National Institute of Child Health and Human Development defined BPD as the need for supplemental oxygen at 36 weeks of PMA, with severity based on oxygen concentration [122]. Infants requiring supplemental oxygen at 28 days of life but not at 36 weeks' PMA were considered to have mild BPD. Infants requiring supplemental oxygen at 28 days and at a fraction of inspired oxygen (FiO_2) < 0.30 at 36 weeks' PMA were considered to have moderate BPD. Infants requiring supplemental oxygen at 28 days, and mechanical ventilation and/or FiO_2 > 0.30 at 36 weeks' PMA were considered to have severe BPD [123]. New BPD is characterized by an "arrest" of alveolar development, with fewer alveoli, little to no airway injury, and no lung fibrosis.

Table 3. BPD severity.

Types of BPD	Gestational age < 32 weeks	Gestational age ≥ 32 weeks
Mild BPD	Breathing room air at 36 weeks' postmenstrual age	Breathing room air by 56 days' postnatal age
Moderate BPD	Need for < 30% oxygen at 36 weeks' postmenstrual age	Need for < 30% oxygen at 56 days' postnatal age
Severe BPD	Need for ≥ 30% oxygen and/or mechanical ventilation at 36 weeks' postmenstrual age	Need for ≥ 30% oxygen and/or mechanical ventilation at 56 days' postnatal age

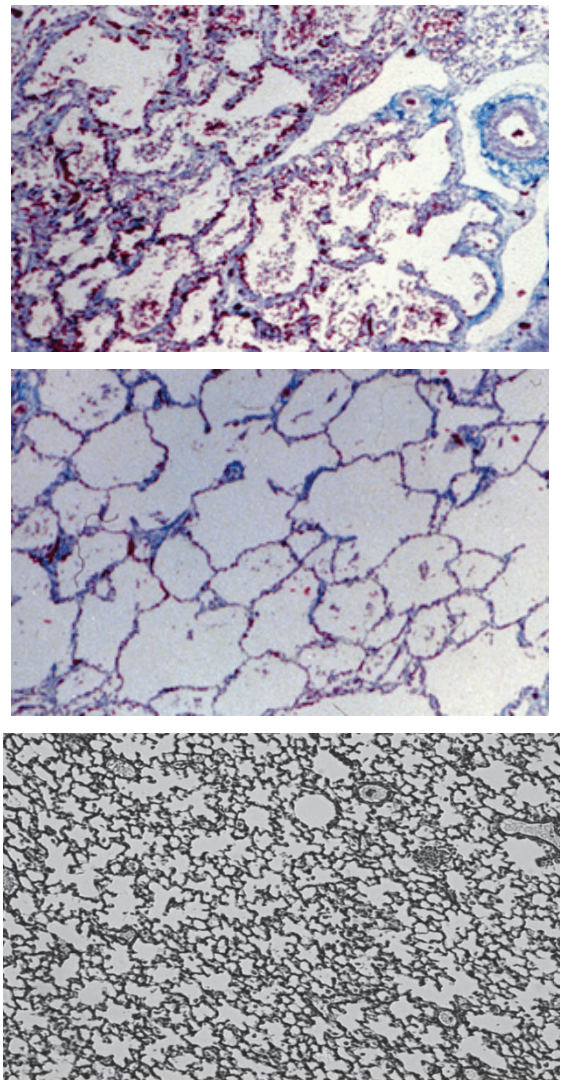
Adapted from Mosca et al.

Table 4. Comparisons between old and new BPD

	Old BPD	New BPD
Gestational age	32 weeks of gestation	24-26 weeks of gestation
Birth weight average	1900 g	600 g
Infants at risk	More mature infant	Extremely low gestational age
Airway injury	Severe	Mild
Fibrosis	Severe	Mild
Alveolar development	Well-developed	Arrested development
Causes	Oxygen toxicity and mechanical ventilation	Interference with development

Adapted from Mosca et al.

It is important to know that these tables summarizing the differences between the two forms of BPD (old vs new) and underlying the causes for BPD have been published before my work. Moreover, my work highlights the clinical importance of PN, which increases the risk for BPD; the arrested alveolar development may be the result of exaggerated apoptosis.



Old BPD: Airway injury, inflammation and fibrosis due to mechanical ventilation and oxygen toxicity.

New BPD: Alveolar hypoplasia leading to fewer and larger alveoli and impaired vascular development.

Normal healthy lung with normal alveoli.

Figure 4. Histological findings comparing old and new BPD.

1.5.1 Consequences of bronchopulmonary dysplasia

Compared to full-term infants, preterm infants with BPD are more susceptible to recurrent wheezing and lower respiratory infections that require frequent hospitalizations during the first 2 years after birth. [125,126] In addition, several studies have shown abnormalities of pulmonary function during infancy in infants who had BPD. [127] Indeed, BPD is associated with a significant reduction in pulmonary function and exercise capacity and an increased risk of respiratory symptoms including cough, wheeze, and asthma in children

aged 7 to 12 years.[128,129] Finally, preterm infants with BPD commonly develop neurodevelopment disorders, growth retardation, and educational difficulties at school age.[130,131]

1.5.2 Treatment and prevention of BPD

Regardless of advances in the treatment of neonatal respiratory disorders such as pulmonary surfactants and corticosteroids, diuretics, ventilatory supports, and in prophylaxis to accelerate lung maturation and reduce neonatal respiratory disorders, BPD remains a major complication for preterm infants [132]. The pathogenesis of BPD is multifactorial and highly complicated. Several factors are known to influence the incidence of BPD, including low gestational age at birth, male sex, inflammation, infection, and oxygen supplementation. [10] Recently, PN has been reported to increase the risk of BPD [133]. Oxidative stress is implicated in the etiology of all these factors.

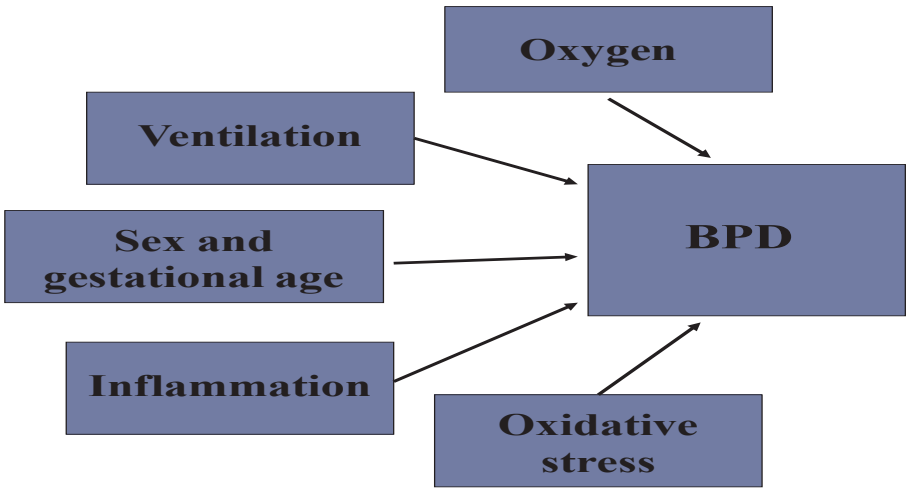


Figure 5. Factors implicated in the incidence of BPD: environmental factors such as inflammation, oxygen supplements, ventilation and oxidative stress; and other internal factors such as sex and gestational age.

Prevention and treatment of BPD will need a comprehensive approach including all the factors and mechanisms leading to this chronic lung disease. Although oxidative stress has been suggested, the causative molecules and mechanisms involved in the development of this disease are still unclear.

The present thesis aims to further our understanding of the mechanistic process of BPD development and the causative molecules involved in this chronic lung disease. This knowledge would help avoid or minimize the extent of this disease, a disease that may have consequences in later life, including persistent lung abnormalities.

In the following section, I will describe oxidative stress in this population, specifically the sources of oxidant molecules and the antioxidant defense system.

1.6 Oxidative stress

Oxidative stress results from an abnormally high level of oxidative molecules in a cell or organ. This may be caused by a low capacity of antioxidant defenses, an excessive production of oxidant molecules, a high load of exogenous oxidant molecules, or all of the above. ROS as shown in Table 5 are produced as a normal consequence of ATP production in the mitochondria and by intracellular specific enzyme systems. Alternatively, they can be derived from external sources such as inhaled oxygen and pollutants. An increase in intracellular levels of these oxidants will eventually damage cellular lipids, proteins, and DNA and lead to impaired cell function and finally, cell death. Thus oxidative stress plays an important role in the development of several pathological complications. [134,135] Recently, oxidative stress has been redefined as an imbalance between pro-oxidants and antioxidants, leading to macromolecular damage and/or disruption of redox signaling (see below) [136]. It is proposed that this latter may induce pathological conditions.

1.6.1 Oxidants

An oxidant can be defined as a substance that accepts electrons in chemical reactions. Oxidants are formed as a normal product of aerobic metabolism. These oxidants can be produced in excessive rates under pathophysiological conditions. They include radical and nonradical oxidants. Radical species include the superoxide anion, hydroxyl radical, and nitric oxide whereas nonradical oxidants include hydrogen peroxide, hydroperoxyl fatty acids, and aldehydes [137].

Table 5. Examples of reactive oxygen species and corresponding antioxidants

Radical	Antioxidant
Superoxide anion (O_2^-)	Superoxide dismutase
Singlet oxygen	B-carotene
Hydrogen peroxide (H_2O_2)	Catalase, glutathione peroxidase, glutathione
Hydroxyl radical ($OH^\bullet$)	Vitamins C and E
Peroxide radical ($LOO^\bullet$)	Vitamins C and E
Hydroperoxyl radical (LOOH)	Glutathione transferase, glutathione peroxidase

Adapted from Davis et al. [138].

1.6.2 Antioxidants

An antioxidant can be defined as any substance that avoids or eliminates oxidative damage to a target molecule [139]. Antioxidants can be classified into 3 categories: primary, preventing oxidant formation; secondary, scavenging the oxidants; and tertiary, repairing or removing damaged molecules.

Antioxidants include enzymatic antioxidants such as catalase, glutathione peroxidase (GPx) and glutathione transferase, and non-enzymatic antioxidants such as vitamin C, vitamin E, glutathione, trace minerals and fatty acids.

1.7 Oxidative stress and antioxidant defense systems of premature infants

At birth, the fetus transfers from an intrauterine hypoxic environment with an arterial partial pressure of oxygen (PaO_2) of 20-25 mm Hg to an extrauterine environment with a PaO_2 of 100 mm Hg. In term infants, there is an increase of protective antioxidant enzyme activity immediately prior to birth. However, the premature infant is prone to oxidant-induced damage principally for two reasons: first, inadequate concentrations of antioxidants; and second, the ability to increase synthesis of antioxidants in response to hyperoxia or other oxidants is insufficient [140]. Frank et al showed that the activity of antioxidant enzymes in the lung, such as GPx and catalase, increases as a function of gestational age in several animal species (rabbit, guinea pigs, rats and hamster) [141]. Therefore, their activity increases in the last 15% of gestation. This finding parallels the premature infant's complications which are inversely proportional to GA. Similarly, serum levels of non-enzymatic antioxidants such as vitamins C and E are reduced in preterm infants as compared to term controls [142,138]. The same is true of glutathione, a key antioxidant: the concentration in cells from endotracheal aspirates increases during gestation [143].

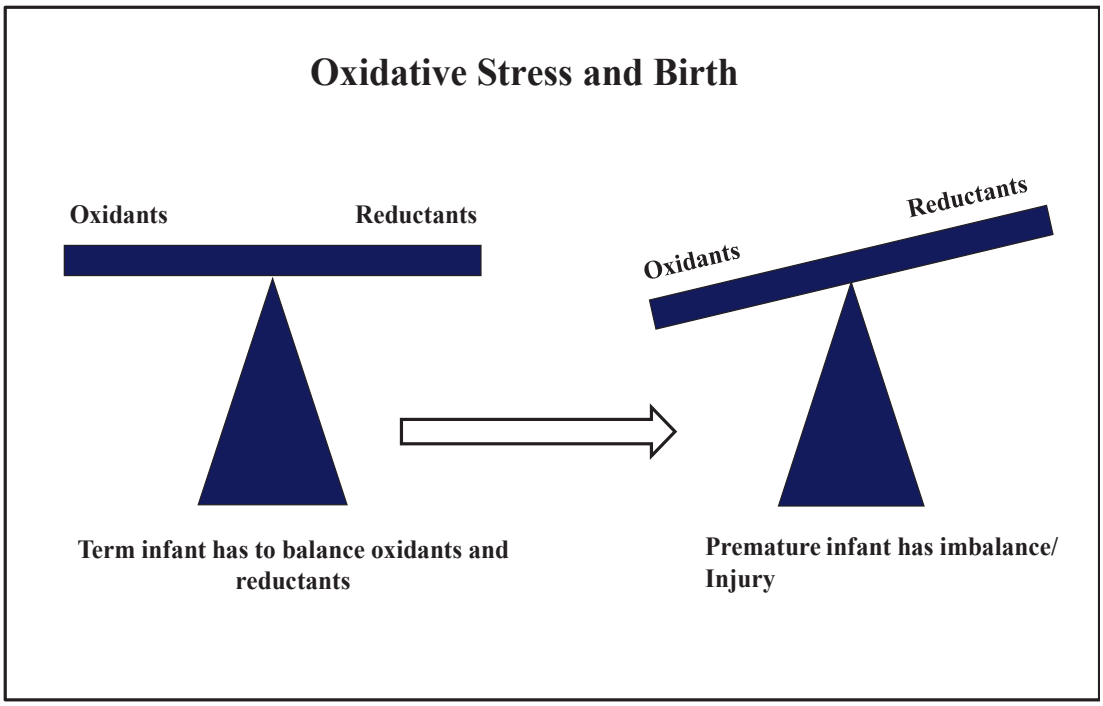


Figure 6. Oxidative stress and birth.

Reductants: catalase, GPx, glutathione and vitamins E, A, C

Oxidants in term infants: inspired oxygen

Oxidants in premature infants: O_2, parenteral nutrition, infection, and inflammation

GSH is considered a key element of antioxidant defenses. It participates in detoxification of peroxides and radicals: first, as an electron donor for the reduction of peroxides through the action of glutathione peroxidases; second, as a recycler of radical scavengers such as ascorbate and tocopherol [144]. In addition, glutathione is recognized as the buffer of the intracellular redox environment [145]. Recently, Chessex and others have reported a strong correlation between the severity of BPD in premature infants and the redox potential of

glutathione measured in their blood [146]. Hence, a perturbation of glutathione metabolism is suspected to induce BPD development.

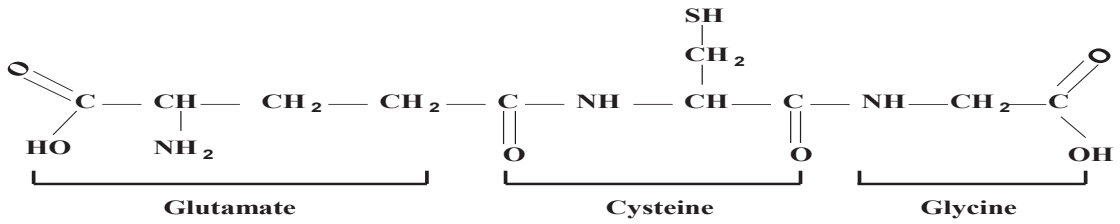


Figure 7.Glutathione structure Glutathione in its reduced form is the tripeptide L-γ-glutamyl-L-cysteinylglycine. Adapted from [147]

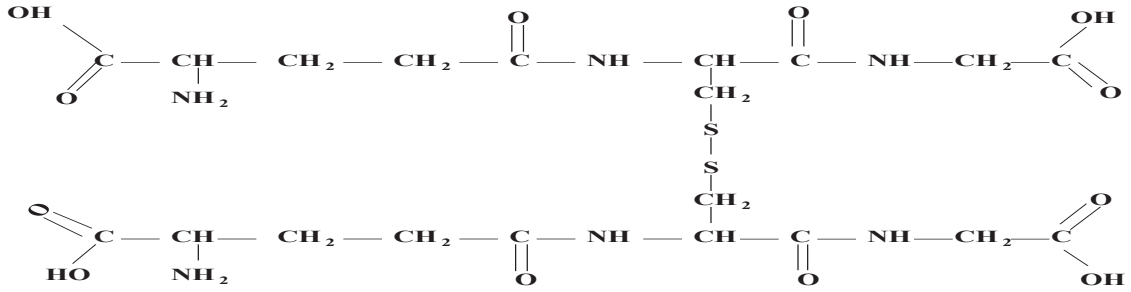


Figure 8. Disulfide (oxidized) form of glutathione. Adapted from [147]

In the next sections, I will discuss the metabolism of glutathione in preterm newborns and the impact of glutathione perturbation on the redox potential.

1.7.1 Glutathione metabolism in premature infants

In vivo, the regulation of GSH (γ-glutamylcysteinylglycine) takes place in the liver [148]. The liver is considered both a synthesizer and exporter of GSH. It provides 90% of the glutathione supply in blood [149,150]. Indeed, the liver has a great capacity of glutathione synthesis for its own needs and for export via the sinusoidal multidrug resistance proteins (MRP3). As GSH does not cross freely through the cellular membrane, γ-glutamyl transpeptidase plays an important role in supplying cells with needed substrates for GSH synthesis. Gamma-glutamyl transpeptidase is an enzyme anchored in the cell membrane with its catalytic site in the extracellular milieu. [151] This omnipresent enzyme transfers the γ-glutamyl moiety of glutathione present in plasma (from the liver) onto a second amino acid, forming two dipeptides (γ-glutamyl amino acid and cysteinylglycine). These dipeptides are absorbed by cells and hydrolysed into free amino acids. Free cysteine availability in cells is a limiting step for *de novo* synthesis of GSH. [152] Indeed, the physiological intracellular concentration of cysteine is close to the Michaelis-Menten kinetics constant (Km) of the first enzyme of the glutathione synthetic pathway, γ-glutamylcysteine synthetase, whereas the concentrations of the two other amino acids (glutamate and glycine) are far higher than the Km of these enzymes. [153] Therefore, in circulating blood, the action of γ-glutamyl transpeptidase on glutathione deriving from the liver favours the increased concentration of cysteine in cells, favouring in turn, the cellular synthesis of glutathione. The activity of γ-glutamyl transpeptidase matures in the first postnatal days of premature infants. [154]

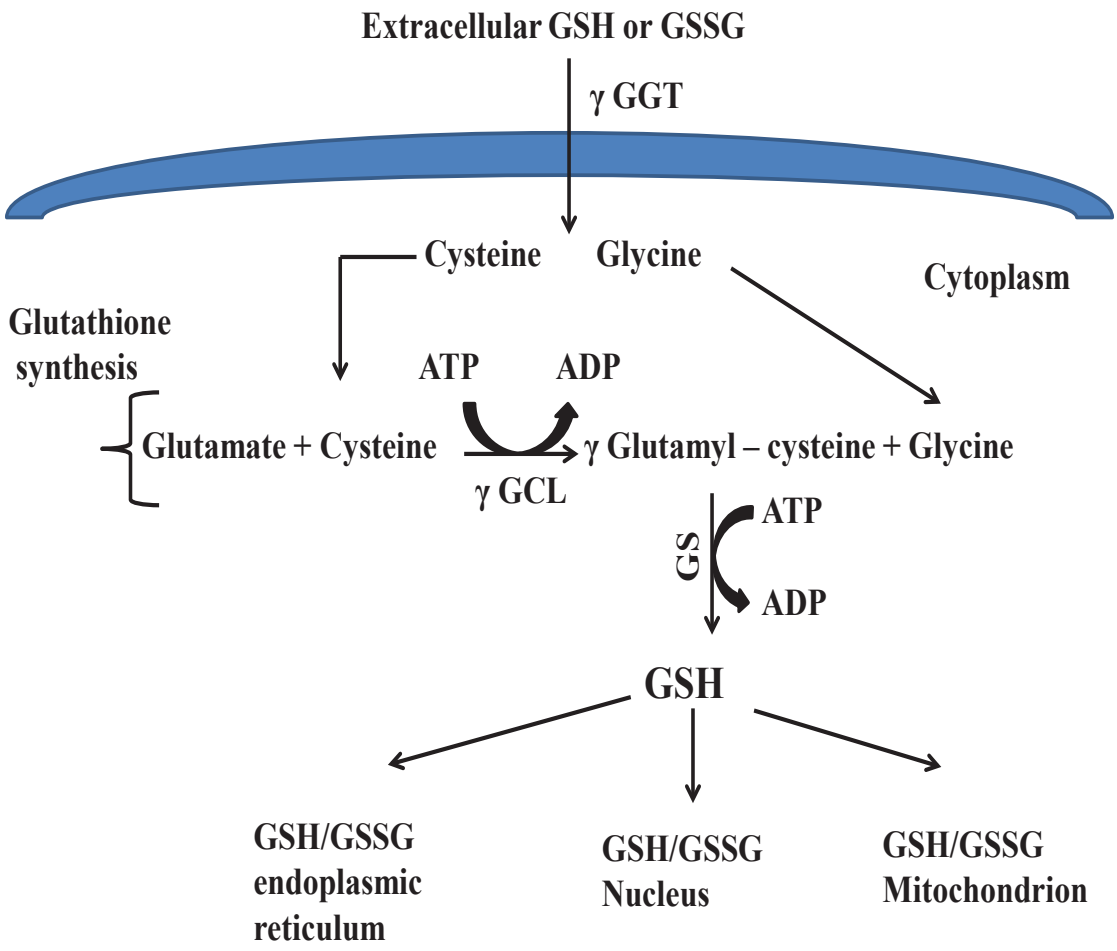


Figure 9. Extracellular GSH hydrolysis is catalysed by γ-glutamyl transpeptidase. The amino acid precursors for GSH synthesis, cysteine and glycine, are transported via membrane carriers for intracellular GSH synthesis. GSH synthesis from glutamate, cysteine and glycine occurs within the cytosol via two ATP-dependent steps that are catalysed by glutamate-cysteine ligase (γ-GCL) and glutathione synthase (GS). Distinct GSH pools are compartmentalized within the cytosol, mitochondria, nucleus and endoplasmic reticulum.

The low glutathione levels observed in premature infants is not explained by an immaturity of the capacity for synthesis, which is fully active even in neonates born at 26 weeks of gestation. [154] The deficiency seems to derive from the availability of cysteine for GSH synthesis or lack thereof. [143]This hypothesis may be explained by an insufficient

concentration of cysteine or glutathione in the circulation. The cysteine can be provided by nutrition. Premature infants < 29 weeks of gestation are essentially fed intravenously, by way of parenteral nutrition. However, the instability of cysteine in solution limits its enrichment in PN. [155] In addition, the cellular uptake of cysteine is immature in this population. [143] Therefore, the main source of cysteine for glutathione synthesis remains the transformation of methionine into cysteine. Indeed, this essential amino acid is known to be primarily metabolized in the liver; it serves as the endogenous source of cysteine and, consequently, of glutathione synthesis. [156]

The first enzyme involved in the transformation of methionine into cysteine is methionine adenosyltransferase (MAT). [148,157] However, PN as used in the clinical setting is contaminated with about 350 μM peroxides and MAT contains redox-sensitive thiols essential for enzymatic activity. I have shown in the course of my Master's thesis that the infusion of H_2O_2 at concentrations similar to those measured in PN induces a lower activity of MAT in animals. Both H_2O_2 and PN lead to a reduction of glutathione levels in the liver as well as in whole blood. This work was published in *Free Radical Biology and Medicine* in 2012. [158]

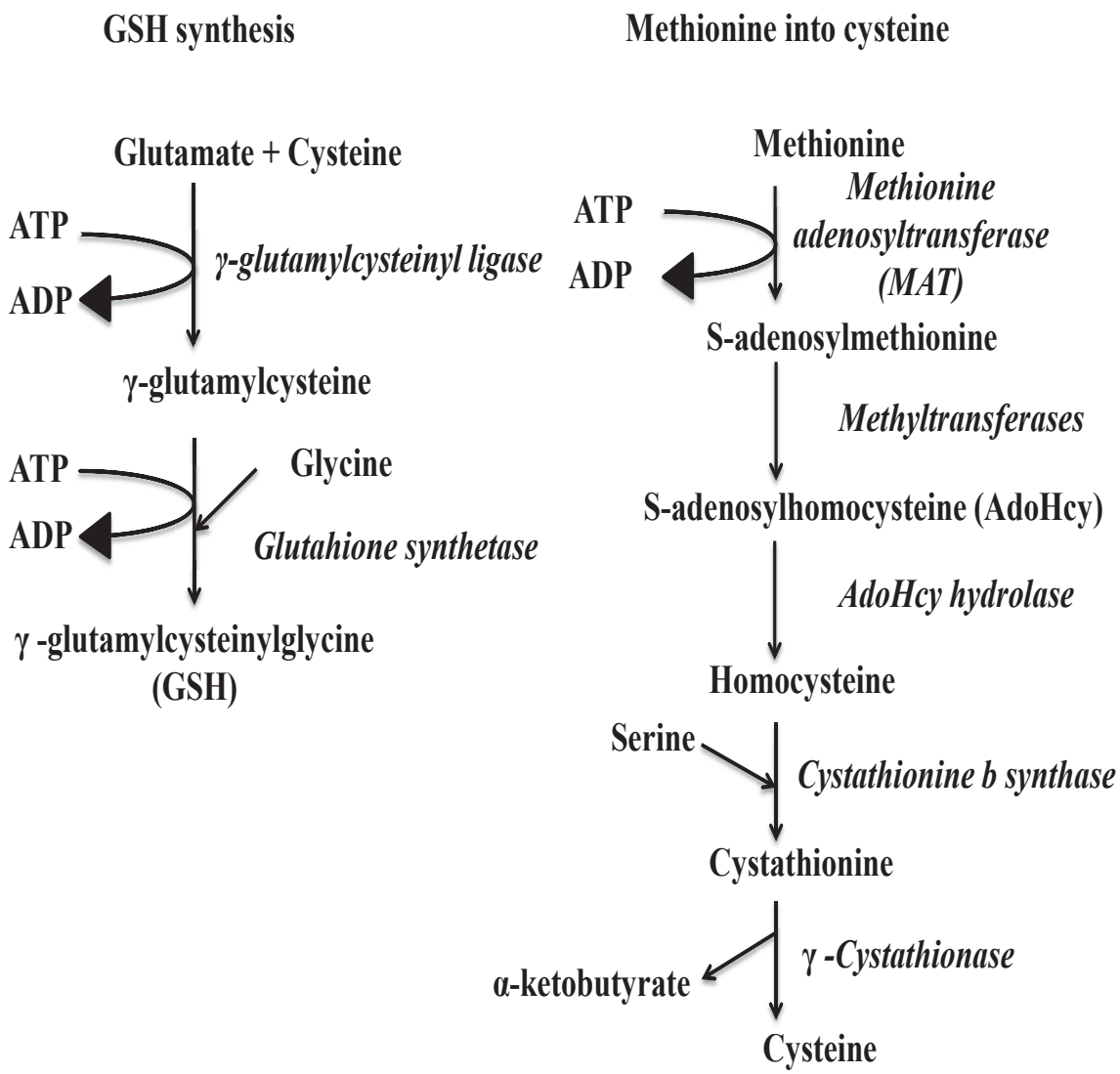


Figure 10. Transformation of methionine into cysteine, the rate-limiting amino acid for glutathione synthesis.

1.7.2 Redox potential of glutathione

In addition to its antioxidant capacity, glutathione is considered the buffer of the intracellular redox potential. The redox potential of the cell influences electron transfer between chemical species and thus affects metabolism to a large degree. Redox potential can be estimated from the concentrations of both GSH and GSSG, with the Nernst equation $\Delta E = \Delta E^{\circ} \cdot (RT/nF) \cdot \log([GSH]^2/[GSSG])$ mV at 25°C and pH 7, where R is the gas

constant, T is the temperature (in Kelvin), n is the number of transferred electrons, F is the Faraday constant, [GSH] is the concentration of reduced glutathione and [GSSG] is the concentration of disulfide glutathione. [159]

The redox potential is associated with the biological status of the cell. During cellular development, cells must pass through several cell cycle stages in order to continue remodeling. These cycle stages are conditioned by the redox potential. For example, cells pass from proliferation to differentiation and apoptosis when the redox potential shifts from a reduced to an oxidized state.[159] During the proliferation phase, where the redox value is highly reduced, cells have a high metabolic rate leading to increased generation of ROS. These ROS shift the redox potential toward an oxidized state, provoking the differentiation phase. An even greater oxidized status induces apoptosis. An oxidized redox potential will activate redox-sensitive factors such as nuclear factor erythroid-derived 2 (Nrf2) which are responsible for the transcription of the gene for γ-glutamylcysteine synthetase, the first enzyme responsible for the glutathione synthesis, and glutathione reductase (enzyme involved in GSSG recycling). In this way, activation of Nrf2 leads to a higher concentration of GSH and a more reduced redox potential, and a new cell cycle begins. Hence oscillation in redox potential is essential for tissue remodeling.

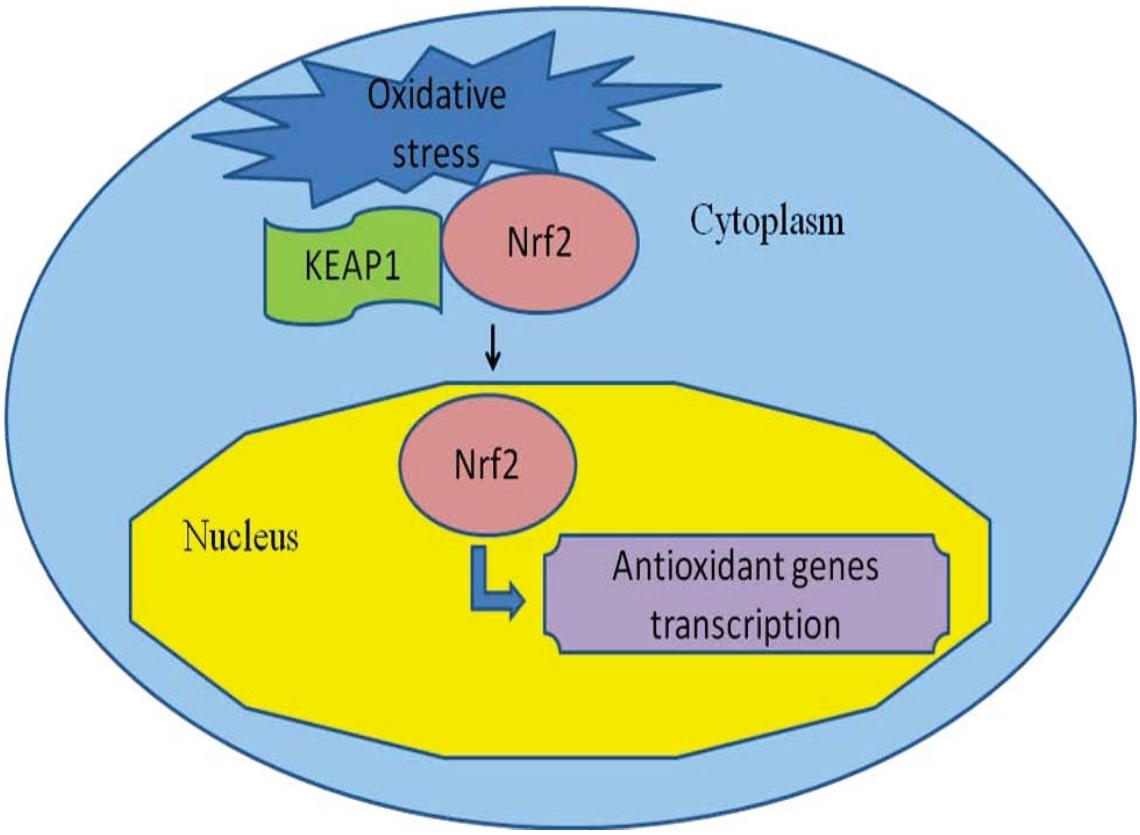


Figure 11. Activation of Nrf2 by oxidative stress

The redox potential is also dependent on the action of GPx and glutathione reductase (GR). GPx plays a very specific role in cellular metabolism, particularly in the removal of LOOH, terminating the lipid peroxidation chain reaction and protecting biological membranes. In mammalian tissues, four isozymes of GPx (GPx 1, GPx2, GPx3 and GPx4) have been identified. Mammalian isozymes GPx1, GPx2 and GPx3 reduce H_2O_2 and peroxides of free fatty acids whereas GPx4 reduces peroxides of phospholipids and cholesterol.

GPx, an enzyme whose catalytic action is dependent on the micronutrient selenium (Se), catalyzes the reduction of H_2O_2 and other organic peroxides to water and corresponding alcohols, respectively. [160] This reaction requires two molecules of GSH as reducing substrate. The product of this reaction is GSSG. The activity of GPx is tightly controlled

since any modification would lead to altered intracellular concentrations of hydrogen peroxide. [161] As a result of this GPx reaction, the intracellular level of GSSG rises. This will affect the cellular redox potential. Normally, GSSG is then reduced back to GSH by the action of GR in the presence of NADPH as electron donor. If the activity of GPx is greater than that of GR, the accumulated GSSG will be actively exported to maintain the intracellular redox potential [162]. In this case, lower intracellular levels of GSH result, affecting the activity of GPx because the normal level of GSH in cells is at the level of the Km of GPx. A lower level of GSH leads to a lower activity of GPx, allowing intracellular levels of H_2O_2 to rise. To prevent loss of glutathione, the cell stimulates de novo synthesis of GSH and recycles the GSSG into GSH by GR [163].

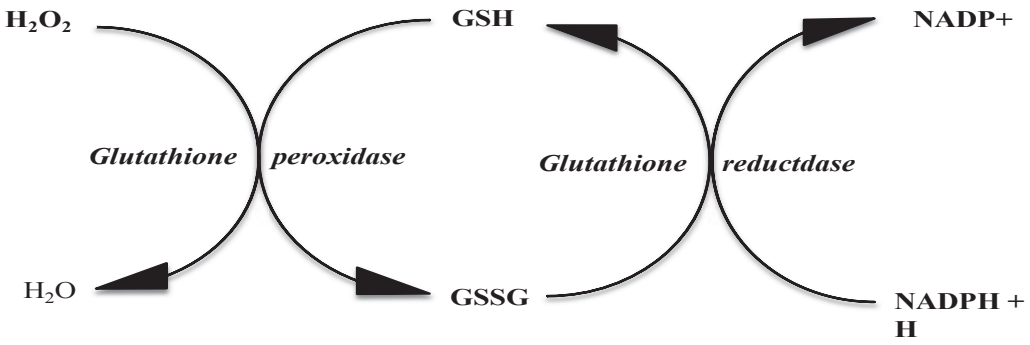


Figure 12. Redox cycle

1.7.3 Main source of oxidative stress in premature infants

1.7.3.1 Oxygen supplementation

Fetal life in utero is maintained under low oxygen pressure. After birth, the baby breathes room air; there is a rapid increase in oxygen intake. Infants born at term are adapted to face this situation [138]. Premature infants, as mentioned above, are born with insufficient antioxidant defenses to cope with this oxygen stress.

Briefly, inspired oxygen (O_2) is a diatomic molecule with two free electrons. The standard redox potential of ½ O_2: H_2O is high in biology (+0.82 V). Oxygen is easily reduced in water through various mitochondrial protein complexes. During these processes, about 1-3% of O_2 will be partially reduced, with generation of superoxide anions ($O_2^{\bullet -}$) [164]. Similarly, dissolved O_2 can accept an electron from several donors such as polyunsaturated fatty acids or ascorbic acid, generating a superoxide anion. This spontaneous reaction results in the formation of oxidized vitamin C and/or by-products of fatty-acid oxidation such as lipid peroxides, aldehydes, and isoprostanes [133]. Superoxide dismutase (SOD) converts two superoxide anions into H_2O_2 and oxygen. H_2O_2 can be reduced to H_2O by two enzymes, catalase or GPx. [165] In premature newborns with immature antioxidant defences, however, the effects of peroxide are disastrous. [166] High intracellular concentrations of H_2O_2 switch the redox potential to a more oxidized status, which could be an early factor leading to the development of several pathological neonatal complications.

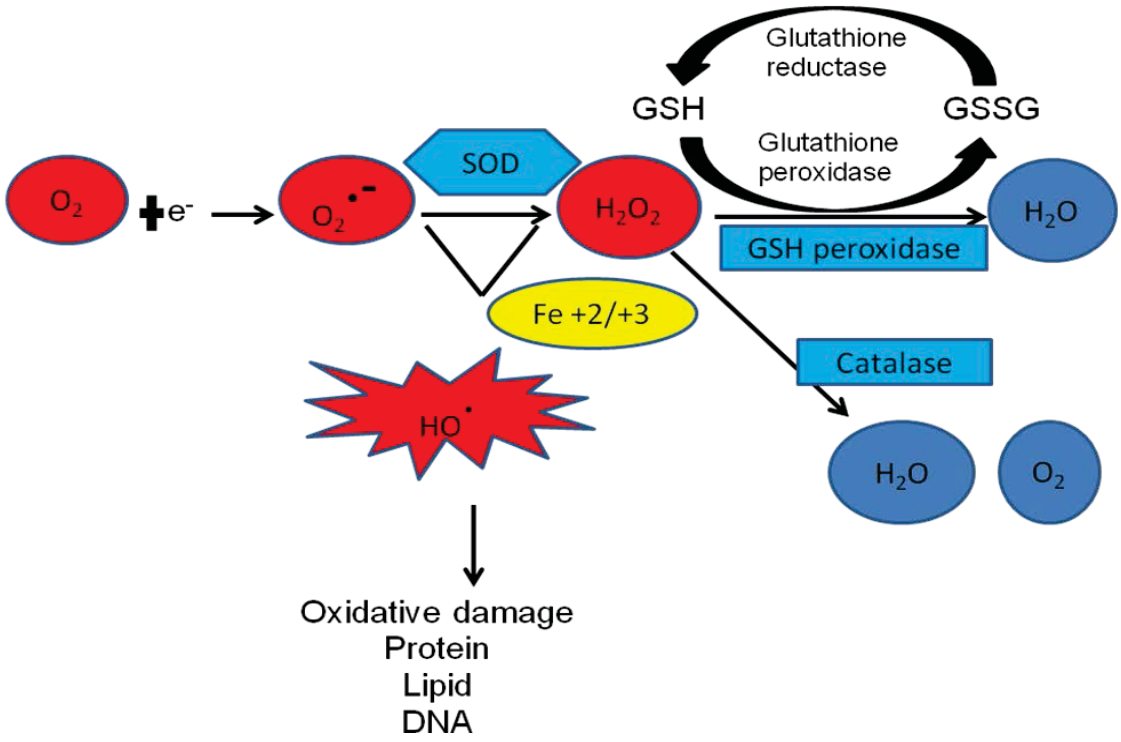


Figure 13. Different oxidants and antioxidant enzymes. O_2: Oxygen, $O_2^{\bullet -}$, Superoxide anions, H_2O_2 : hydrogen peroxide, Fe : iron, HO$^{\bullet}$: Hydroxyl radical , SOD: Superoxide dismutase

Thus, in neonatal units, high oxygen and the administration of PN both have an impact on BPD, sharing H_2O_2 as a common factor. In fact, Mohamed I et al. from our team have shown that high FiO_2 and duration of PN have an additive impact on the prevalence of BPD [167].

1.7.3.2 Parenteral nutrition

As mentioned earlier, premature infants depend on PN to meet their nutritional needs. Although PN is essential for growth and development, PN is also associated with undesirable effects caused by oxidant molecules generated in the solution. [168]

In fact, PN represents the second major source of oxidant molecules in the premature population because PN is contaminated with peroxides. In PN solution, the dissolved oxygen causes the oxidation of polyunsaturated fatty acids, amino acids and vitamin C. Several molecules are formed as a result of these interactions. [169] Among them are aldehydes and hydroperoxides derived from lipid peroxidation, and H_2O_2 [170], as well as ascorbylperoxide (AscOOH) from ascorbic acid. [171] Because PN contains a photo-sensitive compound, riboflavin, these reactions are catalyzed by ambient light. The interaction between oxygen and ascorbate in the presence of photo-excited riboflavin, the last two derived from the parenteral multivitamin preparation, generates H_2O_2 and AscOOH. [172]

Furthermore, these reactions contribute to the loss of fatty acids, amino acids, and antioxidant vitamins such as ascorbate. Even perfect photoprotection of the solution reduces the concentration of peroxides by only half. However, this reduction is sufficient to induce detrimental metabolic changes in children. [173,170] Unfortunately, it is difficult to reach such photoprotection in the clinical setting. Because even a few minutes of light exposure is sufficient to generate significant amounts of oxidant molecules in PN solution, photoprotection must be initiated during PN preparation in the pharmacy without any exposure to light until it reaches the bedside, where the bag and delivery tubes must be fully covered.

1.7.4 Oxidative stress markers in the premature infants

The infusion of PN without adequate photoprotection is associated with several markers of oxidative stress in premature infants, such as high concentrations of peroxides in the urine. [174] Moreover, urinary 8-hydroxy-20-deoxyguanosine has been reported to be higher in preterm than term infants. [138] As with the redox potential of glutathione in whole blood,

urinary dityrosine and isoprostane are elevated in premature infants as compared to adults. [146]

1.7.5 Relation between oxygen supplementation and BPD

Although oxygen is considered a strong oxidant and oxidative stress is suspected to induce BPD, recent reports suggest the absence of a relationship between them. Recently, advances in clinical practice such as the use of surfactant and continuous positive airway pressure have led to a reduction in oxygen supplementation. Stroustrup et al. documented that the reduction in oxygen use in neonatal units between 1993 and 2006 has reduced the incidence of BPD by 30% [175]. Currently, the prevalence of BPD in Canada is 45% in this population (data from Cable News Network (CNN)). In animal studies (newborn preterm baboons), the reduction in the fraction of inspired oxygen had no significant impact on the levels of fibrosis and alveolar hypoplasia. [176] A 2010 clinical study of 1316 infants born at less than 28 weeks of gestation reported a non-significant effect of decreased fraction of inspired oxygen on the incidence of BPD. [177] In the study, oxygen supplementation had been calibrated to reduce blood oxygen saturations from 91-95% to 85-89%.

Briefly, oxygen supplementation remains an important factor in BPD development [167] but its reduction in the clinical setting is at its limit. The second main source of oxidants leading to BPD is PN.

1.7.6 The relation between PN and BPD

The infusion of PN to premature infants without adequate photoprotection is associated with several markers of oxidative stress such as urinary peroxides [174] and an oxidized redox potential in whole blood. [146] This stress is associated with a greater incidence of several pathological complications observed in this population, such as BPD. In clinical studies, photoprotection halves the concentration of peroxides in solutions of PN received

by children and decreases the incidence of BPD in premature infants born before 32 weeks of gestation. [178, 166]

In animal studies, the infusion of PN without adequate photoprotection was associated with a lower level of alveolarization (hypoplasia) [119], the principal characteristic of BPD. Moreover, a higher level of apoptosis was observed in lungs with alveolar hypoplasia.[119] This alveolar hypoplasia was not associated with H_2O_2-contaminated PN but with the interaction between ascorbate and riboflavin. [119] Because this interaction also generated AscOOH [172], this molecule was suspected to induce the loss of alveoli in animals and to induce BPD development in premature infants. What is this molecule?

1.7.7 Ascorbylperoxide (AscOOH)

Vitamin C works as an antioxidant by donating 2 electrons to an oxidant molecule, such as oxygen, thus protecting other compounds from oxidation. [179] Semi-dehydroascorbate, an ascorbyl radical, is formed after the loss of one electron. Loss of a second electron forms dehydroascorbate (DHA). In vivo, DHA is reduced back to ascorbate by many mechanisms within the cell, including direct reduction by GSH and enzymatic reduction by various thiol transferases or NADPH-dependent reductases [180]. In vitro, such as in PN, DHA is hydrolyzed irreversibly to diketogulonate.

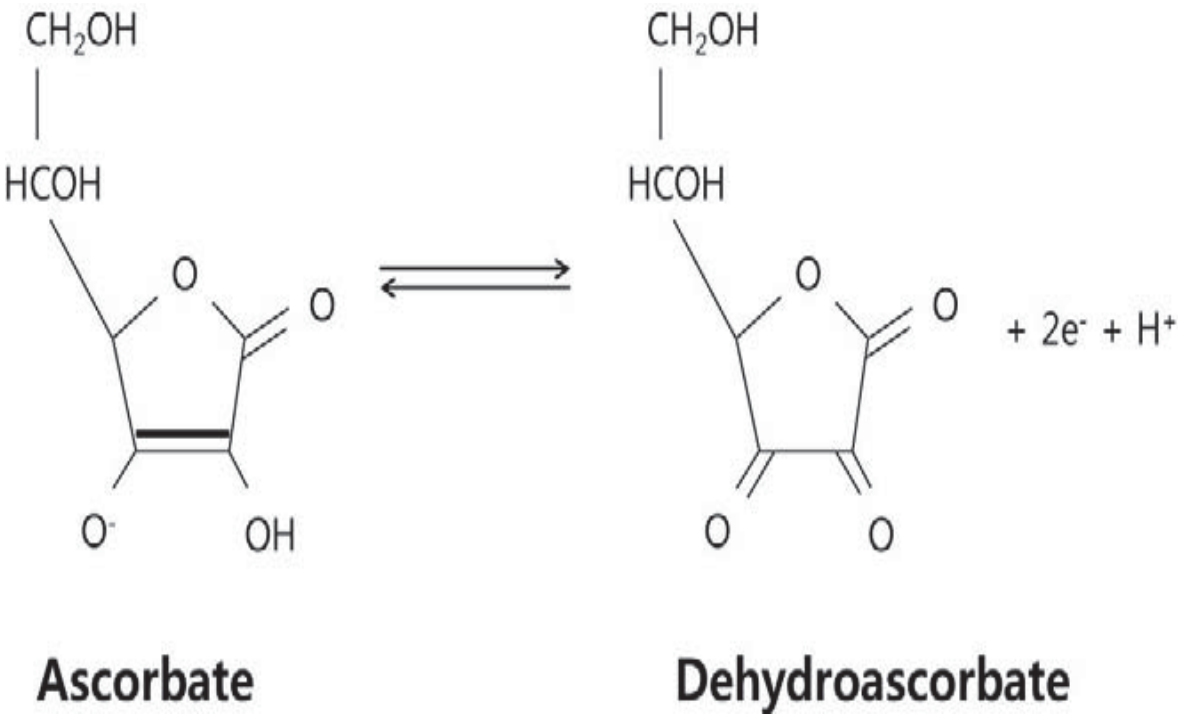


Figure 14. Molecular structure of L-ascorbate and dehydroascorbate, an oxidation product formed by the loss of 2 electrons adapted from [181]

In PN, ascorbate reduces the oxygen to hydrogen peroxide. When exposed to light, the H_2O_2 is split into a hydroxyl anion (HO^-) and HO•. Subsequently, this radical favours the opening of the lactam ring to form a peroxide species, as shown in Figure 15. This new molecule is 2,3-diketo-4-hydroperoxyl-5,6-dihydroxyhexanoic acid [172], or ascorbylperoxide. Because this molecule was suspected to be involved in BPD development, it is at the heart of my work.

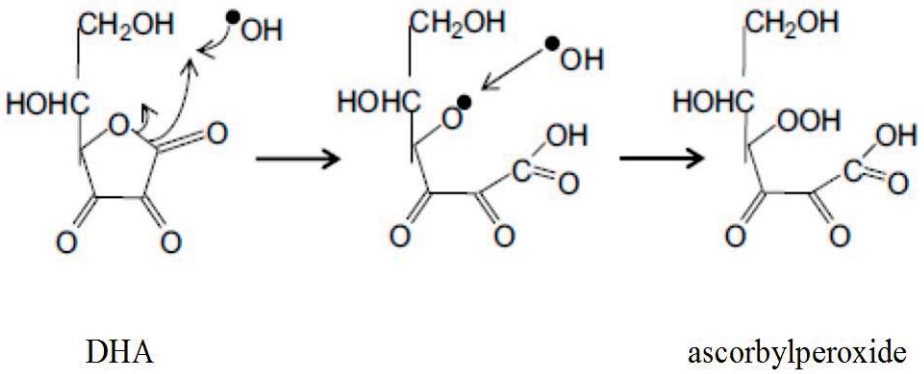


Figure 15. Generation of ascorbylperoxide from dehydroascorbate. Adapted from [172]

2. Hypothesis and objectives

The general hypothesis was that PN as administered in neonatal units is a major source of oxidative stress that contributes to the development of BPD. Based on previous works, the **specific hypothesis** of the thesis was that ascorbylperoxide, an oxidized form of the dehydroascorbic acid spontaneously generated in PN, disrupts alveolar development. The main mechanism of action is an inhibition of the transformation of methionine into cysteine in the liver, leading to a lower glutathione synthesis in the liver as well as in peripheral tissues such as lung. Lower GSH concentrations favour a shift of redox potential to a more oxidized state and consequently, to exaggerated apoptosis. If our hypothesis is correct, the addition of glutathione to PN would help detoxify ascorbylperoxide through the action of glutathione peroxidase and prevent the deleterious impact of PN.

The general objective was to investigate the biochemical mechanisms linking PN to the development of BPD in premature newborns and to propose a nutritional alternative that would prevent the deleterious impacts of PN in lungs of this animal model.

The specific objectives were:

1- To assess, in newborn guinea pigs, the effect of intravenously infused ascorbylperoxide on the metabolic axis redox potential of glutathione in the lung; specifically, apoptosis and the alveolarization index. This objective was reached by infusing increasing doses of ascorbylperoxide in presence or not of H_2O_2 (peroxide generated in PN) during 4 days.

2- To study the impact of ascorbylperoxide and the redox potential on the activity of MAT in the liver; methionine adenosyltransferase is the first enzyme in the metabolic cascade from methionine to cysteine. This objective was reached by investigate mechanisms of inhibition.

3- To try to prevent the deleterious impact of PN or ascorbylperoxide infusions on the lung by improving glutathione status. This objective was reached by adding glutathione in infused solutions.

3.Methods

3.1 Guinea pigs as an animal model

Our model is newborn guinea pig infused during 4 days with various solutions containing PN components (concentrations closed to what is administered in clinical neonatal unit) through a catheter in jugular vein. Guinea pigs and humans are dependent on vitamin C, a strong antioxidant, they are unable to synthesize this vitamin. This is an essential characteristic when we study mechanisms related to oxidative stress [182, 183]. Also it is an accessible animal model for the study of neonatal PN because at three days of life, the size of its body is sufficient for insertion of the smallest catheter of the world in its jugular vein, by this age; their glutathione development is not complete, similarly to humans [184, 185].

The problematic of PN is observed mainly in premature newborns. Clinical studies on the impact of PN are difficult since no control existed, premature infants of the same gestational age without PN or PN without peroxide do not exist. By choosing a term newborn animal, we can separate the impact of PN from prematurity [184]. Similarly to premature infants on PN, several markers of oxidative stress have been measured in this animal model on PN [186]. The absence of photo-protection of PN induced a loss of alveoli, a characteristic feature of the bronchopulmonary dysplasia such observed in premature infants [187].

Additionally, by choice, all the animals in our experiments are males. Lavoie has shown [143], in cells extracted from endotracheal aspirates from the babies, that the increased curve of glutathione levels in function of the gestational age is slower in boys. These results suggesting that the baby girl is potentially better protected against an oxidative stress than boy. Sex is an important determinant of the incidence of BPD among premature infants,

and boys are have much higher incidence to this disease. In addition the recent published results demonstrat that there is a correlation between this disease and the redox potential of the glutathione [146]. Thus this the main reason for choosing male animals to examine our hypothesis with the population that is more vulnerable for oxidative stress.

At three days of life Hartley guinea pigs (Charles River Laboratories, St.Constant, Quebec, Canada) , were anesthetized by using ketamine (50mg/mL;0,18mL/100g) and xylazine (20mg/mL; 0,05mL/100g) in order to fix a catheter (Luther Medical Products, Tustin, CA) in jugular vein. The catheter was placed and externalized in the scapular region, with a branch connected to the infusion system. The studied intravenous solutions were infused continuously through the catheter at rate of 22 mL / 100 g body weight / day. The solutions were changed daily.

After 4 days, at seven days of age, animals were sacrificed for collection of lungs. After ligation of right bronchi, the left lung was filled with a solution of 10% formalin in PBS at a pressure of 10 cm, and stored in the same solution for histological preparation. The right lung was removed, processed, aliquoted and stored at -80^{o}C until biochemical determinations. The urine samples were collected in the last day of the protocol for the determination of ascorbylperoxyde concentration. These protocols, accepted by the Institutional committee for good animal practice in research, were carried out in accordance with the Canadian Council of Animal Care guidelines.

The protocols and statistical method of my thesis are precisely described in the three articles. However, some biochemical and histological methods are abbreviated in those articles here is the details of abbreviated methods of the articles.

3.2 Ascorbylperoxide generating system

Knafo et al., [172] has identified ascorbylperoxyde in solution of parenteral nutrition. This molecule is new and the pure molecule does not commercially exist. Ascorbylperoxyde can be obtained by mixing the following molecules: 1.8 mM ascorbate + 400 μM H_2O_2 + 30 μM riboflavin (pH 7). The solution was incubated at room temperature with strong stirring under ambient light exposure (75 foot-candle) for 48 hours. The control solution was obtained by a similar system, but without ascorbate. The solutions were treated 20 min with100 U/mL catalase and filtered against a 30 kDa filter Centricon Plus-20 (Millipore Corporation); FOX assay was used to ensure the absence of H_2O_2. This generating system produced a solution of 1800 μM ascorbylperoxide. Following dilutions in HPLC water solutions of 20, 60 and 180 μM ascorbylperoxide have been made.

3.3 Ascorbylperoxide measurement

The quantification of ascorbylperoxide in these solutions as well as in animals urine were assessed by using Agilent LC/MS 1100 mass spectrometer as mentioned in Knafo et al., [172]. Urine samples were centrifuged 1 min at 7000 g. Five μL of 2.5 mM OTC (an internal standard in this assay, because its molecular weight is near to the molecular weight of ascorbylperoxide moreover this molecule is not produced in humans) was added to 95 μL of sample and 4 μL of this solution was injected on LC column ZORBAX Eclipse XDB C18 (Agilent). The elution was performed with isocratic mixture of ammonium acetate 10 mM (pH 7): acetonitril (1:1) at 0.4 mL/min. Retention time was 2.6-2.8 min for ascorbylperoxide and 4.0 – 4.3 min for OTC. Ascorbylperoxide and OTC was quantified by monitoring ion abundance at m/z 207 (ascorbylperoxide), 147 (OTC) and 293 (OTC dimer) from electrospray ionization mass spectrometry (Agilent 1100 single quadrupole) (negative mode, 25V, source temperature of 350°C, nitrogen nebulizer gas flow of 12 L/min). Total

OTC abundance (abundance at 147 m/z + 2 fold abundance at 293) was used as internal standard [171].

3.4 Determination of peroxides

Total peroxides were determined by following at 560 nm the generation of chromophore from the complex Fe^{3+}-xylenol orange [188,189]. The principle is based on the Fenton reaction in which ferrous ion (Fe2+) is oxidized in presence of H_2O_2, or other peroxides, in its ferric form (Fe3+). Thus, diluted PN was mixed with reactive solution containing 22.5 mM H_2SO_4, 90 μM xylenol orange, 225 μM $FeCl_2$, and 3.6 mM 2,6-di-*tert*-butyl-4-methylphenol in methanol. After 30 min of incubation, at room temperature, the solutions were centrifuged at 5500 × g for 3 min). The absorbency of the supernatant was read at 560 nm. The results were expressed in μM. H_2O_2 was used for the external standard curve.

3.5 Protein determination

Protein was measured by Bradford method, the principle of this technique is a dye-binding reaction to protein with a differential color change of a dye (Coommassie) occurs in response to the protein concentrations. The absorbance was detected at 595nm. The pellets from different tissues were solubilized with 1N NaOH at 37 °C for 2 hours. Addition dilution (1 /250) was made and the diluted samples were transferred to spectrophotometric cuvettes. Bradford reagent previously diluted (1 / 5) with pure water was added. Samples were incubated 10 minutes at room temperature. The absorbance values were compared to the curve of bovine serum albumin (BSA; 0 – 90 pg/μL).

3.6 Alveolarization index

At the time of the sacrifice the right lung and left lung were separated. The right lung was isolated by ligation of the right bronchus following an incision in the trachea. The left lung was filled with 10% formalin at 10 cm pressure; the lungs were stored in formalin until histological preparation. Histological slides were prepared by the pathology service of the CHU Sainte-Justine. Hematoxilin-eosin coloration was used. The mean number of intercepts between a standardized straight-line and histological structure (200X magnification) on four different fields were used as an index of alveolar counts [119]. The right lung was collected, sampled, prepared and frozen at -80 ^{o}C until biochemical determinations.

3.7 GSH and GSSG levels

Immediately after sampling, 0.25 g of lung was mixed with 5 volumes of 5% (w/v) freshly prepared metaphosphoric acid. The lung sample was homogenated on ice during 20 seconds with Polytron (*Biospec Products, Bartlesville, OK, USA*). Thereafter, they were centrifugated for 3 min at 10000 RPM. Supernatants (for glutathione determinations) and pellets (for protein determination) were separated and frozen at -80°C until the day of the assay. Reduced (GSH) and disulfide (GSSG) forms of glutathione were separated by capillary (75 μm×50 cm silica) electrophoresis (75 mM boric acid +25 mM Bis–Tris buffer, pH 8.4, 28°C, 18 kV) and were detected at 200 nm on a P/ACE MDQ system (Beckman Coulter). GSH and GSSG were used as external standard curves. The redox potential was calculated by using the Nernst equation (25ºC, pH 7): Ehc = -240-(59.1/2) log ([GSH] 2/ [GSSG]) mV [158]. For this calculation, we assumed that the density of the tissue is one.

3.8 Determination of glutathione peroxidase activity

Glutathione peroxidase activity was measured by mixing the solution with reactive solution (250 mM Tris, 0.1mM EDTA-Na $_2$, 1 mM GSH, different solutions of AscOOH, 4.8 U/mg GR, and 0.1mM NADPH, pH 7.6). Determination of the glutathione peroxidase activity is based on the following principle

2GSH + TBH→ GSSG + BOOH

Then GR reduces the GSSG to complete the cycle:

GSSG + NADPH + H+ → 2 GSH + NADP+

The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPx activity. Slope of the drop of absorbance recorded during 5 minutes was quantified using the NADPH molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. The activity was expressed as μmol NADPH oxidized/min/mg protein at 30C° [143].

3.9 Nuclear and cytoplasmic extraction

Lung tissue weight is ranging 0.6-0.9 g for one animal. It is too small to do several determinations such as redox potential, enzyme's activities and Western blot of several proteins. Thus I have made one extractions (0.1 g of lung tissue) to do several protein expression by Western blot. The extractions were made as follow.

Cytoplasmic and nuclear extracts were isolated by using a commercially nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). According to the manufacturer's protocol, tissue was thawed and washed with PBS (pH 7.4). Then 0.1 g of lung tissue was homogenated in 1000 μL of ice cold cytoplasmic extraction reagent-1 for 10 min, and 55 μL of ice-cold cytoplasmic extraction reagent-2 for 1 min,. After centrifugation at 16,000*g* for 5 min, the supernatant (cytoplasmic extract) was stored on ice, while the insoluble pellet fraction was resuspended in 55 μL of nuclear-extraction reagent.

After a 40-min incubation period, with vortexing every 10 min and another centrifugation at 16,000*g* for 10 min, the supernatant (nuclear extract) was collected. Cytoplasmic and nuclear protein concentrations were measured by Bradford method as mentioned above. Extracts were aliquoted and stored at -80^{o} C until the day of the determination.

3.10 Apoptosis level

Western blot was used to quantify the cytoplasmic caspase 3 and cleaved caspase 3 in the lung tissues in response to treatments. Briefly, samples (cytoplasmic extracts) were` adjusted to equal amounts of protein (50 µg protein/lane). Proteins were run on electrophoresis (SDS-PAGE) gel. After electrophoresis proteins were transferred (90 V, 2 hours, 4ºC) to a 0.45-µm pore size polyvinylidene difluoride membrane (BioRad) in tris-glycine transfer buffer. After transfer, the membrane was blocked in tris-buffered saline(TBS) containing 2.5% milk powder (Bio-Rad) for 1 h, then the membrane was incubated overnight at 4ºC with a 1:1000 dilution of polyclonal anti-rabbit caspase-3 (Cell Signaling Technology). Detection was performed by using horseradish peroxidase–conjugated secondary anti-rabbit (Cell Signaling Technology) at a 1:2000 dilution, and the enhanced chemiluminescence reagent system (Femto, Pierce, U.S.A), using Kodak BioMax light film. To evaluate the levels of reference protein (alpha tubulin), the blots were stripped in Western-blot stripping buffer (Pierce) for 15 min, at room temperature. After being blocked in TBS containing 5% milk powder for 1 h at room temperature, the blots were incubated with a polyclonal mouse antihuman (US Biological) at a dilution 1:500, and with a secondary anti-mouse horseradish peroxidase-conjugated antibody (R&D Systems) at a dilution 1:2000, for 1 hour at room temperature, with detection as described above.

To quantitatively assess the amount of cleaved caspase-3 in the cytoplasm, the films were digitally imaged. The pixel intensity of each band determined, by using image analysis

software (UN-scan-it gel 6.1). The activation of caspase-3 and cleaved caspase 3 were calculated relative to tubulin [190].

3.11 Activation of Nrf2

Western blot was used to quantify the cytoplasmic and nuclear Nrf2 in the lung tissues in response to treatments. Briefly, samples (cytoplasmic and nuclear extracts) were adjusted to equal amounts of protein (30 μg protein/lane). Proteins samples were run on electrophoresis (SDS-PAGE) gel. After electrophoresis proteins were transferred (90 V, 2 hours, 4ºC) to a 0.45-μm pore size polyvinylidene difluoride membrane (BioRad) in Tris-glycine transfer buffer. After transfer, the membrane was blocked in phosphate-buffered saline (PBS) containing 5% milk powder (Bio-Rad) for 1 h, then the membrane was incubated overnight at 4ºC with a 1:1000 dilution of anti-human Nrf2 monoclonal IgG (Novus Biological) . Detection was performed by using a secondary anti-rabbit horseradish peroxidase-conjugated antibody (Cell Signaling Technology) at 1:2000, for 1 hour at 23ºC, followed by the enhanced chemiluminescence reagent system (Plus-ECL,PerkinElmer, U.S.A), using Kodak BioMax light film. To evaluate the levels of nuclear protein in the nuclear extracts, the blots with nuclear extracts were incubated with anti-human Nrf2 monoclonal IgG (Novus Biological) at a 1:1000 dilution, and with a secondary anti-rabbit horseradish peroxidase-conjugated antibody (Cell Signaling Technology) at 1:2000, for 1 hour at 23ºC [191], with detection as described above.

To evaluate the levels of reference protein (alpha tubulin), the blots were incubated with a polyclonal mouse antihuman (US Biological) at a 1:500 dilution, and with a secondary anti-mouse horseradish peroxidase-conjugated antibody (R&D Systems) at a dilution 1:2000, for 1 hour at 23ºC, with detection as described above .

To quantitatively assess the amount of Nrf2 in the cytoplasm and nucleus, the films were digitally imaged. The pixel intensity of each band determined, by using image analysis software (UN-scan-it gel 6.1). The quantity of Nrf2 was determined relative to tubulin.

3.12 Activation of NFκB

Western blot was used to quantify the level of cytoplasmic and nuclear NFκB in lung tissues in response to treatments. Briefly, samples (cytoplasmic and nuclear extracts) were adjusted to equal amounts of protein (50 μg protein/lane). Proteins were run on electrophoresis (SDS-PAGE) gel. After electrophoresis proteins were transferred (90 V, 2 hours, 4ºC) to a 0.45-μm pore size polyvinylidene difluoride membrane (BioRad) in Tris-glycine transfer buffer. After transfer, the membrane was blocked in PBS containing 5% milk powder (Bio-Rad) for 1 h, then the membrane was incubated overnight at 4ºC with a 1:1000 dilution of mouse polyclonal NFκB antibody (MaxPab). Detection was performed by using a secondary anti-mouse horseradish peroxidase-conjugated antibody (R&D Systems) at a dilution 1:2000, for 1 hour at 23ºC, following by the enhanced chemiluminescence reagent system (Plus-ECL, PerkinElmer, U.S.A), using Kodak BioMax light film. To evaluate the levels of nuclear protein in the nuclear extracts, the same as described above.

To evaluate the levels of reference protein (alpha tubulin), the blots were incubated with a polyclonal mouse antihuman (US, Biological) at a dilution 1:500, and with a secondary anti-mouse horseradish peroxidase-conjugated antibody (R&D Systems) at a dilution 1:2000, for 1 hour at 23ºC, with detection as described above.

To quantitatively assess the amount of NFκB in the cytoplasm and nucleus the films were digitally imaged. The pixel intensity of each band was determined, by using image analysis software (UN-scan-it gel 6.1). The level of NFκB was determined relative to tubulin.

3.13 Determination of Methionine adenosyl transferase

Liver sample was homogenated on ice during 20 seconds with Polytron (*Biospec Products, Bartlesville, OK, USA*) in 4 volumes of (10 mM Tris/HCL, 0.3 M sucrose, 1 mM benzamidine, 0.1 mM phenylmethanesulfonylfluoride, and pH 7.5). The homogenate was centrifuged at 40000 g for 2h30 at 4 C°. The supernatants were taken for determination of MAT activity and protein level. The supernatants were incubated at 37C° for 30 min with (75m M Tris/HCL, 250 mM KCl, 9 mM $MgCl_2$, 60 µM methionine and 5 mM [2-^{3}H]ATP (1Ci/mol), pH 7.8). The reaction was stopped by adding 3 ml of cold water and immediately applied onto the cation exchanger Dowex AG5OW columns (0.5 ml) that was previously prepared and balanced with water, adjusted pH at 7 with NaOH. The chromatography was made at room temperature. Subsequently, the column was washed with 20 ml water to get rid of the unused radioactive substrate. The H^3-S-adenosyl methionine was eluted by using 4 ml of 3M NH_4OH. The radioactivity of this fraction was measured by liquid scintillation by a beta counter (Beckman Co, Fullerton, CA, USA). The activity was expressed as nmol-S-adenosyl methionine formed/min/mg protein at 37°C. [192, 193,194]

Contributors' Statement:

Ascorbylperoxide from parenteral nutrition induces an increase of redox potential of

glutathione and loss of alveoli in newborn guinea pig lungs. (Redox Biol. 20;2:725-31,2014).

Wesam Elremaly: I contributed to the study design, help in animal manipulations, performed redox potential analyses, Western blots and histology, carried out the initial analyses, drafted the initial manuscript, and I approved the final manuscript as submitted.

Dr. **Ibrahim Mohamed:** Dr Mohamed contributed to the analysis and interpretation, reviewed and approved the final manuscript as submitted.

Dr Tiphaine Mialet-Marty: Dr Marty performed PGJ2 analyses, critically reviewed the manuscript, and approved the final manuscript as submitted

Thérèse Rouleau: Ms. Rouleau performed the animal surgeries and care, supervised the biochemical determinations, critically reviewed the manuscript, and approved the final manuscript as submitted.

Jean-Claude Lavoie: Dr Lavoie is the author of the study conception; he has supervised the team's work, finalized analyses and interpretation of the data. He has finalized the writing of the manuscript.

Ascorbylperoxide from parenteral nutrition induces an increase of redox potential of glutathione and loss of alveoli in newborn guinea pig lungs (Redox Biol. 20;2:725-31,2014).

Wesam Elremaly, Ibrahim Mohamed, Tiphaine Mialet-Marty, Thérèse Rouleau and Jean-Claude Lavoie.

Highlights

- Oxidative stress is suspected to induce bronchopulmonary dysplasia.

- We investigate the role of ascorbylperoxide contaminating parenteral nutrition.

- This molecule induces oxidation of redox potential, apoptosis and loss of alveoli.

- The alveolar loss is independent of the redox potential.

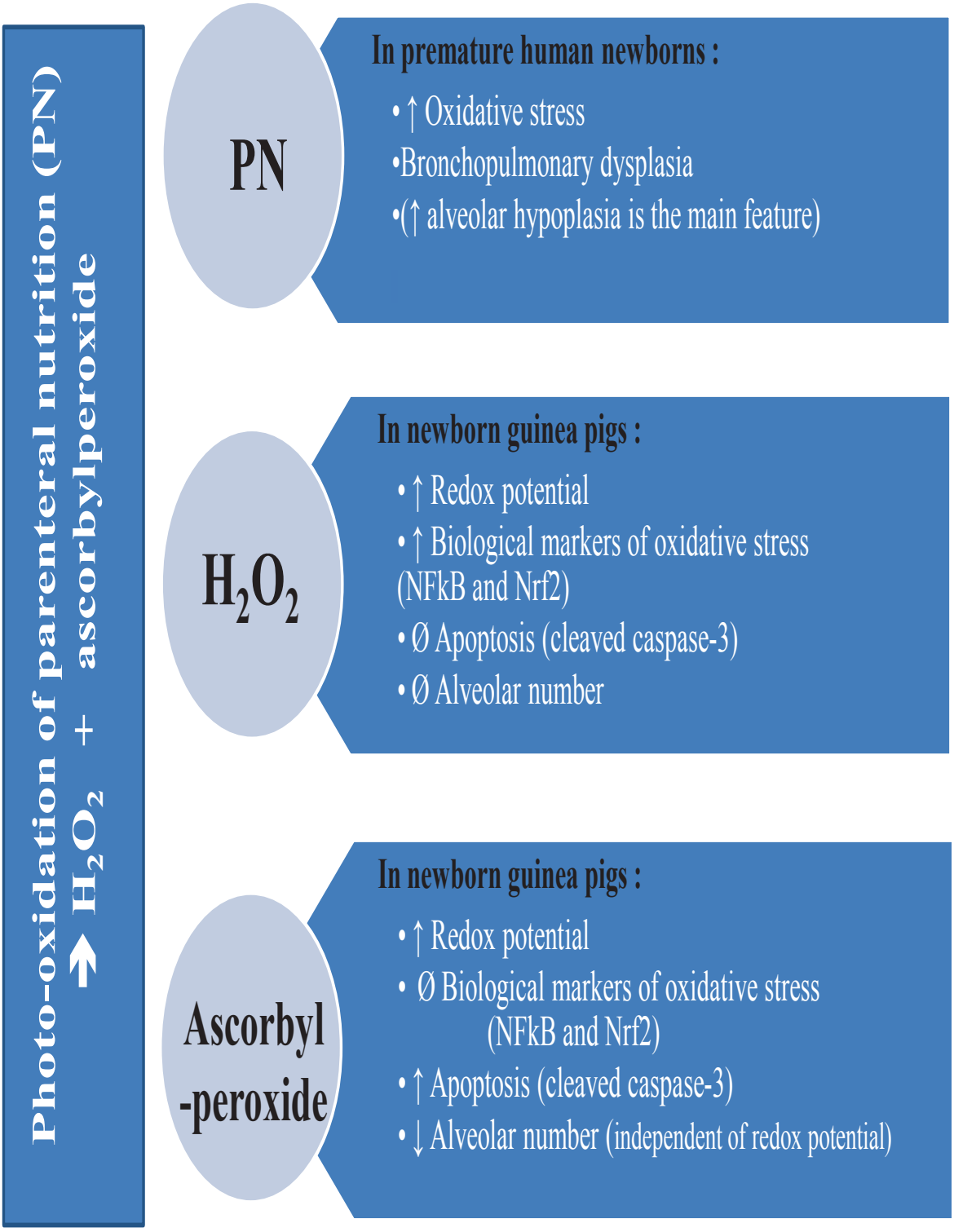


Photo-oxidation of parenteral nutrition (PN)
➔ H_2O_2 + ascorbylperoxide
PN
In premature human newborns :
• ↑ Oxidative stress
•Bronchopulmonary dysplasia
•(↑ alveolar hypoplasia is the main feature)
H_2O_2
In newborn guinea pigs :
• ↑ Redox potential
• ↑ Biological markers of oxidative stress (NFkB and Nrf2)
• Ø Apoptosis (cleaved caspase-3)
• Ø Alveolar number
Ascorbyl -peroxide
In newborn guinea pigs :
• ↑ Redox potential
• Ø Biological markers of oxidative stress (NFkB and Nrf2)
• ↑ Apoptosis (cleaved caspase-3)
• ↓ Alveolar number (independent of redox potential)

Abstract

Background: Bronchopulmonary dysplasia is one of the main complications associated with extreme prematurity. Oxidative stress is suspected to be a trigger event of this lung disease, which is characterized by impaired alveolar development. Peroxides, mainly ascorbylperoxide and H_2O_2, are known contaminant of parenteral nutrition. We hypothesize that these oxidant molecules induce bronchopulmonary dysplasia development. The aim was to determine if the infusion of ascorbylperoxide, whether in presence or absence of H_2O_2, is associated with oxidative stress, apoptosis and loss of alveoli in the lungs of newborn guinea pigs.

Method: Three days-old guinea pigs received parenteral solutions containing 0, 20, 60 or 180 μM ascorbylperoxide in the presence or not of 350 μM H_2O_2 (concentrations similar to those measured in parenteral nutrition). After 4 days, the lungs were collected for determination of glutathione's redox potential, caspase-3 activation (an apoptosis marker), alveolarization index (by histology), activation of Nrf2 and NFκB (biological markers of oxidative stress), and IL-6 & PGJ_2 levels (markers of NFκB activation). Groups were compared by ANOVA, $p < 0.05$.

Results: Loss of alveoli was associated with ascorbylperoxyde in a dose-dependent manner, without an influence of H_2O_2. The dose-dependent activation of caspase-3 by ascorbylperoxide was lower in the presence of H_2O_2. Ascorbylperoxide induced an increase of redox potential in a dose-dependent manner, which reached a plateau in presence of H_2O_2. Nrf2 and NFκB were activated by H_2O_2 but not by ascorbylperoxide.

Conclusion: Results suggest that ascorbylperoxide, generated in parenteral nutrition, is involved in the development of bronchopulmonary dysplasia, independently of the increase

of the redox potential. This study underlines the importance of developing a safer formulation of parenteral nutrition.

Key words: Premature newborn; oxidative stress; bronchopulmonary dysplasia; chronic lung disease; glutathione.

Introduction

Decreased alveolar number and impaired vascular development are characteristic features of bronchopulmonary dysplasia (BPD) (1, 2). Extremely premature infants (<29 weeks of gestation) are affected by BPD in 50% of cases. Amongst the multiple consequences of BPD, lung function impairment, which can persist until adulthood (3-5), and altered neurodevelopment are the most worrisome (6, 7). Requirement in oxygen supplementation, with its associated oxidative stress, has long been recognized as a component in the aetiology of BPD (1, 2, 8). Recently, parenteral nutrition (PN) has been suggested to play an important role in BPD development. It has been reported that peroxides, which contaminate PN, induce oxidative stress in newborn animals (9) as well as in premature infants (10). Light-protection of PN reduces by half the peroxide generation in intravenous solution (10-12), decreases the incidence of BPD (13) and chronic lung diseases (12) in premature infants and prevents the alveolar loss in newborn guinea pigs (14). An animal study by Lavoie JC *et al.* (15) reported that an exaggerated apoptosis is associated with the loss of alveoli, independently of the H_2O_2 level present in PN. This study also described that the loss of alveoli is reproduced by the infusion of a solution containing vitamin C and riboflavin, components found in PN. Interaction between these two vitamins, in presence of ambient light, generates a new organic peroxide derived from the peroxidation of dehydroascorbate by H_2O_2 (16, 17). We suspect that this molecule (2,3-diketo-4-hydroxyperoxyl-5,6-dihydroxyhexanoic acid), named in the current report as "ascorbylperoxide", participates in the development of BPD.

Peroxides are detoxified by the glutathione metabolism, in which GSH is oxidized into GSSG. The concentration of both forms of glutathione impacts on the cellular redox environment. Because cell cycle (proliferation, differentiation and apoptosis) is influenced

by the redox environment (18), the presence of peroxides in PN can induce arrest of pulmonary development or even apoptosis. Chessex *et al.* (19) have recently reported that the level of redox potential of glutathione measured in blood of premature infants was strongly correlated with the severity of BPD (19). We hypothesize that ascorbylperoxide infused with PN induces oxidative stress characterized by a higher redox potential, which stimulates apoptosis and leads to alveolar loss. Therefore, the aim of this study was to document that the infusion of increasing concentrations of ascorbylperoxide is associated with an elevation of redox potential of glutathione, activation of an apoptosis marker and a reduced number of alveoli in the lungs of newborn guinea pigs Because PN contains also H_2O_2, which can potentially exacerbate the oxidation of glutathione, the intravenous solutions infused to animals in this study will contain or not H_2O_2 (ascorbylperoxide ± H_2O_2).

Materials and Methods

Sixty Hartley guinea pigs (Charles River Laboratories, Saint-Constant, Quebec, Canada) have been used in eight groups (6-9 animals per group). At three days of life, a catheter (SAI Infusion Technologies, Lake Villa, IL) was fixed in the right jugular vein as previously described (13, 14, 20) to inject the experimental intravenous solutions freshly prepared each day. The solutions, which were continuously infused at a rate of 19 mL/100g/d (21), contained: 8.7% (w,v) dextrose + 0.3% (w,v) NaCl + 1 U/mL heparin + 0, 20, 60 or 180 μM ascorbylperoxide ± 350 μM H_2O_2 (concentrations previously measured in total parenteral nutrition) (11, 14). A usual total parenteral nutrition containing 1% of a multivitamin preparation (Multi-12 pediatric, Sandoz, Boucherville, QC, Canada) generates 36 ± 1 μM ascorbylperoxide (J.C. Lavoie, personal communication). Because

ascorbylperoxide does not commercially exist, it was generated *in vitro*, as previously described (21). After 4 days, lungs were collected as per the protocol previously explained in (14) and (15). The right lung was used for biochemical measurements and the left one was used for histology. Urine was collected directly from the bladder for determination of ascorbylperoxide concentration. Forty-eight animals had urine in their bladder at the time of sacrifice. The protocols were approved by the Institutional Committee for Good Practice with Animals in Research, in accordance with the Canadian Council of Animal Care guidelines.

Alveolarization index represents the mean value for each animal of the number of intercepts between a standardized straight-line (1 mm) and histological structures calculated from four different fields of the same lung (200X magnification) (14, 15).

Ascorbylperoxide concentration in intravenous solutions, as well as in urine, was quantified by using a LC/MS methodology previously described (16, 21). Because the internal standard was L-2-oxothiazolidine-4-carboxylate (OTC), results are expressed in OTC equivalent.

Glutathione (GSH and GSSG) was determined by capillary electrophoresis, as previously described (20-22), whereas the redox potential was calculated (25ºC, pH 7) by using the Nernst equation.

Apoptosis was defined by calculating the proportion of active (cleaved caspase-3) on total caspase-3 (cleaved + non-cleaved). These two forms of the protein were quantified by Western blot as described in (22).

Nrf2 and NFκB: In order to qualify the oxidative stress, in addition to glutathione redox state, protein levels of Nrf2 and NFκB have also been measured. Nrf2 is a nuclear factor

involving antioxidant defences. It favours the transcription of genes encoding for, among others, glutathione synthesis. $NF_{\kappa}B$ is a nuclear factor favouring inflammation. Determination by Western blot (23) of their cytosolic level and nuclear fraction, using a commercially nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA), allowed calculation of the proportion present in the nucleus (active form). They were reported as activated protein (nuclear level / nuclear + cytosolic levels). The Western blot method described by Hübner RH *et al.* (23) was used, except for the antibody against $NF_{\kappa}B$, for which we have used the MaxPab mouse polyclonal $NF_{\kappa}B$ antibody from Abnova (Taipei, Taiwan).

IL-6 and PGJ_2: From lung homogenate (1 in 4 volume of water), concentration of IL-6 was evaluated by the Human IL-6 ELISA kit (Anogen Mississauga, Ontario) whereas the 15-deoxy-$\Delta^{12,14}$-PGJ_2 EIA kit (Enzo, Plymouth, PA, USA) was used to measure PGJ_2 levels after a C-18 extraction (24).

Statistical analysis: Data from each group are presented as mean ± s.e.m, and have been compared by factorial ANOVA (4 concentrations of ascorbylperoxide x H_2O_2). All comparisons were orthogonal. To meet the homoscedasticity, which was verified by using the Bartlett's Chi squared test, data for GSH, Nfr2 and $NF_{\kappa}B$ have been logarithmically transformed. The level of significance was set at p-value < 0.05. Comparison between groups receiving the solutions devoid of ascorbylperoxide and groups infused with the three concentrations of this peroxide was used to statistically document the effect of ascorbylperoxide. The use of coefficients for linear and quadratic polynomials allowed analysis of the dose-response effect of ascorbylperoxide. A significant interaction between H_2O_2 and ascorbylperoxide led us to analyse the impact of ascorbylperoxide separately of the presence or absence of H_2O_2.

Results

There was no statistical difference between initial mean body weight among groups (104 ± 2 g (n=60); H_2O_2 effect: $F_{(1,52)} < 0.1$; ascorbylperoxide effect: $F_{(3,52)} = 1.3$). The urinary concentration of ascorbylperoxide (**Figure 1**) increased ($F_{(1,40)} = 12.6$, $p<0.01$) linearly ($F_{(1,40)} = 37,3$, $p<0.01$) in function of the dose of ascorbylperoxide received and was 25% lower in animals infused with solutions containing H_2O_2 ($F_{(1,40)} = 5.9$, $p<0.05$). The total amount of intravenous solution received was similar ($F_{(1,40)} = 0.3$) in the group receiving H_2O_2 (72.3 ± 1.3 mL) and in the group without H_2O_2 (71.9 ± 0.8 mL) .

The ANOVA revealed a significant interaction between ascorbylperoxide and H_2O_2 ($F_{(3,49)} = 3.1$, $p<0.05$) on the redox potential of glutathione in the lungs (**Figure 2A**). In the absence of H_2O_2, the ascorbylperoxide has induced an increase of the redox potential ($F_{(1,49)} = 7.7$, $p<0.01$) that was linearly dependent on the dose ($F_{(1,49)} = 4.4$ $p<0.05$), whereas in presence of H_2O_2 the redox potentials were not influenced by ascorbylperoxide ($F(3,49) = 0.7$). The mean value of redox in H_2O_2 groups was of -203 ± 1 mV (n = 28). This value was similar to the one measured in the groups 60 and 180 μM ascorbylperoxide without H_2O_2. The modification in redox potential was caused by modification in GSH rather than a change in GSSG value. Indeed, there was no modification in GSSG levels between groups (0.61 ± 0.03 nmol/mg prot, n= 57) whereas a significant interaction ($F_{(1,49)} = 4.0$ $p<0.05$) between ascorbylperoxyde and H_2O_2 was observed for GSH values (**Figure 2B**). The level of GSH was lower in the lung of animals infused with a solution containing ascorbylperoxide or H_2O_2. The actions of these peroxides were not additive (significant interaction) and the effect of ascorbylperoxide was not dose-dependent ($F_{(1,49)} < 2.1$) (**Figure 2B).**

Ascorbylperoxide and H_2O_2 influenced the levels of activated caspase-3 (**Figure 3**). The absence of significant interaction ($F_{(3,52)} = 1.0$) suggested an additive effect. In fact, data

show that ascorbylperoxide was a linear dose- dependent ($F_{(1,53)}$ = 5.3, $p < 0.05$) inducer ($F_{(1,53)}$ = 24.7, $p < 0.01$), whereas H_2O_2 had a 15% inhibitory effect ($F_{(1,53)}$ = 11.3, $p < 0.01$). The activated caspase-3 is defined as the ratio of cleaved caspase-3 on the total caspase-3 (cleaved + non-cleaved) (**Figure 4**). Ascorbylperoxide ($p < 0.01$) and H_2O_2 ($p < 0.05$) had a similar impact on cleaved caspase-3 and on activated caspase-3 whereas they did not influence the level of the non-cleaved enzyme. The low level of non-cleaved caspase-3 in the group 180 μM ascorbylperoxide without H_2O_2 remains without explanation.

Despite the negative impact of H_2O_2 on the apoptosis marker (Figure 3), the alveolarization index (**Figure 5A**) was influenced only by ascorbylperoxide ($F_{(1,47)}$ = 6.8, $p < 0.05$), not by H_2O_2 ($F_{(1,47)}$ = 0.1), without interaction ($F_{(3,47)}$ = 0.6). The decreasing in alveolarization index responded to a linear dose-effect of ascorbylperoxide ($F_{(1,47)}$ = 9.0, $p<0.01$). Examples of histology of lungs are shown on the **Figure 5B**.

Due to the difference in effect between both peroxides on alveolarization index, a doubt persisted on the value of the observed redox potentials and on the real oxidative capacity of each peroxide infused during the four days of the experiment. Thus, biological markers of oxidative stress, such as NFκB and Nrf2, were measured. Both peroxides were associated with a similar impact on activated NFκB and Nrf2 (**Figure 6A** and **6B,** respectively). Their levels were higher in animals receiving H_2O_2 ($F_{(1,49)}$ = 14.9, $p < 0.01$) and ascorbylperoxide ($F_{(1,49)}$ = 8.2, $p < 0.01$), without a dose-effect ($F_{(1,49)}$ = 1.0). The interactions did not reach statistical significance ($F_{(3,49)}$= 1.8).

Examples (n = 3 for each group) of Western blot of proteins measured in cytosolic and nuclear fractions are shown in **Figure 7**. Complete values are reported in **Figures 8 and 9**. NFκB levels in nucleus (Figure 8A) and in cytosol (Figure 8B) were higher in animals infused with solutions containing ascorbylperoxide ($F_{(1,49)}$= 4.9, $p < 0.05$), independently of

the presence of H_2O_2 (no interaction, $F_{(3,49)} = 2.8$). The effect of ascorbylperoxide was without dose-effect ($F_{(1,49)}=2.0$). NFκB was lower in the cytosolic fractions of lungs from animals infused with solution containing H_2O_2 ($F_{(1,49)} =23.1$, $p < 0.01$). H_2O_2 had no effect on values in nucleus ($F_{(1,49)} = 1.5$).

In the nucleus, the interaction between peroxides for Nrf2 was significant ($F_{(3,49)} = 3.0$, $p < 0.05$) (**Figure 9A**). The levels of Nrf2 were higher in animals receiving solutions containing both peroxides ($F_{(1,49)} = 9.1$, $p < 0.01$) without dose-effect of ascorbylperoxide ($F_{(1,49)} = 3.9$). Without H_2O_2, ascorbylperoxide was without significant effect ($F_{(1,49)} = 1.6$). In the cytosol, the levels of Nrf2 were lower in animals infused with solutions containing H_2O_2 ($F_{(1,49)} = 4.5$, $p < 0.05$), whereas ascorbylperoxide was without significant effect on Nrf2 levels ($F_{(1,49)} = 2.0$). There was no interaction between H_2O_2 and ascorbylperoxide on the cytosolic Nrf2 levels ($F_{(3,49)} = 0.9$).

The efficiency of activation of NFκB by peroxides was evaluated by measuring two molecules derived from his activation. The four-day exposure to the peroxides could induce inflammation. Thus, IL-6 level (a pro-inflammatory marker) was measured, as well as and PGJ_2 level (an anti-inflammatory marker). There was no significant effect on IL-6 (mean ± sem = 0.69 ± 0.06 pg/mg prot; $F_{(1,20)} < 3.9$), whereas PGJ_2 levels (**Figure 10**) were higher in the H_2O_2 groups ($F_{(1,51)} = 9.4$, $p < 0.01$). Ascorbylperoxide was without significant effect ($F_{(1,51)} = 0.02$) on PGJ_2 level. There was no significant interaction ($F_{(1,51)} = 0.3$).

Discussion

This study highlights the toxic effect of ascorbylperoxide, a by-product of peroxidation of dehydroascorbate generated in the solution of parenteral nutrition, on the lungs of our animal model. After four days of infusion, this molecule has induced one of the main features of BPD (a lower alveoli number), independently of the presence of H_2O_2.

Consistent with our hypothesis, this study demonstrated that ascorbylperoxide has produced 1) an oxidation of the redox potential, 2) an activation of caspase-3, a marker of apoptosis, and 3) a lower alveolarization index. The second part of the hypothesis was that H_2O_2, a contaminant of parenteral nutrition, exacerbates the oxidative stress and, consequently, influences the impact of ascorbylperoxide. Indeed, H_2O_2 has induced an oxidation of the redox potential that has reached a plateau. The mean redox value measured in animal infused with solutions containing H_2O_2 was similar to that observed in animals infused with solutions containing the two greatest concentrations of ascorbylperoxide (without H_2O_2). This observation suggests that the redox potential might not be the main trigger of the apoptosis. Indeed, although the animals infused with H_2O_2 had the highest redox potential, independently of the presence of ascorbylperoxide, the activation of caspase-3 by ascorbylperoxide was lower by 15% in H_2O_2 groups. Despite this negative effect of H_2O_2 on caspase-3, the activation remained sufficient to induce a decrease in the alveolar number. The effect of ascorbylperoxide on alveolarization index was similar to the values previously reported in studies investigating the effect of parenteral nutrition (14, 15).The discrepancy between redox potential values and activation of caspase-3, especially in H_2O_2 groups, suggests that the modification of the redox potential could be not enough to induce biologically pertinent oxidative stress. The study of the impact of both peroxides on

the activation of NFκB and Nrf2 is instructive. The effects of ascorbylperoxide on redox potential, caspase-3 and alveolarization index were linearly dependent to the dose infused. In contrast, the positive impact of this peroxide on activation of NFκB and Nrf2 is not dose-dependant and reached a plateau at the lowest concentration. The levels of NFκB in cytosol and nucleus also reached a plateau, whereas no activation of Nrf2 by ascorbylperoxide was documented in cytosol and nucleus. One may question the relevance of the activation of NFκB by ascorbylperoxide considering the absence of dose-effect between those two molecules. Indeed, levels of IL-6 and PGJ_2, two products of this activation, were not affected by ascorbylperoxide. Thus, the biological oxidative capacity of infused ascorbylperoxide in lungs is limited. In addition, the action of ascorbylperoxide on redox potential of glutathione is linked to a lower level of GSH rather than elevation of GSSG, an oxidized form of glutathione.

The statistically significant effect of H_2O_2 on activation of NFκB resulted from a lower level of NFκB in the cytosol rather than from a higher level in the nucleus. However, the activation is suggested by the positive impact of H_2O_2 on PGJ_2 levels. Thus, in contrast to ascorbylperoxide, the oxidative effect of infused H_2O_2 is confirmed by the activation of NFκB, the increase of PGJ_2 and the activation of Nrf2. The similarity between the histogram of nuclear Nrf2, shown in **Figure 9A**, and the results for PGJ_2 level (**Figure 10**) suggest that the activation of Nrf2 in H_2O_2 groups is induced by PGJ_2 (25) rather than by the direct oxidative property of H_2O_2. Thus, independently of its effects on the redox potential of glutathione, ascorbylperoxide seemed to be without biological oxidative capacity in the lungs. Ascorbylperoxide has induced alveolar loss following activation of caspase-3. H_2O_2 exerted a biological oxidative stress, but without an effect on

alveolarization index. Those results put into question the relation between oxidative stress and the effect of PN on BPD

From a previous report by our group, it is known that H_2O_2 infused with parenteral nutrition inhibits the hepatic pathway of transformation of methionine into cysteine, of which the availability is a rate-limiting step in the glutathione synthesis (20). This is in accordance with the current study in which the increase of redox potential of glutathione was related to a lower GSH rather than to a higher GSSG. The inhibition of hepatic transformation of methionine by PN containing H_2O_2 led to a lower glutathione concentration in liver and in blood (20). Glutathione in blood circulation is an important source of substrates for the *de novo* cellular synthesis of GSH. Indeed, by-products of the γ-glutamyltranspeptidase action on the glutathione that is in blood circulation are captured by the cell and are processed to a new synthesis of glutathione. A similar inhibition by ascorbylperoxide could also explain the lower level of GSH in lungs from animal infused with ascorbylperoxide alone.

The discrepancy between the positive effect of H_2O_2 on Nrf2 and its negative effect (increase) on redox potential is surprising. Nrf2 is well known to be the nuclear factor that favours a new synthesis of GSH (26). The absence of concordance between them could be explained by a limiting availability of substrate, specifically cysteine, for the *de novo* synthesis of glutathione. The experimental design involved the use of a solution containing only ascorbylperoxide ± H_2O_2, without amino acids or lipids. However, glucose was the only carbon substrate added as an energy source. The levels of glutathione in animals infused with these solutions, which were devoid of substrates for GSH synthesis, ranged from 21 to 33 nmol/mg prot (**Figure 2**). These levels were similar (16 to 23 nmol/mg prot) to those reported in a previous study where animals received total parenteral nutrition that

included amino acids (14). Thus, in the present study, the GSH pool seems in equilibrium. Lack of amino acids in nutrition of animals may still limit the *de novo* protein synthesis following gene transcription.

The association between the redox potential of glutathione (measured in lungs from animals infused with solutions containing ascorbylperoxide) and the alveolarization index is in accordance with the association reported by Chessex *et al*. (19). Indeed, the aforementioned study described a relationship between the severity of BPD and the redox potential of glutathione measured in whole blood of premature newborns. However, our results suggest that these two parameters are parallel events rather than being interconnected. The discrepancy between pulmonary redox potential and alveolar count has already been reported in newborn guinea pigs infused with parenteral nutrition, despite presence or absence of photo-protection (14).

Activation of caspase-3 leads to cleavage of cellular proteins, a process of irreversible apoptosis (27). This protease is activated by caspase-8, caspase-9 or caspase-10. Following impairment of mitochondria, cytochrome c is released and transforms the pro-caspase-9 in an active protease (28). Caspases-8 and 10 are activated following stimulation of receptors such as Fas receptor or Tumour necrosis factor receptor 1 (29). The redox potential does not seem to play a key role in the activation of caspase-3 and H_2O_2 reduces the activation of caspase-3. These observations suggest that ascorbylperoxide effect on caspase-3 is through the death receptors (29). From activation of these receptors until the final endpoint of apoptosis: caspase-8 is firstly activated, followed by the activation of caspase-3 by caspase-8. The fact that the later is inhibited by H_2O_2 (30) might explain the negative effect of H_2O_2 on caspase-3 activation, as found in our animal model.

Levels of ascorbylperoxide in urine were lower in animals infused with solutions containing H_2O_2. These animals received intravenous solutions in similar amount to the ones who were infused with solutions devoid of H_2O_2. These findings suggest that animals receiving solutions containing H_2O_2 had a better capacity to detoxify ascorbylperoxide and that the concentration of ascorbylperoxide is important for the activation of caspase-3. The detoxification of ascorbylperoxide could be due to a higher activity of glutathione S-transferases, secondary to the greater activation of Nrf2 in the H_2O_2 group. Further studies are needed to clarify the interactions between ascorbylperoxide, Nrf2 activation, glutathione S-transferases and their effects on alveolar loss.

Ascorbylperoxide is considered a xenobiotic due to its *in vitro* formation (in the parenteral nutrition solution) (17). However, in animals infused with a solution devoid of ascorbylperoxide but containing H_2O_2, the level of ascorbylperoxide is measurable in the urine (**Figure 1**). This could be explained by the interaction between H_2O_2 and dehydroascorbate, elements that are both present in the urine and are known to be precursor of ascorbylperoxide production (16). The relation between the urinary concentrations of ascorbylperoxide in function of the quantity infused could be useful for a further clinical investigation on the impact of ascorbylperoxide on BPD development.

Conclusion

Ascorbylperoxide generated in parenteral nutrition appears to be related to the loss of alveoli associated to apoptosis induction. Although this molecule induces an increase of the redox potential, findings of the current study suggest that this is a parallel phenomenon to apoptosis. Indeed, the shift in the redox potential toward a more oxidized status, as observed with ascorbylperoxide and with H_2O_2, is perhaps not sufficient to induce

apoptosis (18). However, this modification of the redox value should not be taken lightly. The increase of the redox potential obtained with ascorbylperoxide or H_2O_2 could influence the proliferation and the differentiation of cells (18). Pulmonary development, such as in premature newborns, must go through cellular proliferation, differentiation and apoptosis for the remodelling of the lungs, until they reach their final maturity. Bronchopulmonary dysplasia may result from the perturbation of several of these cellular states. In our experimental model, we have exposed the association between the loss of alveoli, a feature of bronchopulmonary dysplasia, and ascorbylperoxide. The impact of ascorbylperoxide on other cellular stages and on the nature of the affected cells should be the focus of future investigations. Nevertheless, since the generation of ascorbylperoxide is dependent on light exposure (17), the present study supports the beneficial effect of photo-protection of parenteral nutrition on chronic lung disease (12), such as BPD (13) in neonates. Our results add an important piece of information regarding the mechanism by which parenteral nutrition, as presently compounded, can induce BPD development.

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Figure 1

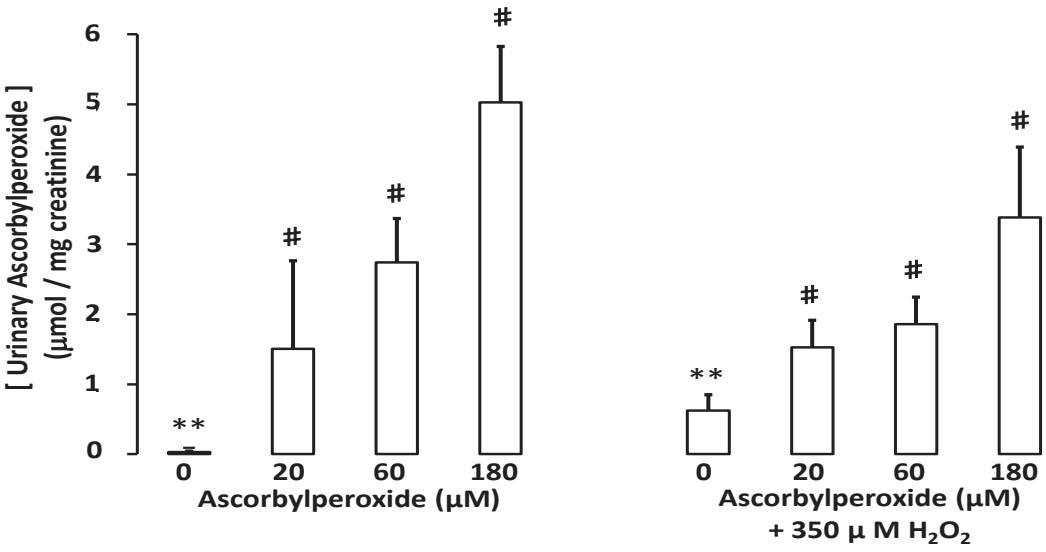

[Urinary Ascorbylperoxide]
(µmol / mg creatinine)
6
5
4
3
2
1
0
**
#
#
#
0
20
60
180
Ascorbylperoxide (µM)
**
#
#
#
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µ M H2O2

Figure 2

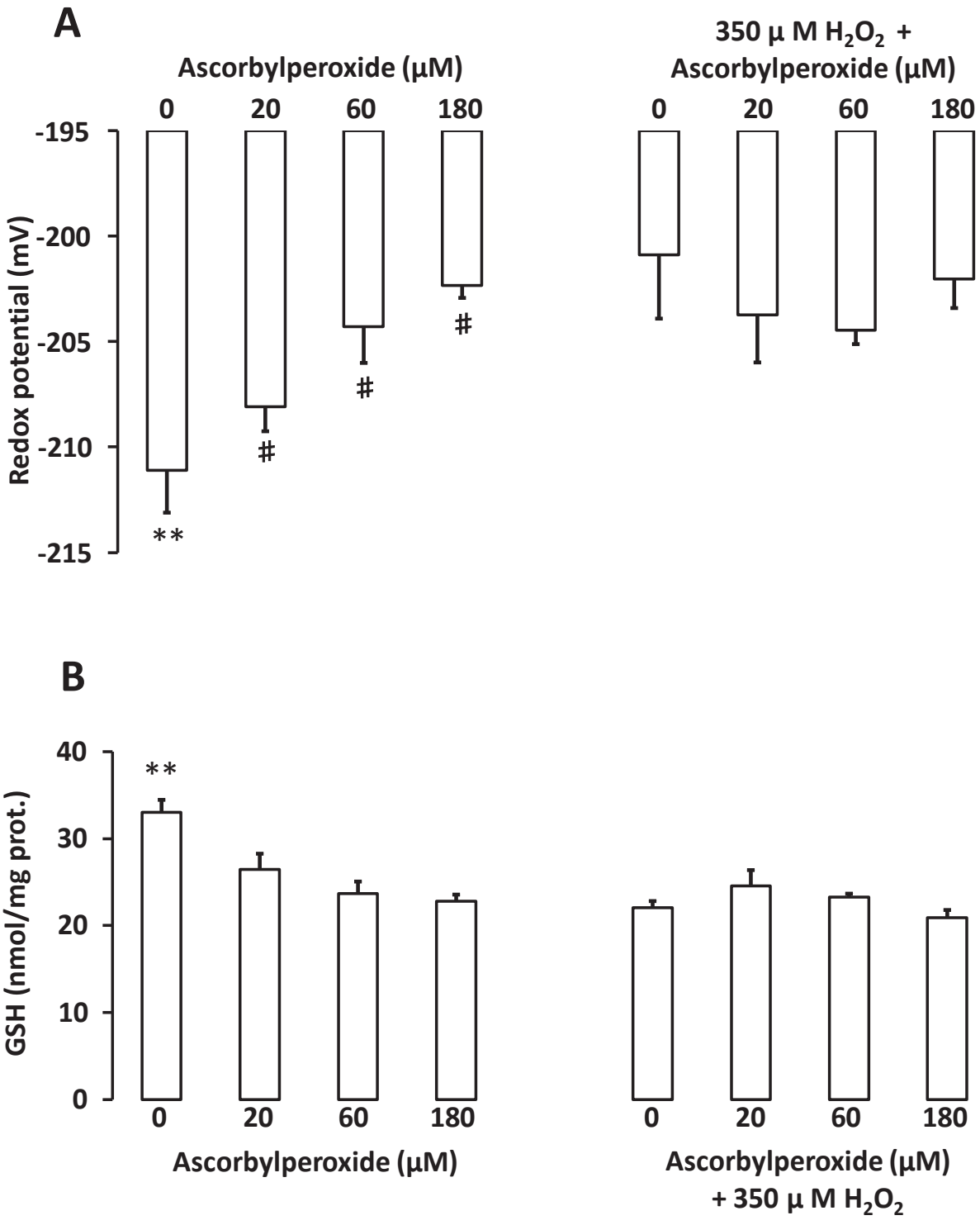

A
Ascorbylperoxide (µM)
0
20
60
180
-195
-200
-205
-210
-215
Redox potential (mV)
**
#
#
#
350 µ M H2O2 +
Ascorbylperoxide (µM)
0
20
60
180
B
40
30
20
10
0
GSH (nmol/mg prot.)
**
0
20
60
180
Ascorbylperoxide (µM)
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µ M H2O2

Figure 3

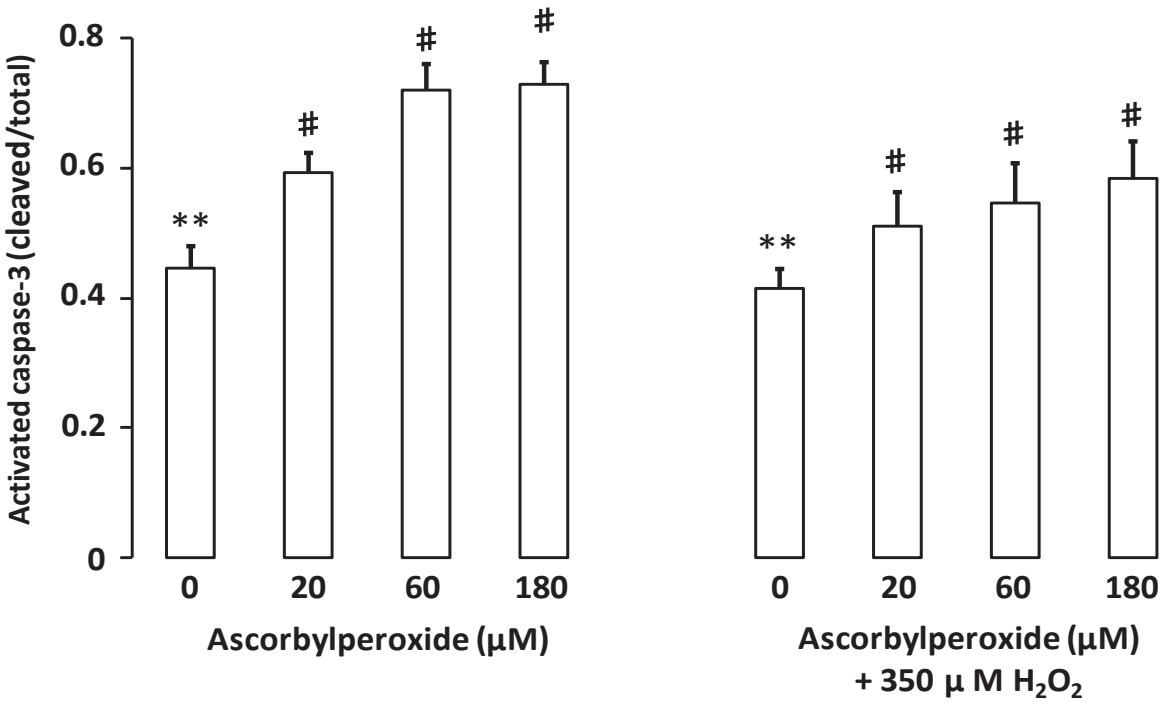

Activated caspase-3 (cleaved/total)
0.8
0.6
0.4
0.2
0
**
#
#
#
0
20
60
180
Ascorbylperoxide (µM)
**
#
#
#
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µ M H2O2

Figure 4

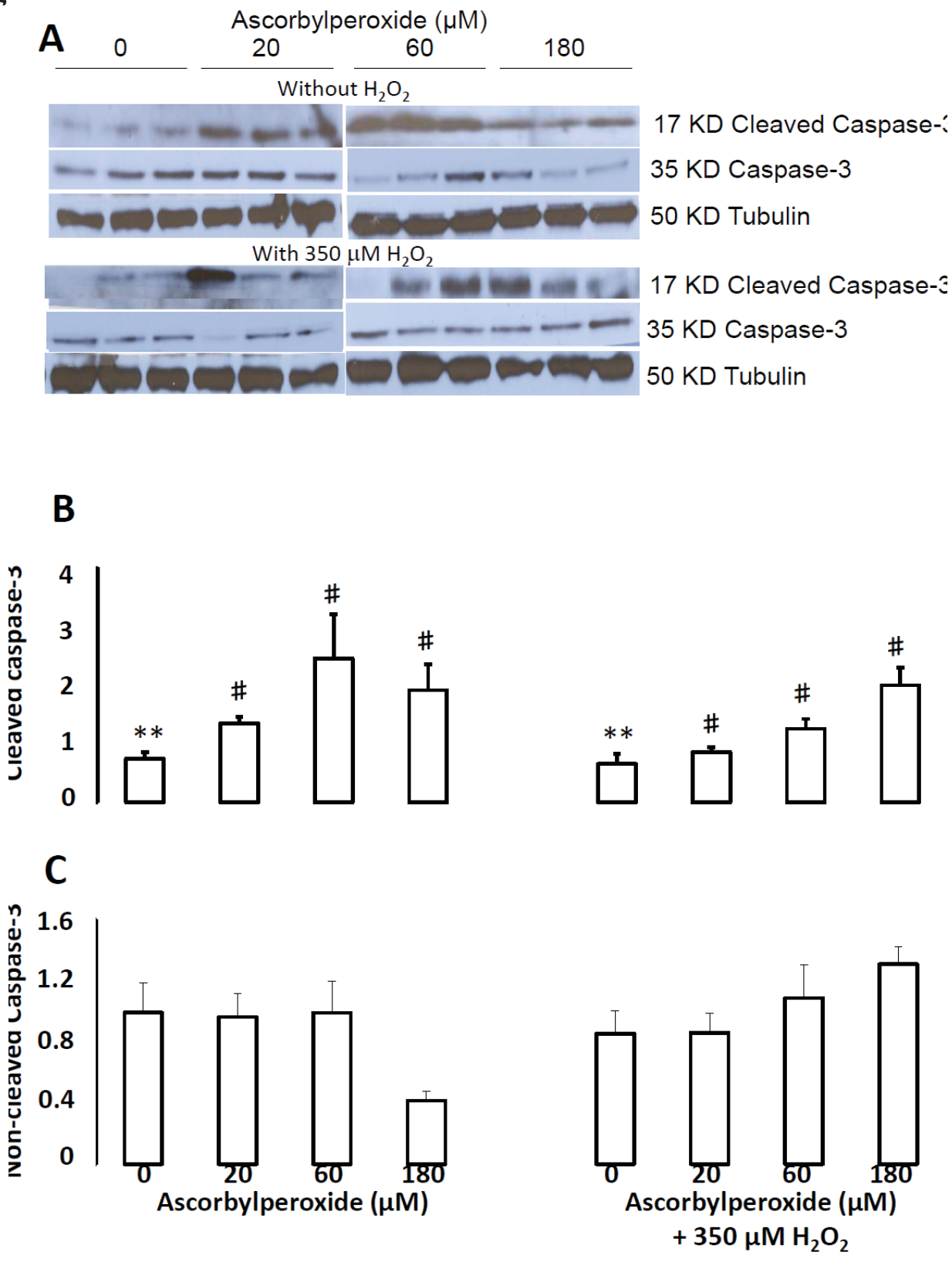

A
Ascorbylperoxide (μM)
0
20
60
180
Without H_2O_2
17 KD Cleaved Caspase-3
35 KD Caspase-3
50 KD Tubulin
With 350 μM H_2O_2
17 KD Cleaved Caspase-3
35 KD Caspase-3
50 KD Tubulin
B
Cleaved caspase-3
4
3
2
1
0
**
#
#
#
**
#
#
#
C
Non-cleaved caspase-3
1.6
1.2
0.8
0.4
0
0
20
60
180
Ascorbylperoxide (μM)
0
20
60
180
Ascorbylperoxide (μM)
+ 350 μM H_2O_2

Figure 5

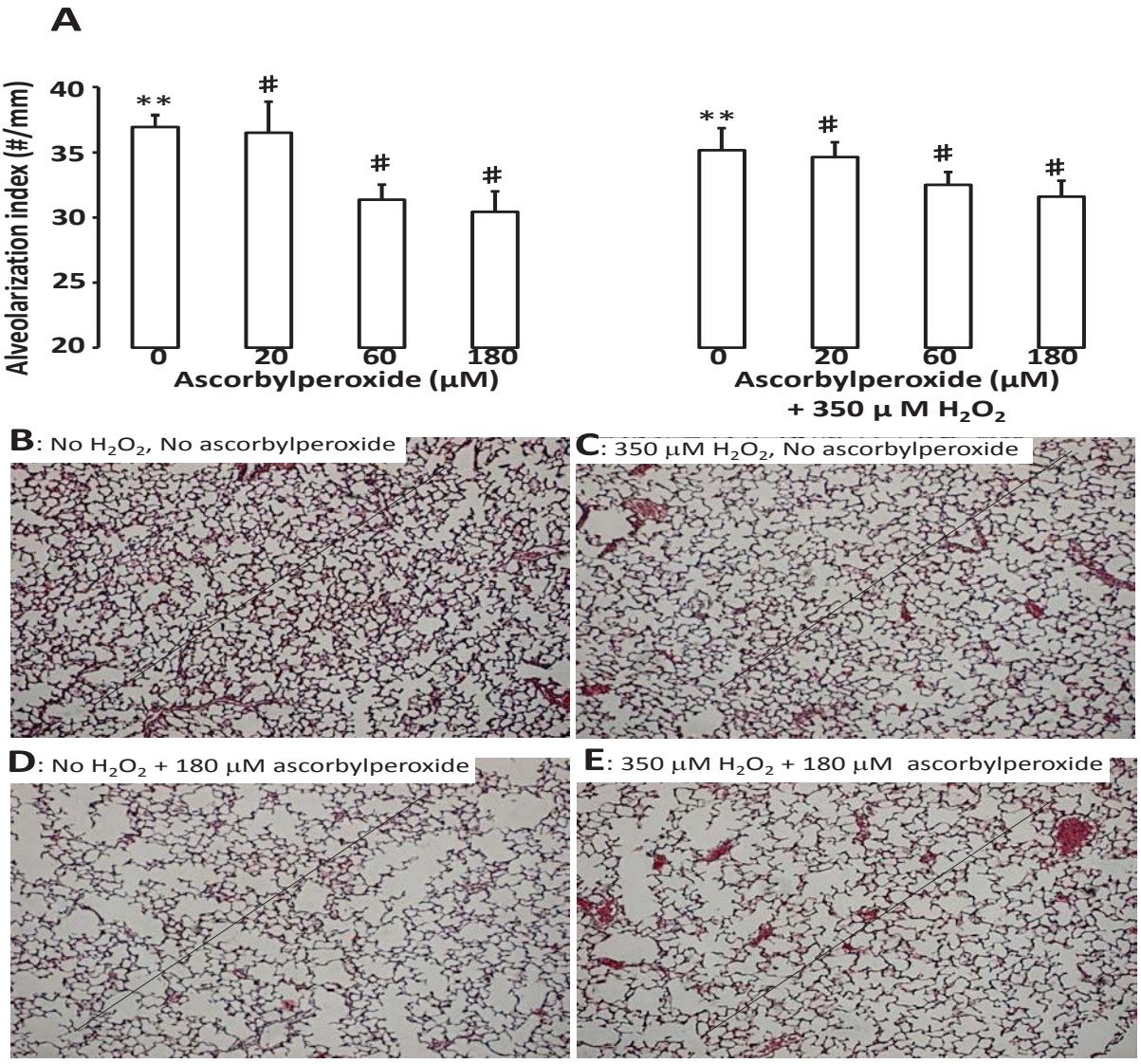

A
Alveolarization index (#/mm)
40
35
30
25
20
**
#
#
#
0
20
60
180
Ascorbylperoxide (μM)
**
#
#
#
0
20
60
180
Ascorbylperoxide (μM)
+ 350 μ M H2O2
B: No H2O2, No ascorbylperoxide
C: 350 μM H2O2, No ascorbylperoxide
D: No H2O2 + 180 μM ascorbylperoxide
E: 350 μM H2O2 + 180 μM ascorbylperoxide

Figure 6

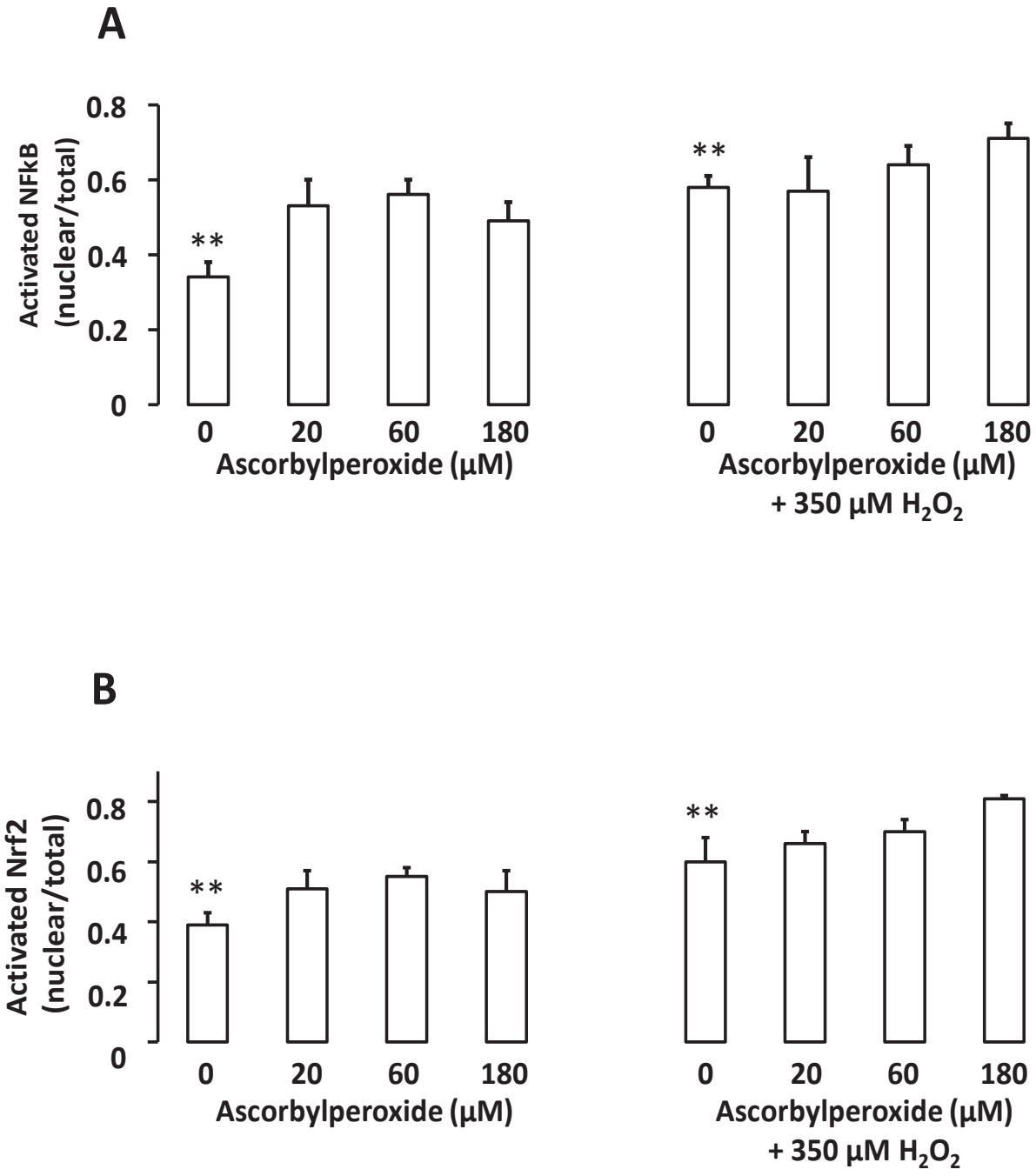

A
Activated NFkB (nuclear/total)
0.8
0.6
0.4
0.2
0
**
0
20
60
180
Ascorbylperoxide (µM)
**
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µM H2O2
B
Activated Nrf2 (nuclear/total)
0.8
0.6
0.4
0.2
0
**
0
20
60
180
Ascorbylperoxide (µM)
**
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µM H2O2

Figure 7

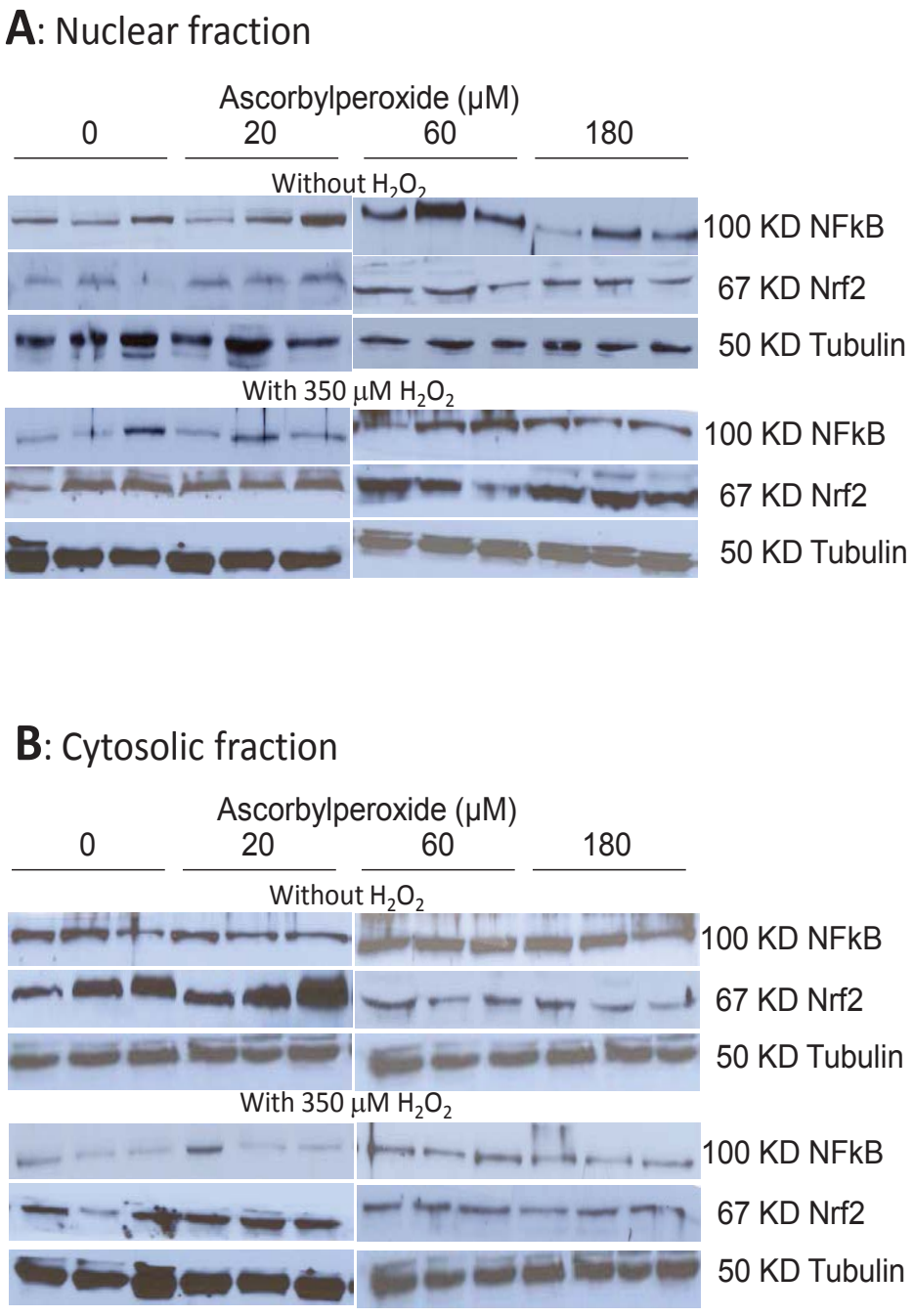

A: Nuclear fraction
Ascorbylperoxide (μM)
0
20
60
180
Without H_2O_2
100 KD NFkB
67 KD Nrf2
50 KD Tubulin
With 350 μM H_2O_2
100 KD NFkB
67 KD Nrf2
50 KD Tubulin
B: Cytosolic fraction
Ascorbylperoxide (μM)
0
20
60
180
Without H_2O_2
100 KD NFkB
67 KD Nrf2
50 KD Tubulin
With 350 μM H_2O_2
100 KD NFkB
67 KD Nrf2
50 KD Tubulin

Figure 8

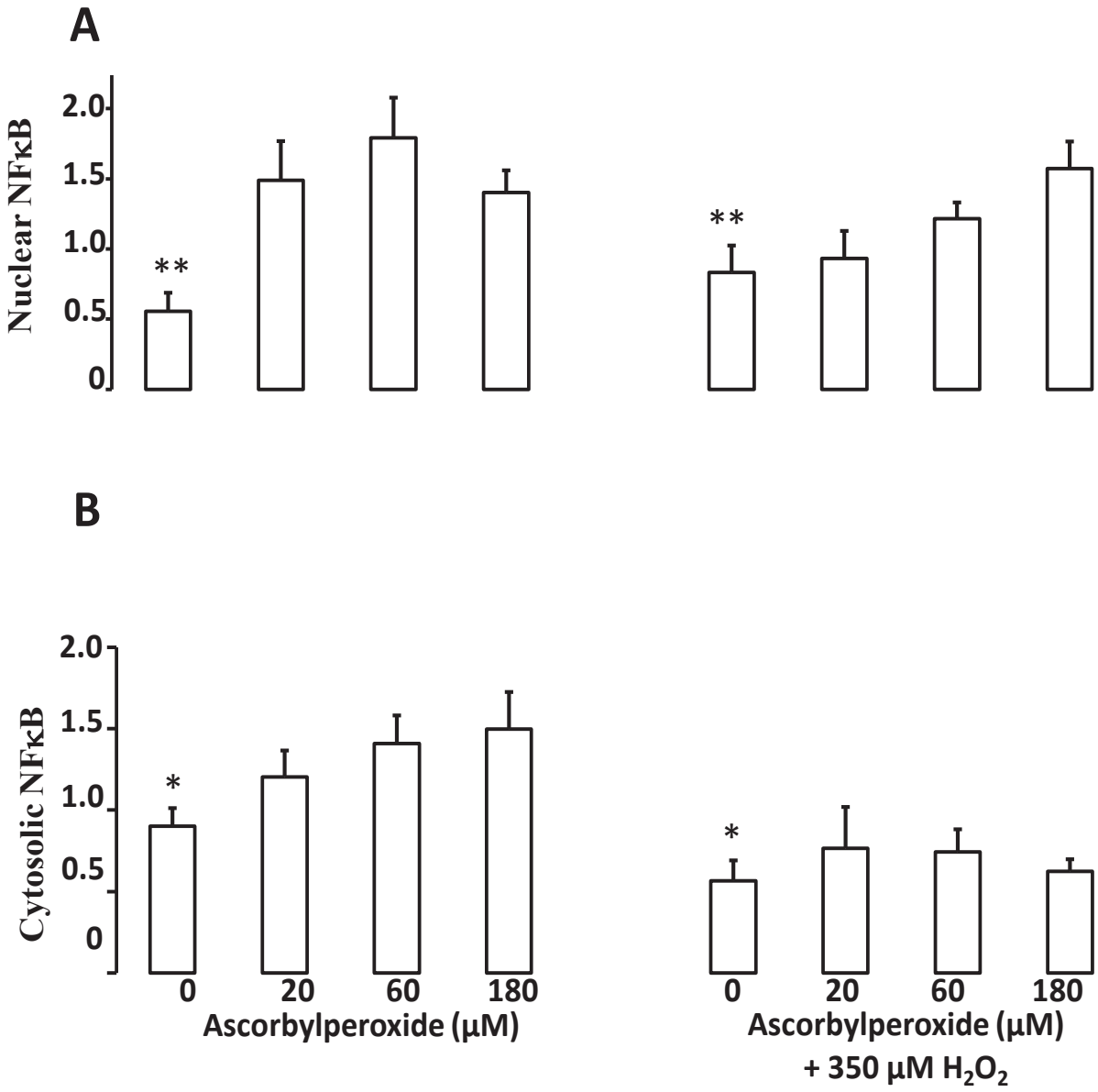

A
Nuclear NFκB
2.0
1.5
1.0
0.5
0
**
**
B
Cytosolic NFκB
2.0
1.5
1.0
0.5
0
*
*
0
20
60
180
Ascorbylperoxide (μM)
0
20
60
180
Ascorbylperoxide (μM)
+ 350 μM H2O2

Figure 9

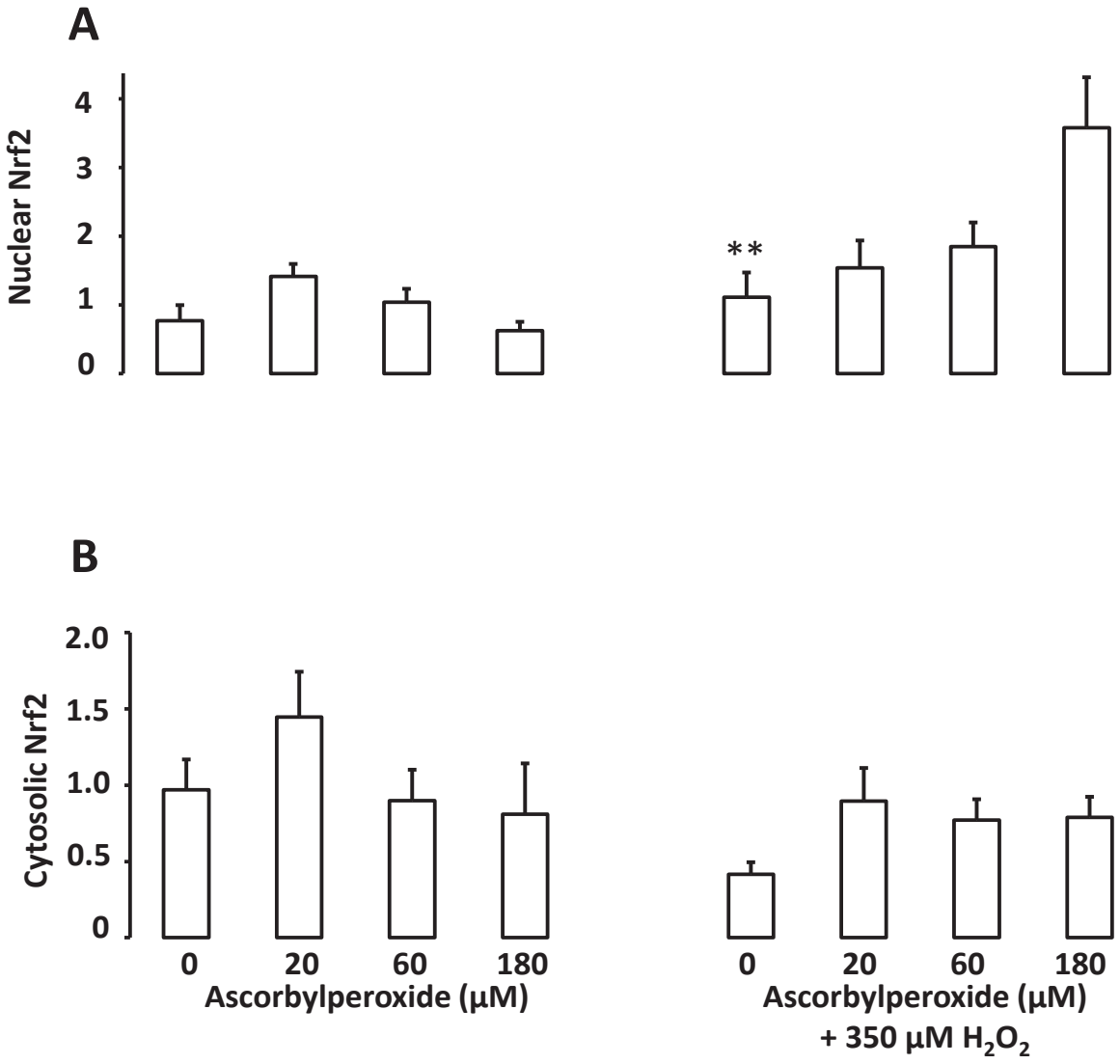
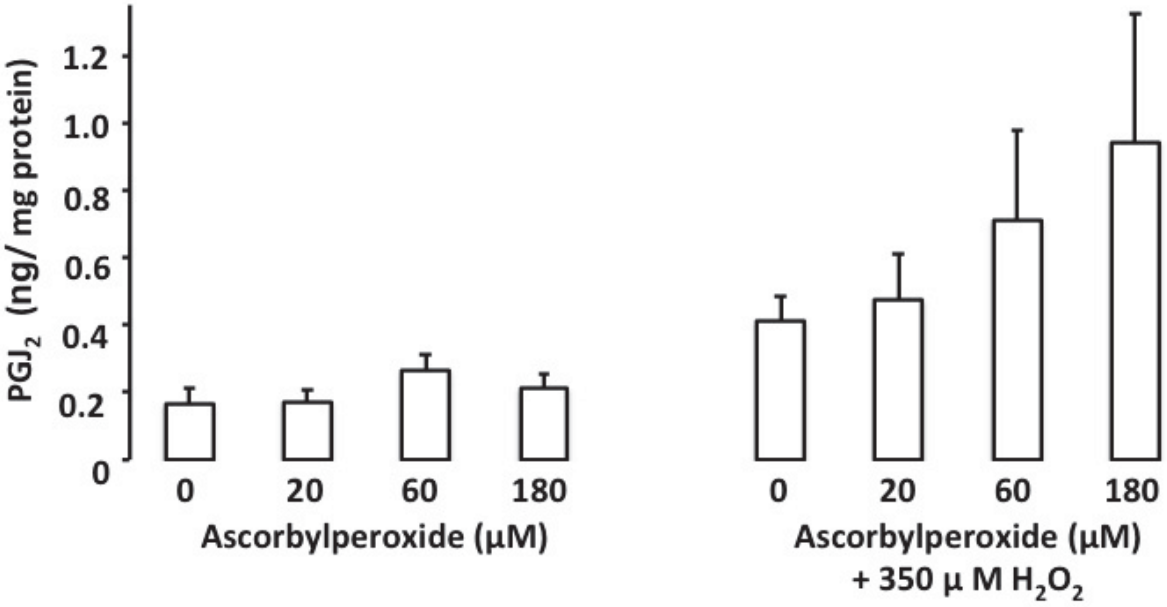

A
Nuclear Nrf2
4
3
2
1
0
**
B
Cytosolic Nrf2
2.0
1.5
1.0
0.5
0
0
20
60
180
Ascorbylperoxide (µM)
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µM H2O2

Figure 10


PGJ2 (ng/ mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
60
180
Ascorbylperoxide (µM)
0
20
60
180
Ascorbylperoxide (µM)
+ 350 µ M H2O2

Legends for Figures

Figure 1: Influence of ascorbylperoxide and H_2O_2 on the urine concentration of ascorbylperoxide.

The level of ascorbylperoxide in urine increased (**: 0 vs. 20, 60,180 μM; $p<0.01$) linearly in function of the received dose of ascorbylperoxide (#: between 20, 60,180 μM; $p<0.01$) and was lower in groups infused with solutions containing H_2O_2 (with vs. without H_2O_2; $p<0.05$). Mean ± s.e.m.; n = 4-7.

Figure 2: Influence of ascorbylperoxide and H_2O_2 on the redox potential of glutathione and GSH level in lungs.

Due to the significant interaction ($p<0.05$) between ascorbylperoxide and H_2O_2, the effects of the ascorbylperoxide on the redox potential and on GSH have been analyzed according to the presence or not of H_2O_2. Panel A: In absence of H_2O_2, the redox potential was lower in the group devoid of ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$). The impact of the ascorbylperoxide was linearly dose- dependent (#: between 20, 60,180 μM; $p<0.05$). There was no modification of redox potential by the ascorbylperoxide in animals infused with solution containing H_2O_2. Panel B: In absence of H_2O_2, the GSH level was higher in the group devoid of ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$). The impact of the ascorbylperoxide was independent of the dose (no difference between 20, 60,180 μM). There was no modification of the GSH value in animals infused with solution containing H_2O_2. Mean ± s.e.m.; n = 6-9.

Figure 3: Influence of ascorbylperoxide and H_2O_2 on the activated caspase-3.

The level of activated caspase-3 was increased (**: 0 vs. 20, 60,180 μM; $p<0.01$) linearly in function of the received dose of ascorbylperoxide (#: between 20, 60,180 μM; $p<0.05$) and was lower in groups infused with solutions containing H_2O_2 (with vs. without H_2O_2; $p<0.01$). Mean ± s.e.m.; n = 4-7

Figure 4: Influence of ascorbylperoxide and H_2O_2 on cleaved and non-cleaved caspase-3.

Panel A: Representative examples (n=3 per group) of Western blot obtained for the cleaved and non-cleaved caspase-3, relatively to tubulin. Panel B: The level of cleaved caspase-3 was lower in groups devoid of ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$) but increased linearly in function of the dose of ascorbylperoxide (#: between 20, 60,180 μM; $p<0.05$). The level of cleaved caspase-3 was not affected by the presence of H_2O_2. Panel C: Non-cleaved caspase-3 was not affected by ascorbylperoxide or H_2O_2, with exception of the 180 μM ascorbylperoxide group ($p<0.05$). Mean ± s.e.m.; n = 6-10.

Figure 5: Influence of ascorbylperoxide and H_2O_2 on alveolarization index.

Panel A: The alveolarization index was higher in groups devoid of ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.05$). The impact of the ascorbylperoxide was linearly dose-dependent (#: between 20, 60,180 μM ; $p<0.01$). The alveolarization index was not affected by the presence of H_2O_2. Panels B-E: Representative examples of histological pictures of lungs from animals infused with a solution (B) devoid of H_2O_2 and of ascorbylperoxide,

(C) containing 350 μM H_2O_2 without ascorbylperoxide, (D) containing 180 μM ascorbylperoxide without H_2O_2, or (E) containing 350 μM H_2O_2 and 180 μM ascorbylperoxide. The alveolarization index is based on the number of histological structures crossing the calibrated (1 mm) line. Mean ± s.e.m.; n = 6-10.

Figure 6: Influence of ascorbylperoxide and H_2O_2 on the activated NFκB and Nrf2 in lungs.

Activation of NFkB (panel A) and of Nrf2 (panel B) were lower on groups without ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$). However, the impact of ascorbylperoxide was without dose-effect (no difference between 20, 60,180 μM). There was an additive effect of H_2O_2 ($p<0.01$) on activation of both factors. Mean ± s.e.m.; n = 6-8.

Figure 7: Representative examples of Western blot results for NFκB and Nrf2 determined in cytosol and nucleus.

Three examples of Western blot results (relative to tubulin) for each group of animals infused with 0 to 180 μM ascorbylperoxide ± 350 μM H_2O_2 on the nuclear (Panel A) and cytosolic fraction (Panel B) of Nrf2 and NFkB in lungs. Full data are shown in figure 8.

Figure 8: Influence of ascorbylperoxide and H_2O_2 on nuclear and cytosolic NFκB.

Panel A: Nuclear levels of NFκB were lower on group without ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$). The impact of ascorbylperoxide was without dose-effect (no difference between 20, 60,180 μM). The presence of H_2O_2 had no impact on results. Panel

B: Cytosolic levels of NFκB were lower on group without ascorbylperoxide (*: 0 vs. 20, 60,180 μM; $p<0.05$). The impact of ascorbylperoxide was without dose-effect (no difference between 20, 60,180 μM). Cytosolic levels of NFκB were lower in presence of H_2O_2 ($p<0.01$). Mean ± s.e.m.; n = 6-10.

Figure 9: Influence of ascorbylperoxide and H_2O_2 on nuclear and cytosolic Nrf2.

Panel A: Due to the significant interaction ($p<0.05$) between ascorbylperoxide and H_2O_2, the effects of the ascorbylperoxide on nuclear levels of Nrf2 have been analyzed according to the presence or not of H_2O_2. In absence of H_2O_2, there was no difference between groups. In presence of H_2O_2, the nuclear level of Nrf2 was lower in the group devoid of ascorbylperoxide (**: 0 vs. 20, 60,180 μM; $p<0.01$). Panel B: Ascorbylperoxide was without effect on cytosolic Nrf2 levels whereas they were lower in animals infused with solutions containing H_2O_2 ($p<0.05$). Mean ± s.e.m.; n = 6-10.

Figure 10: Influence of ascorbylperoxide and H_2O_2 on PGJ_2.

Ascorbylperoxide was without effect on PGJ_2 levels whereas they were higher in animals infused with solutions containing H_2O_2 ($p<0.01$). Mean ± s.e.m.; n = 6-10.

Contributors' Statement:

Impact of glutathione supplementation of parenteral nutrition on hepatic methionine adenosyltransferase activity. (Redox Biol. 17;8:18-23. 2015)

Wesam Elremaly: I contributed to the study design, help in animal manipulations, performed biochemical determinations, carried out the initial analyses, drafted the initial manuscript, and I approved the final manuscript as submitted.

Dr. **Ibrahim Mohamed:** Dr Mohamed contributed to the analysis and interpretation, reviewed and approved the final manuscript as submitted.

Thérèse Rouleau: Ms. Rouleau performed the animal surgeries and care, supervised the biochemical determinations, critically reviewed the manuscript, and approved the final manuscript as submitted.

Jean-Claude Lavoie: Dr Lavoie is the author of the study conception; he has supervised the team's work, finalized analyses and interpretation of the data. He has finalized the writing of the manuscript.

Impact of glutathione supplementation of parenteral nutrition on hepatic methionine adenosyltransferase activity (Redox Biol. 17;8:18-23. 2015)

Wesam Elremaly, Ibrahim Mohamed, Thérèse Rouleau, Jean-Claude Lavoie.

Highlights

1- Methionine adenosyltransferase (MAT) is essential for healthy liver.

2- Parenteral nutrition (PN) inhibits hepatic MAT.

3- The inhibition is caused by intrinsic peroxides and by unknown component of PN.

4- Adding glutathione in PN is not sufficient to prevent PN-associated liver diseases.

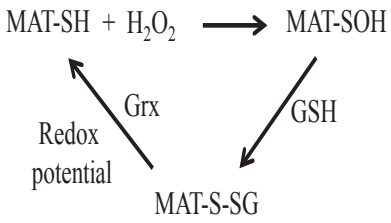
CONTEXT

Liver disorders associated with PN (parenteral nutrition).
Importance of MAT (methionine adenosyltransferase) for the hepatic health.

RESULTS

In newborn guinea pigs, PN inhibits MAT activity by a double mechanisms:

1) by intrinsic peroxides (contaminating) of PN that oxidize
   i) thiol functions of MAT leading to its inhibition, and
   ii) redox potential of glutathione, limiting the recycling of oxidized MAT



Grx: glutaredoxin. GSH: reduced glutathione

2) by an unknown component of PN that inhibits MAT by a peroxide independent mechanism.

CONCLUSION

Correction of redox potential by adding glutathione in PN is not enough to restore MAT activity

Abstract

Background & Aims: The oxidation of the methionine adenosyltransferase (MAT) by the combined impact of peroxides contaminating parenteral nutrition (PN) and oxidized redox potential of glutathione is suspected to explain its inhibition observed in animals. A modification of MAT activity is suspected to be at origin of the PN-associated liver disease as observed in newborns. We hypothesized that the correction of redox potential of glutathione by adding glutathione in PN protects the MAT activity. Aim: to investigate whether the addition of glutathione to PN can reverse the inhibition of MAT observed in animal on PN.

Methods: Three days old guinea pigs received through a jugular vein catheter 2 series of solutions. First with methionine supplement, 1) Sham (no infusion); 2) PN: amino acids, dextrose, lipids and vitamins; 3) PN-GSSG: PN+10 μM GSSG. Second without methionine, 4) D: dextrose; 5) D+180 μM ascorbylperoxide; 6) D+350 μM H_2O_2. Four days later, liver was sampled for determination of redox potential of glutathione and MAT activity in presence or not of 1 mM DTT. Data were compared by ANOVA, $p<0.05$.

Results: MAT activity was 45±4% lower in animal infused with PN and 23±7% with peroxides generated in PN. The inhibition by peroxides was associated with oxidized redox potential and was reversible by DTT. Correction of redox potential (PN+GSSG) or DTT was without effect on the inhibition of MAT by PN. The slope of the linear relation between MAT activity and redox potential was two fold lower in animal infused with PN than in others groups.

Conclusion: The present study suggests that prevention of peroxide generation in PN and/or correction of the redox potential by adding glutathione in PN are not sufficient, at least in newborn guinea pigs, to restore normal MAT activity.

Key words: Parenteral nutrition; Peroxide; Newborn; Methionine adenosyltransferase; Thiol oxidation; Redox potential of glutathione.

Introduction

The intravenous nutritional support for individuals who have impaired or immature gastrointestinal tract such as extreme premature infants is essential for their development and health. However, several hepatic complications are associated with this mode of nutrition. In adults, parenteral nutrition (PN) induces hepatic steatosis (1,2) whereas the intra-hepatic cholestasis is frequent in premature infants (3,4). Animal data suggest that peroxides, H_2O_2 and ascorbylperoxide (2,3-diketo-4-hydroxyperoxyl-5,6-dihydroxyhexanoic acid), that contaminating parenteral nutrition (5,6) are involved in these disorders (7,8). Peroxides can lead to perturbation of the metabolism following oxidation of the redox-sensitive thiol functions of specific proteins (**Figure 1**). Hence, the activity of the hepatic methionine adenosyltransferase (MAT) is inhibited by PN or infused H_2O_2 (9).

MAT is at the crossroads of several metabolic pathways (Figure 1). For instance, MAT catalyzes the formation of S-adenosylmethionine, the main methyl donor of the organism (10,11). Perturbation in the generation of S-adenosylmethionine is frequently associated with hepatic disorders such as intrahepatic cholestasis (12,13). The activity of MAT is the first step in the transformation of methionine into cysteine of which the availability is a limiting step for glutathione synthesis (14). Intracellular concentration of GSH affects the activity of glutathione peroxidase during detoxification of peroxides (17). Thus, peroxides generated in the PN can induce a vicious cycle by inhibiting MAT that leads to a lower GSH (9), and therefore to a lower capacity to detoxify the infused peroxides.

The oxidation of thiol into sulfenic acid (MAT-SOH) by peroxide is reversible (15). The mixed disulfide (MAT-SSG) formed following interaction of MAT-SOH with GSH is recycled into the native protein by the glutaredoxin using glutathione as electron donor. We

expect that the inhibition of MAT by PN occurs by this mechanism. The influence of the redox potential in the regeneration of MAT-SH, from MAT-SSG, is explained by its participation in the Gibbs equation (16) explaining a better efficiency of glutaredoxin in a more reduced environment.

Recently we have reported that addition of glutathione into PN led to a more reduced status of redox potential in lungs of newborn guinea pigs (17). We hypothesize that the addition of glutathione in PN will decrease the redox potential value (to a more reduced status) in the liver, and consequently, will improve the regeneration of MAT activity. Therefore, the objectives of the study were 1) to compare the redox potential values as well as the hepatic MAT activities in newborn guinea pigs receiving a PN enriched or not with glutathione, or intravenous solutions containing peroxides, 2) to assess that the inhibition is caused by oxidation of thiols by using dithiothreitol (DTT), and 3) to document the relation between the redox potential and the activity of MAT.

Methods

Animal model

At three days of life, Hartley guinea pigs (Charles River Laboratories, St-Constant, QC, Canada) were anaesthetized by using ketamine and xylazine in order to fix a jugular catheter (Lake Villa, IL, USA). The catheter was placed and externalized in the scapular region, and connected to the infusion system. The studied solutions were infused continuously through the catheter at rate of 22 mL / 100 g body weight / d. The solutions were changed daily.

Experimental designs

We examined the stability of both GSH and GSSG in parenteral nutrition solution to decide which molecule should be used in further experiments. In plasma, γ-glutamyltranspeptidase uses GSSG and GSH with the same efficiency to enrich the tissues into cysteine (essential for the cellular synthesis of GSH) (17,18). Twenty μM GSH or 10 μM GSSG (20 μM GSH equivalent) were added to PN (without lipid). After 1, 3, 5 and 24 hours incubation at room temperature, samples were collected for the determination of total glutathione (GSH+GSSG) using a colorimetric method (19) as previously described (17).

Based on the report documenting that diets with different intakes in methionine influence the activity of MAT (20), two different protocols were used to assess the impact of PN or peroxides on the MAT activity and on redox potential. For the first protocol three groups of animals, in which methionine was included in the nutrition, were compared:

1- Sham: The catheter was closed and animals were fed the regular laboratory food for Guinea pigs.

2- PN: Animal were exclusively on intravenous solution containing 2% (w,v) amino acids (Primene, Baxter, Toronto, ON, Canada) 8,7% (w,v) dextrose, 1% (v,v) multivitamin preparation (Multi-12/K_1 pediatrics, Sandoz, Boucherville, QC, Canada), 1.6% (w,v) lipid emulsion (Intralipid20%, Fresenius Kabi, Mississauga, ON, Canada) and 1 U/mL heparin .

3- PN +10 μM GSSG: Animals were exclusively on PN containing 10 μM GSSG. This form of glutathione was choice to avoid interactions with other components of PN (17).

For the second protocol three other groups of animals were compared. In order to isolate the effect of peroxides, the only carbon source for energy was dextrose (no amino acids or lipids):

4- D: Animals were infused with a solution containing 8.7% (w,v) dextrose, 0.3% (w,v) NaCl and 1 U/mL heparin.

5- AscOOH: Animals were infused with D containing 180 µM ascorbylperoxide; a concentration inducing perturbation of hepatic lipid and glucose metabolism (8) as well as redox potential in liver (8) and in lung (21) of newborn guinea pigs.

6- H_2O_2: Animals were infused with D containing 350 µM H_2O_2; similar concentration of peroxides reported as contaminant in PN (21).

Four days later, at seven days of age, all animals were sacrificed. The liver samples were removed, processed and stored at -80°C until biochemical determinations.

In accordance with the principles of the Canadian Council, the Institutional committee for good practice with animals in research of CHU Sainte-Justine approved the present protocol.

Determinations of redox potential of glutathione:

Briefly, as previously described (9,21), 0.5 g of liver was mixed with 5 volumes of 5% (w/v) freshly prepared metaphosphoric acid and homogenized on ice during 20 seconds with Polytron (Biospec Products, Bartlesville, OK, USA). After centrifugation 3 min at 10000 RPM, supernatants were isolated for glutathione determination and pellets were used

for protein measurement. GSH and GSSG were separated by capillary electrophoresis (Beckman Coulter). Assuming a density of 1.0 for the liver, the redox potential was calculated (25ºC, pH 7) by using the Nernst equation.

Determination of MAT activity:

The activity of MAT was quantified on the cytosolic fraction of liver as previously described (9). Briefly, 300 µg of protein (measured with BSA as standard) were suspended in the buffer (75 mM Tris/HCL, 250 mM KCl, 9 mM $MgCl_2$, pH 7.8) that contained substrates (5 mM methionine + 5 mM [2,8-^{3}H]ATP (1Ci/mol)) for a final volume of 150 µl , and were incubated 30 min at 37°C. To validate whether the inhibition of MAT was caused by oxidation of thiol functions of the protein, the buffer used for livers from Sham, PN and H_2O_2 groups contained or not, 1 mM dithiothreitol (DTT). Immediately after stopping the reaction by adding 3 ml of ice water, the total 3.15 ml were applied onto a 0.5 ml Dowex AG5OW column (BioRad laboratories). The column was treated with 20 ml water followed by 4 ml of 3M NH_4OH to displace the tritiated S-adenosylmethionine. The activity was calculated and expressed as nmol S-adenosylmethionine formed/min/mg protein.

Statistical analysis

Results were expressed as mean ± S.E.M. and were orthogonally compared by ANOVA after verification of the homogeneity of the variances by the Bartlett's Chi squared. Paired ANOVA was used to analyse the impact of DTT on the MAT activity. The Pearson's correlations between MAT activity and redox potential value were reported. The significance of the difference was set at $p < 0.05$.

Results

The stability of glutathione in PN (**Figure 2**) differed according to its redox form. After 3 hours incubation the concentration of GSH was 50 ± 2% of the initial value whereas it remained 82±1% of GSSG. After 24 hours, it was 11± 2% of GSH and 72±2% of GSSG.

The initial body weights (108± 1.7 g n=43) as well as the relative liver weights (3.5± 0.1 g/100 g body weight) were similar between groups.

Compared to the Sham group, MAT activity (**Figure 3A**) was lower ($p < 0.01$) in groups that received PN or PN+GSSG. There was no difference ($F_{(1,17)}=1.1$) between PN and PN+GSSG groups. Compared to the Control group (D), the MAT activity was lower ($p < 0.01$) in groups that received H_2O_2 or ascorbylperoxide. There was no difference ($F_{(1,18)}=0.3$) between H_2O_2 or ascorbylperoxide groups. The presence of DTT (**Figure 4**) in assay has increased the activity of MAT only in the H_2O_2 group. DTT was without effect in the Sham ($F_{(1,14)}=1.4$) and PN ($F_{(1,14)}=2.2$) groups.

The redox potential of glutathione (**Figure 3B**) was lower ($p < 0.01$) in PN+GSSG group compared to the Sham group and PN groups. There was no difference ($F_{(1,15)}=0.4$) between Sham and PN groups. Compared to the Control group (D), redox potential of glutathione was higher ($p < 0.01$) in groups that received H_2O_2 or ascorbylperoxide. There was no difference ($F_{(1,15)}=0.01$) between H_2O_2 and ascorbylperoxide groups.

The GSH values (**Table 1**) were higher ($p < 0.01$) in PN+GSSG group compared to the Sham group and PN groups. There was no difference ($F_{(1,15)}=0.5$) between Sham and PN

groups. Compared to the Control group (D), GSH levels were lower ($p < 0.01$) in groups that received H_2O_2 or ascorbylperoxide. There was no difference ($F_{(1,15)}=0.06$) between H_2O_2 or ascorbylperoxide groups. Levels of GSSG (**Table 1**) did not statistically differ between groups ($F_{(1,15)} < 3.1$).

The influence of the redox potential on MAT activity (**Figure 5**) was linear and significant ($r^2= 0.70$, $p<0.01$) among the groups Sham, D, H_2O_2 and ascorbylperoxide (AscOOH) (-0.080 $U \bullet mV^{-1} \bullet x$ – 14.3 U; U = nmol/min/mg prot). The relation was also significant ($r^2= 0.56$, $p<0.01$) for the groups PN and PN+GSSG (-0.032 $U \bullet mV^{-1} \bullet x$ – 5.3 U). However, the slopes were statistically different ($p<0.01$, respectively (IC_{95}): -0.080 (-0.105 to -0.056) and -0.032 (-0.050 to -0.014) $U \bullet mV^{-1}$).

Discussion

The main finding of the study is that, at least in newborn guinea pig, the mechanism of inhibition of the hepatic methionine adenosyltransferase (MAT) by parenteral nutrition (PN) is not explained solely by the classical and reversible oxidation of its thiol functions by the peroxides present in the nutritive solution. Therefore, the prevention of peroxides formation in PN or improving the redox potential value in liver by adding glutathione would not be enough to eliminate hepatic metabolic complications associated with this mode of nutrition.

The results confirm the induction of a lower activity of MAT (9) and a higher (more oxidized) redox potential of glutathione (9,21) in animals infused with PN or with peroxides that contaminate the nutritive solution. A classical way of inhibition of MAT is the reversible oxidation of its redox sensitive thiol functions by peroxides (Figure 1). It is through this mechanism that the H_2O_2 inhibits MAT, since the activity is recovered by

using DTT. The failure to obtain a rescue with DTT in tissues from PN group suggests that the inhibition is caused by a different way than oxidation of thiols by peroxide.

The metabolic importance of the activity of MAT led us to investigate a way to reverse its inhibition. Previously, we have added glutathione in PN to correct the low level of glutathione that is observed both in newborn animals (22) and in premature infants (23). The rational was that 1) the hepatic transformation of methionine into cysteine by MAT is low in premature infants (24) and in individuals on PN (9), 2) the availability of cysteine being a limiting step for the glutathione synthesis, the liver produces and releases a lower quantity of glutathione in blood stream (25), 3) the presence of glutathione in PN allows to reach a higher plasma concentration of glutathione (17), 4) cellular γ-glutamyltranspeptidase uses GSH as well as GSSG present in plasma to enrich cells in cysteine for a *de novo* synthesis of GSH. Thus the addition of GSSG in PN has prevented the oxidation of redox potential in lung of animal on PN (17). With the same strategy, we wanted to improve the hepatic redox potential in order to improve the capacity of the liver to recycle the oxidized MAT (Figure 1). However, the results shown that even with an enhanced GSH level as well as a more reduced redox potential in animals fed with PN+GSSG, the activity of MAT remained inhibited. Yet, the value of the redox potential influences the MAT activity (figure 5). Nevertheless, there was a strong difference according if animals received or not PN. Seventy percent (r^2=0.70) of the activity of MAT was explained by the redox potential in animals without PN. Data from all groups but PN (Sham, D, H_2O_2, ascorbylperoxide) were aligned on the same correlation, where the activity increases by one unit (nmol/min/mg prot) at each 12.5 mV reduction in the redox potential. Fifty-six percent (r^2=0.56) of the activity of MAT was explained by the redox potential in animals on PN. However, here, the efficiency of redox potential on MAT is

lower; a reduction of 33 mV in redox potential is required to observe a one-unit increase in MAT activity. This 2.5 fold magnitude of change required in redox to obtain a same impact on MAT activity underlines that the inhibition observed with PN is from another kind in addition to oxidative. Of course, the relation between MAT and the redox potential is bi-directional; the activity of MAT has also an influence on glutathione synthesis. Nevertheless, the difference between slopes remains and supports the presence of a different mechanism of inhibition between peroxides and PN.

In liver, MAT activity is regulated by the oxidative state of their numerous thiols (10 per subunit (26)) that are present in active site (cysteinyl residue 121(11)) or are involved in oligomerization of the enzyme (11). MAT I is a homo tetramer whereas MAT III is a homo dimer (26). MAT I has a greater affinity for methionine (Km about 30 μM in rat liver (27)) than MAT III (11,27) (Km about 200 μM (27)). At physiological concentration of methionine (60 μM), the activity of MAT I is 10 fold greater than MAT III (26). The global activity of MAT as measured here is dependent of the proportion of MAT I and III. Their oligomerization involves several thiol functions (11,26). Sanchez-Perez GF *et al* (26) reported that, *in vitro*, the oligomerization of MAT differs according to the presence of DTT (10 mM) or a mixture of GSH (10 mM) and GSSG (1 mM). DTT favours the formation of MAT III whereas GSH/GSSG favours a stable mixture of MAT I and III. Thus, with the glutathione system, we expect to have a greater global activity of MAT. The ratio GSH/GSSG is known to regulate the MAT activity (26,28). The calculated redox potential of glutathione used by Sanchez-Perez GF *et al* is -210 mV, a value close to that observed in our animals infused with H_2O_2, ascorbylperoxide or PN (Figure 3). The redox potential of the medium containing DTT is certainly more reduced (approximately -330 mV). The figure 5 shows that the activity of MAT increased in function of reduction of

redox environment, whereas data from Sanchez-Perez GF *et al* (26) suggest the opposite, a greater activity (MAT I + III) in a more oxidized redox environment (-210 mV) compared to the lowest activity (mainly MAT I) in the most reduced redox environment (-330 mV) obtained with DTT. This discrepancy suggests that the inhibition observed with PN, or with peroxides, does not occur at the level of oligomerization.

The regulatory function of redox potential of glutathione on MAT activity observed in the present study supports the fact that the inhibitions are obtained following the oxidation of a thiol function, probably on the C121 at the active site as suggested by Pajares MA *et al* (11). However, in animal infused with PN, the inhibition cannot be explained only by oxidation of this thiol function, since DTT was without effect. Other molecules must be involved. 4-Hydroxynonenal, from the lipid peroxidation of cellular membranes or from the lipid moiety of the PN, is a possible candidate. It is a well known that this aldehyde has a strong reactivity with several amino acids such as histidine, lysine and cysteine (29). These kinds of involved reactions (Schiff-base formation or Michael addition) are irreversible and led to inactivation of several proteins (30).

Conclusion

The present report suggests that prevention of peroxide generation in PN and/or correction of the redox potential by adding glutathione in PN (17) are not sufficient, at least in newborn guinea pigs, to restore normal MAT activity. Further studies should be undertaken to identify all chemical players, in addition of peroxides, present in PN that influence the activity of the various isoforms of MAT. The knowledge of the molecules and pathways

implied is the first step in the prevention of deleterious effects of PN on hepatic metabolism.

Figure 1

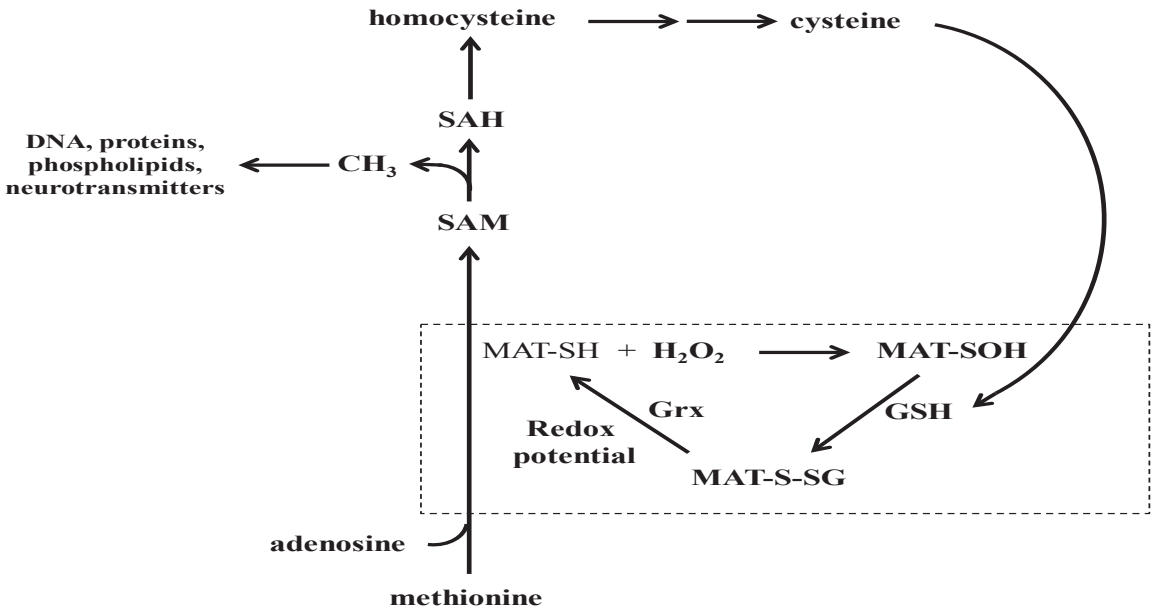

homocysteine
cysteine
SAH
DNA, proteins,
phospholipids,
neurotransmitters
CH3
SAM
MAT-SH + H2O2
MAT-SOH
Grx
GSH
Redox
potential
MAT-S-SG
adenosine
methionine

Figure 2

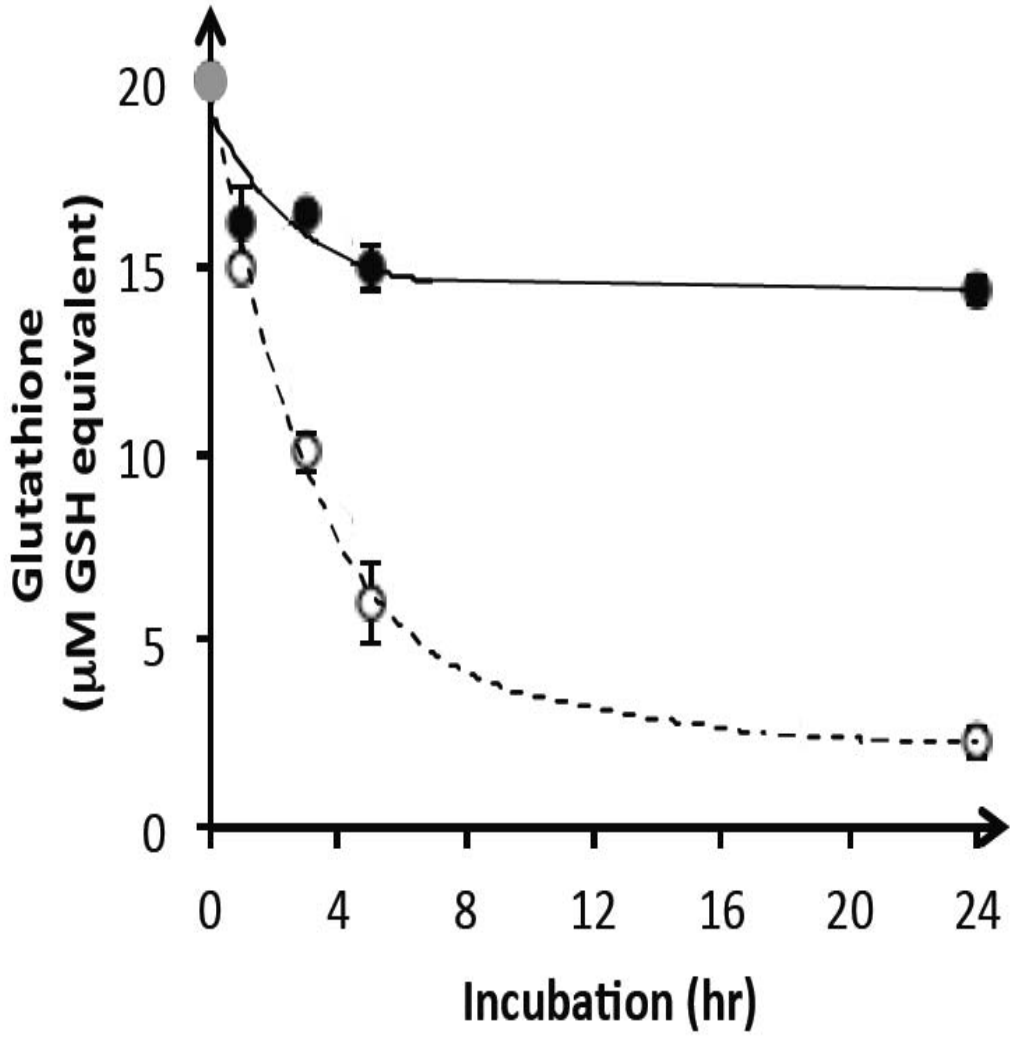

Glutathione
(µM GSH equivalent)
20
15
10
5
0
0
4
8
12
16
20
24
Incubation (hr)

Figure 3

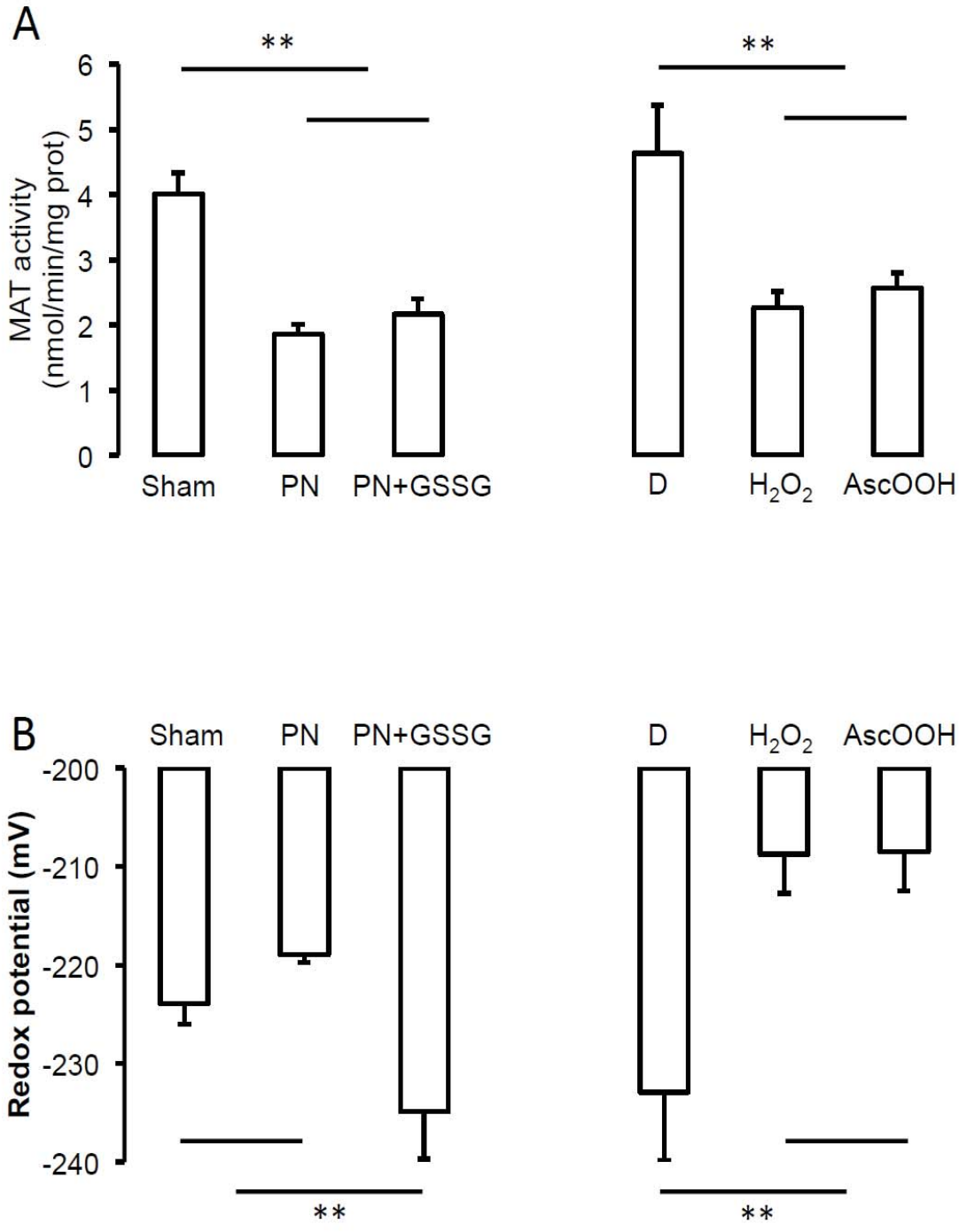

A
**
**
MAT activity (nmol/min/mg prot)
6
5
4
3
2
1
0
Sham
PN
PN+GSSG
D
H_2O_2
AscOOH
B
Sham
PN
PN+GSSG
D
H_2O_2
AscOOH
Redox potential (mV)
-200
-210
-220
-230
-240
**
**

Figure 4

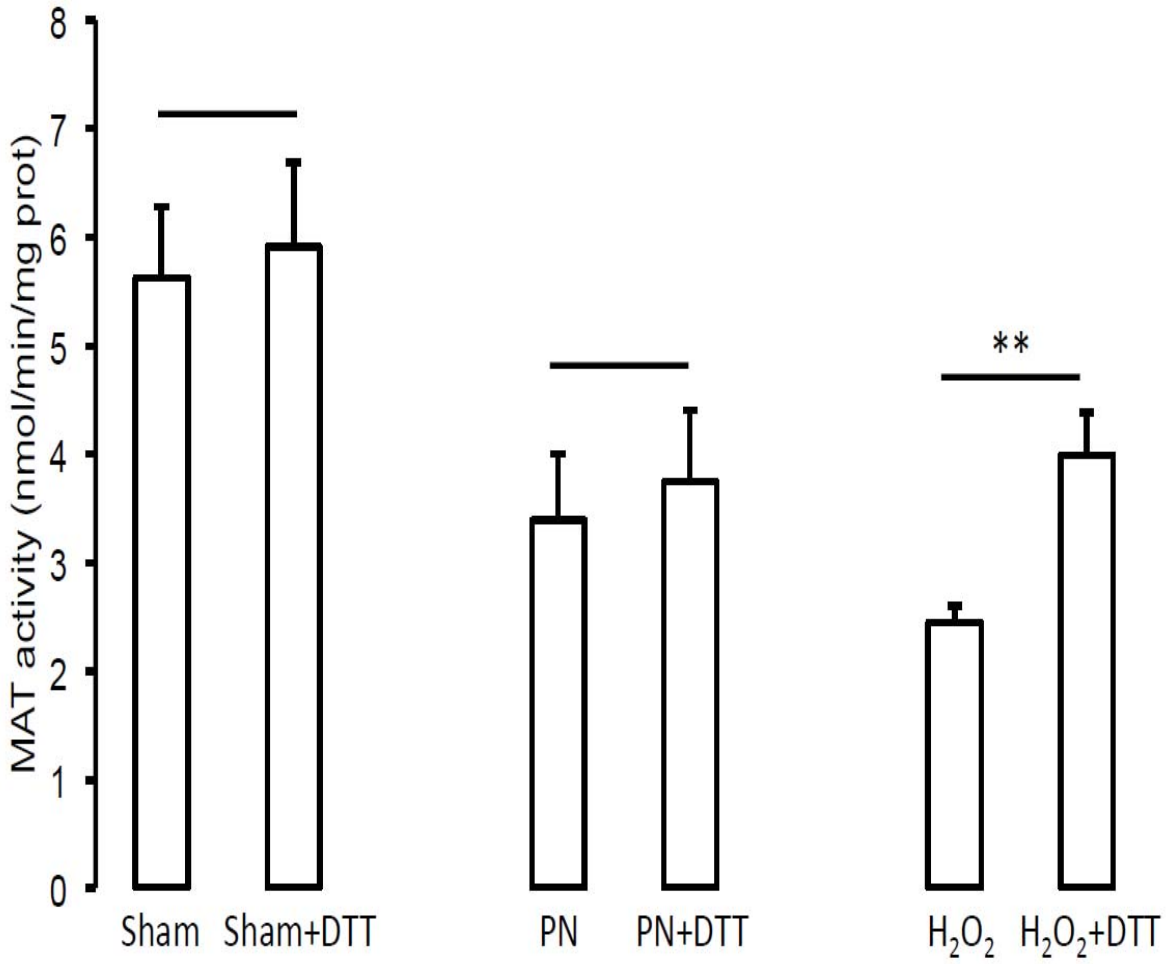

MAT activity (nmol/min/mg prot)
0
1
2
3
4
5
6
7
8
**
Sham
Sham+DTT
PN
PN+DTT
H2O2
H2O2+DTT

Figure 5

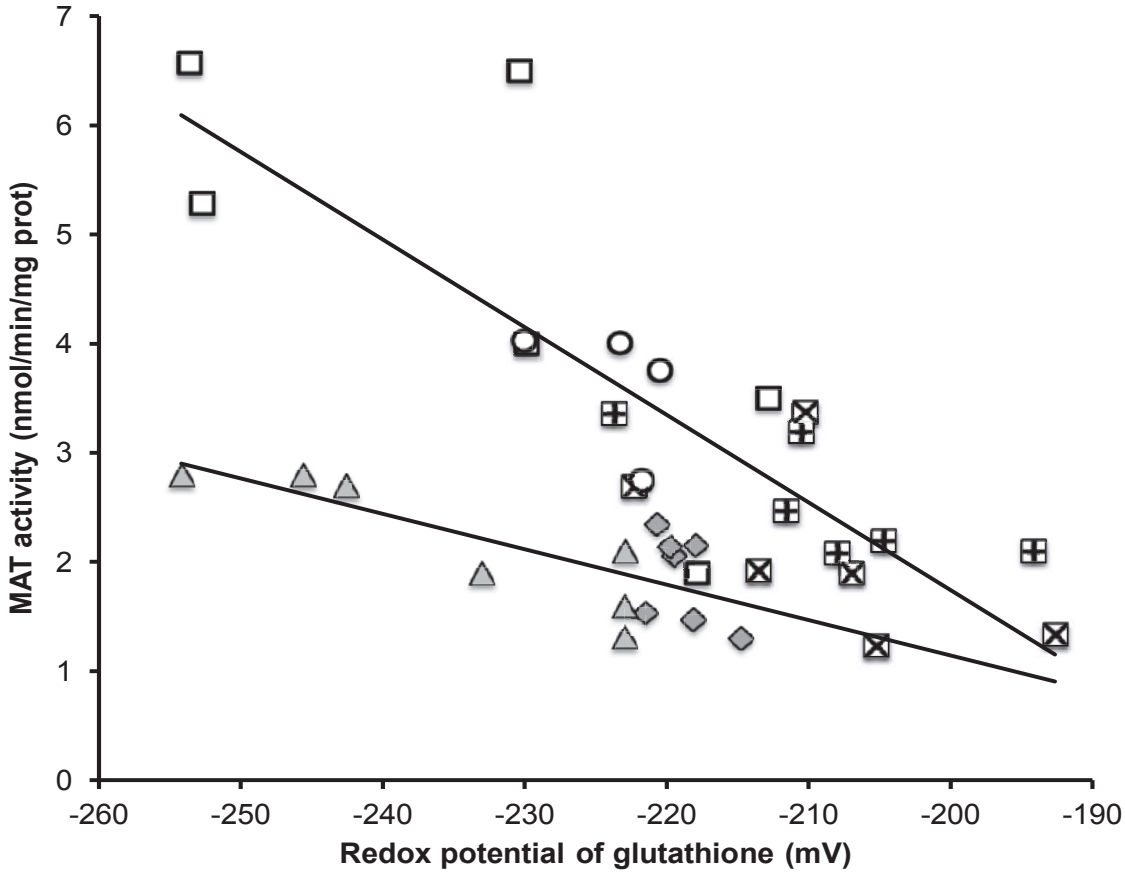

MAT activity (nmol/min/mg prot)
0
1
2
3
4
5
6
7
-260
-250
-240
-230
-220
-210
-200
-190
Redox potential of glutathione (mV)

Table 1: **Hepatic GSH and GSSG values as a function of treatments**.

	Sham	PN	PN + GSSG	D	H_2O_2	AscOOH
GSH (nmol/mg prot)	45±2	34±4	76±14*	79±18†	21±1	23±3
GSSG (nmol/mg prot)	0.58±0.07	0.71±0.12	0.58±0.07	0.67±0.10	0.44±0.11	0.5±0.03

Sham: animals with closed catheter and enterally fed with chow; PN: animals exclusively on parenteral nutrition; PN+GSSG: 10 μM GSSG added to PN; D: animals infused with a solution of dextrose; H_2O_2: D + 350 μM H_2O_2; AscOOH: D + 180 μM ascorbylperoxide. Two sets of statistical analyses were used according of the presence of methionine into their nutrition (Sham, PN, PN+GSSG). 1) There was no statistical difference in GSH between Sham and PN, both are different from PN+GSSG (*: $p<0.01$). 2) There was no statistical difference in GSH between H_2O_2 and AscOOH, both are different from D (†: $p<0.01$). The GSSG levels did not differ between groups. Mean ± s.e.m., n= 4-7/group.

Figure Legends

Figure 1: Interrelation between MAT activity, peroxides and glutathione.

The active form of MAT (MAT-SH) is responsible of the transformation of methionine in cysteine in order to sustain the synthesis of glutathione (GSH). In a context of PN, when MAT is inhibited (MAT-SOH) by peroxides (H_2O_2) generated into PN, a vicious cycle occurs. The low activity of MAT induces a low synthesis of GSH that is essential for the MAT recovery. By a compromised generation of the methyl donor S-adenosylmethionine (SAM), several metabolisms (proteins, DNA, phospholipids, neurotransmitters, etc.) are altered. SAH: S-adenosylhomocysteine.

Figure 2: Stability of GSH and GSSG in PN.

PN contained 2% (w,v) amino acids, 8,7% (w,v) dextrose, 1% (v,v) multivitamin preparation and 1 U/mL heparin. The gray circle represents the initial concentration of GSH and GSSG added in PN. The concentration of total glutathione (GSH+GSSG, expressed in GSH equivalent) decreased in function of time. The drop was greater with GSH (open circle) than with GSSG (dark circle). Mean ± s.e.m. (some s.e.m. are smaller than the symbol), n= 3.

Figure 3: Impact of intravenous infusion of isolated peroxides or of the presence or not of glutathione in PN on the activity of methionine adenosyltransferase (MAT) and on the redox potential of glutathione in liver.

Sham: animals with closed catheter and enterally fed with chow; PN: animals exclusively on parenteral nutrition; PN+GSSG: 10 μM GSSG added to PN; D: animals infused with a solution of dextrose; H_2O_2: D + 350 μM H_2O_2; AscOOH: D + 180 μM ascorbylperoxide. Two sets of statistical analyses were used according of the presence of methionine into their nutrition (Sham, PN, PN+GSSG). Panel A: The MAT activities were lower ($p < 0.01$) in groups PN and PN+GSSG compared to the Sham group and in groups H_2O_2 and AscOOH compared to the Control group (D). Panel B: The redox potential of glutathione was more reduced (lower) ($p < 0.01$) in PN+GSSG group than in Sham and PN and in D group compared to H_2O_2 and AscOOH groups. Mean ± s.e.m., n= 4-9/group. **: $p<0.01$.

Figure 4: Impact of DTT on the activity of methionine adenosyltransferase (MAT).

Sham: animals with closed catheter and enterally fed with chow; PN: animals exclusively on parenteral nutrition; H_2O_2: animals infused with a solution of dextrose containing 350 μM H_2O_2. The presence of 1 mM DTT in the assay has improved ($p<0.01$) the activity of MAT only in the H_2O_2 group. Mean ± s.e.m., n= 4-7/group. **: $p<0.01$.

Figure 5: Activity of hepatic methionine adenosyltransferase (MAT) in function of the redox potential of glutathione.

The relation between MAT activity and the redox potential was statistically different ($p<0.01$) according to that the animals were infused with PN (±GSSG) or not. Without PN, the redox potential explained 70% of the variation in MAT activity ($r^2 = 0.70$, $p<0.01$). With PN, the redox potential explained 56% of the variation in MAT activity ($r^2 = 0.56$,

$p<0.01$). However, with PN groups, the slope of the linear relationship was half ($p<0.01$) that observed with animals without PN. Open circles: Sham; open squares: D; x: H_2O_2; +: AscOOH; gray diamonds: PN; gray triangles: PN+GSSG.

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Contributors' Statement:

Adding glutathione to parenteral nutrition prevents alveolar loss in newborn guinea pig (Free Radic Biol Med.06.040, 2015)**.**

Wesam Elremaly: I contributed to the study design, help in animal manipulations, performed redox potential analyses, Western blots and histology, carried out the initial analyses, drafted the initial manuscript, and I approved the final manuscript as submitted.

Dr. **Ibrahim Mohamed:** Dr Mohamed contributed to the analysis and interpretation, reviewed and approved the final manuscript as submitted.

Thérèse Rouleau: Ms. Rouleau performed the animal surgeries and care, supervised the biochemical determinations, critically reviewed the manuscript, and approved the final manuscript as submitted.

Jean-Claude Lavoie: Dr Lavoie is the author of the study conception; he has supervised the team's work, finalized analyses and interpretation of the data. He has finalized the writing of the manuscript.

Adding glutathione to parenteral nutrition prevents alveolar loss in newborn guinea pig (Free Radic Biol Med.06.040, 2015)**.**

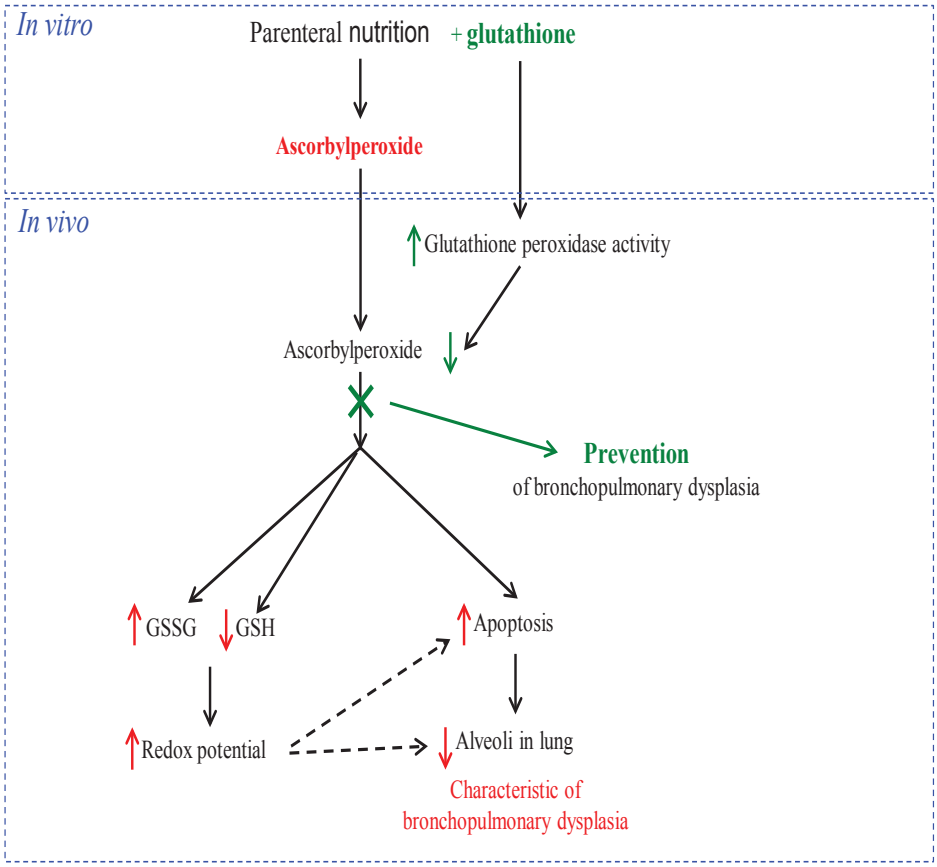
Wesam Elremaly, Ibrahim Mohamed, Thérèse Rouleau and Jean-Claude Lavoie.

Key words: Premature newborn; bronchopulmonary dysplasia; ascorbylperoxide; apoptosis; hypoplasia; redox potential of glutathione.

Highlights

- Incidence of bronchopulmonary dysplasia (hypoplasia) is high in premature infants
- Ascorbylperoxide from parenteral nutrition (PN) induces loss of alveoli in animal
- Glutathione level is low in premature infants
- Adding glutathione in PN prevents apoptosis and loss of alveoli in newborn guinea pig
- Proposed mechanism: detoxification of ascorbylperoxide by glutathione peroxidase

PARENTERAL NUTRITION **WITHOUT** OR **WITH GLUTATHIONE**



Abstract

Bronchopulmonary dysplasia, a main complication of prematurity, is characterized by an alveolar hypoplasia. Oxidative stress is suspected to be a trigger event in this population who has a low level of glutathione, a main endogenous antioxidant, and who receives high oxidative load, particularly ascorbylperoxide from their parenteral nutrition. Hypothesis: the addition of glutathione (GSSG) in parenteral nutrition improves detoxification of ascorbylperoxide by glutathione peroxidase and therefore prevents exaggerated apoptosis and loss of alveoli.

Methods: Ascorbylperoxide is assessed as substrate for glutathione peroxidase in Michaelis-Menten kinetics. Three-days old guinea pig pups were divided in 6 groups to receive, through a catheter in jugular vein, the following solutions: 1) Sham (no infusion); 2) PN(-L): parenteral nutrition protected against light (low ascorbylperoxide); 3) PN(+L): PN without photo-protection (high ascorbylperoxide); 4) 180 μM ascorbylperoxide; 5) PN(+L) + 10 μM GSSG; 6) ascorbylperoxyde + 10 μM GSSG. After 4 days, lungs were sampled and prepared for histology and biochemical determinations. Data were analysed by ANOVA, $p<0.05$.

Results: The Km of ascorbylperoxide for glutathione peroxidase was 126±6 μM and Vmax was 38.4±2.5 nmol/min/ U. The presence of GSSG in intravenous solution has prevented the high GSSG, oxidized redox potential of glutathione, activation of caspase-3 (apoptosis marker) and loss of alveoli induced by PN(+L) or ascorbylperoxide.

Conclusion: A correction of the low glutathione levels observed in newborn animal on parenteral nutrition, protects lungs from toxic effect of ascorbylperoxide. Premature infants having a low level of glutathione, this finding is of high importance because it provides hope in a possible prevention of bronchopulmonary dysplasia.

Introduction

Bronchopulmonary dysplasia (BPD) is a main complication of prematurity. The exact aetiology of this chronic lung disease, characterized by alveolar hypoplasia, is unknown. Nonetheless there is a general consensus that oxidative stress is a triggering event. In this population, the two main oxidative sources are oxygen supplement because pulmonary immaturity and parenteral nutrition (PN) because gastrointestinal track immaturity. The link between oxygen supplement and development of BPD is well described [1]. In the last two decades, physicians were more careful with oxygen administration. Despite this caution in the use of supplemental oxygen, the incidence of BPD remains high [2].

The other main source of oxidant molecules is PN. The interactions between electron donors such as vitamin C and dissolved oxygen in the intravenous solution generate peroxides [3, 4]. A typical PN administered to premature infants, containing 1% of multivitamin preparation generates between 300 and 400 μM peroxides [3,5], of which close to 80% are hydrogen peroxide [3]. The absence of adequate protection against ambient light accelerates the reaction. By using the light energy, the photo-excited riboflavin favours the transfer of electrons between ascorbate and oxygen. Full light protection reduces by half the generation of peroxides [5,6]. Since the photo-protection of PN has been reported to reduce the incidence of BPD [7,8], these peroxides are suspected to involve in BPD development. We have reproduced the effect of light exposure of PN on pulmonary alveolarization in newborn animal [9,10]. The administration on four days of PN that is devoid of light protection induces a loss of alveoli following an exaggerated apoptosis [10,11]. Recently we have shown that this

loss is not the effect of H_2O_2 but of a new molecule named ascorbylperoxide (2,3-diketo-4-hydroxyperoxyl-5,6-dihydroxyhexanoic acid). This molecule is derived from peroxidation of dehydroascorbate by H_2O_2 [12,13].

Because an adequate photo-protection of PN, from pharmacy service, where the intravenous solution is compounded, until the bedside is practically impossible in clinical routine, we have investigated the possibility to improve the *in vivo* capacity to detoxify the ascorbylperoxide. The peroxide characteristic of this molecule suggested that it could be reduced by the action of glutathione peroxidase. Therefore, the first objective was to demonstrate the relationship between ascorbylperoxyde and glutathione peroxidase. The success of this approach suggested that the tissue concentration of glutathione (GSH) is important for this detoxification.

At birth, the level of glutathione is dependent on gestational age [14]. In extreme premature newborns, the level of glutathione remains low for at least the first three weeks of life [14]. A lack of substrate for the *de novo* synthesis of glutathione explains this fact [15]. A low cellular availability of cysteine is a known limiting factor for glutathione synthesis [16]. This condition can be explained by the immaturity of capture of cysteine by cells [17] and by low capacity of liver to deliver glutathione in bloodstream. An important role of glutathione is to serve as pool of cysteine for cells [18]. γ-Glutamyltranspeptidase on cellular membranes transfers the γ-glutamyl moiety of glutathione to another amino acid in circulation thereby generating two dipeptides that are up-taken by the cells. Following dipeptidase action, amino acids are released for a new synthesis of glutathione. The activities of γ -glutamyltranspeptidase and of synthetic enzymes are mature in preterm infants [19]. Glutathione present in

bloodstream derives from the liver. This organ has high capacity to synthesize glutathione because it uses the transformation of methionine to generate cysteine. However, the first enzyme of this transformation, the methionine adenosyltransferase, is inhibited by peroxides from PN as shown in animal [20] and the last enzymatic step, the cystationase, is immature in premature infants [21,22]. We hypothesized that the addition of glutathione in PN bypasses the liver process of glutathione production, increases the cellular capacity to detoxify ascorbylperoxide and consequently prevents the loss of alveoli following infusion of PN. Therefore, the second aim of the study was to test in newborn animals, the impact of an addition of glutathione in PN on the pulmonary level of glutathione (GSH, GSSG and redox potential), apoptosis and alveolar development.

Materials

Guinea pigs were purchased from Charles River (St-Constant, Montréal, QC, Canada). Catheters were obtained from SAI Infusion Technologies (Lake Villa, IL, USA). Intralipid20% was acquired from Fresenius Kabi Canada (Mississauga, ON, Canada). Multivitamin preparation (Multi-12/K_1 paediatrics), amino acids (Primene) and dextrose were provided by Baxter (Toronto, ON, Canada). GSH, GSSG, L-ascorbic acid, riboflavin, catalase and glutathione peroxidase were bought from Sigma-Aldrich (Oakville, ON, Canada). Glutathione reductase was obtained from Roche Diagnostics (Indianapolis, IN, USA). L-2-oxo-thiazolidine 4-carboxilic acid (OTC) was acquired from Clintec (Deerfield, IL, USA). Hydrogen peroxide 30%, and ammonium acetate HPLC grade were purchased from Fisher (Fair Lawn, NJ, USA). Boric acid was obtained from J.T.Baker Chemical (Phillipsburg, NJ, USA). The Enzyme Immunoassay

Kit for the determination of 8-Isoprostane-F2α was provided by Cayman Chemical (Ann Arbor, MI, USA). Sep Pak C18 column were purchased from Water limited (Mississauga, On, Canada).

Methods

In vitro study

Glutathione peroxidase reduces peroxides in their corresponding alcohol. Into the reaction, two electrons from GSH are transferred to the peroxide. The formed GSSG is recycled by glutathione reductase using NADPH as electron donor. In our assay, the loss of NADPH monitored at 340 nm is used to assess the activity of glutathione peroxidase. The enzymatic kinetic of glutathione peroxidase corresponds to the Michaelis-Menten model. Such kinetic using increasing concentrations of ascorbylperoxide allows the confirmation that ascorbylperoxide is detoxified by glutathione peroxidase. The reaction medium contained 50 mM GSH, 5 mM NADPH, 0 to 540 µM ascorbylperoxide, 0.25 U glutathione reductase in a buffer (250 mM TRIS, 0.1 mM EDTA-Na_2) at pH 7.6. After 3 minutes at 25ºC, the reaction was started with the addition of 0.1 U glutathione peroxidase (from human erythrocytes - according to Sigma - thus glutathione peroxidase-1). After one minute of reaction, the loss of NADPH was monitored at 340 nm. The velocity of the reaction (v_0) was obtained using the molar extinction coefficient of NADPH (6.22 $mM^{-1} \bullet cm^{-1}$) and was expressed as µmol NADP generated / minute / U glutathione peroxidase.

In vivo study

A catheter was fixed in jugular vein of 36 guinea pigs aged of 3 days as previously described [10,20]. The studied intravenous solutions were infused continuously through

the catheter at a rate of 200 mL/kg/d. The solutions were changed daily. Animals were divided in six groups as followed:

- Sham: the catheter was closed by a node. Animals did not receive any intravenous solution. They were fed with regular food for guinea pig.
- PN(-L): animal were fed exclusively by intravenous solution containing 4 g/kg/d amino acids, 17.4 g/kg/d dextrose, 2 mL/kg/d multivitamin preparation, 3.2 g/kg/d lipid emulsion and 1 U/mL heparin [20]. The solution was photo-protected (-L) with opaque materials. We previously reported that this solution contains about 20 μM ascorbylperoxide [11].
- PN(+L): PN without photo-protection (about 75 feet-candle). We previously reported that this solution contains about 35 μM ascorbylperoxide [11].
- PN(+L)+GSSG: PN(+L) enriched with 10 μM GSSG.
- Ascorbylperoxide: animals were fed exclusively by intravenous solution containing 17.4 g/kg/d dextrose, 0.6 g/kg/d NaCl, 1 U/mL heparin and 180 μM ascorbylperoxide. This concentration of ascorbylperoxide is about five time higher than the measured concentration in PN(+L) and served to test the robustness of the concentration of GSSG used.
- Ascorbylperoxide+GSSG: ascorbylperoxide enriched with 10 μM GSSG.

GSSG was used rather than GSH because of its relative low reactivity with other components of parenteral nutrition. γ-Glutamyltranspeptidase has similar affinity for GSSG and GSH [23]. The purpose of using a concentration of 10 μM was to reach the normal plasma concentration that range between 1 and 10 μM [24,25].

Animals were kept on a 12h/12h light cycle. After four days of infusion, animals were sacrificed for collection of the lungs and of the urine in bladder. The left lung was filled with a solution of 10% formalin in PBS at a pressure of 10 cm water and was stored in a same solution for the histological preparation [10]. The right lung samples were removed, processed, aliquot and stored at -80ºC until biochemical determinations. The protocols were approved by the Institutional Committee for Good Practice with Animals in Research, in accordance with the Canadian Council of Animal Care guidelines.

Ascorbylperoxide generation

Because ascorbylperoxide does not commercially exist, it was generated *in vitro*, as previously described [11,12]. Briefly, ascorbylperoxide was generated by a solution containing 14.4 mM ascorbate + 3 mM H_2O_2 + 30 µM riboflavin (pH 7). The solution was incubated at room temperature with strong stirring under ambient light exposure (75 foot-candle) for 48 hours. Thereafter, the solutions were treated 20 min with 100 U/mL catalase and filtered against a 30 kDa filter Amicon Ultra-15 (Millipore Corporation); FOX assay [26] has used to ensure the absence of H_2O_2.

Determinations

Ascorbylperoxide concentrations in the urine were determined by the Regional Centre of Mass Spectrometry of the Université de Montréal as followed. Centrifuged urine was directly injected on HPLC coupled to MS. Data were acquired on a 6224 TOF-LC/MS coupled to a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, USA). Mass Hunter 6.0 software (Agilent) was used to control the system and process the data. Separations were carried out on an Eclipse XDB-C18 analytical column (5 µm particles,

150 mm × 4.6 mm) maintained at 40ºC. The eluents consisted of 10 mM Ammonium Acetate in water (eluent A) and acetonitrile (eluent B). The following gradient elution was applied at a flow rate of 500 µL/min: 0% B from 0 to 3 min; 0 to 80% B from 3 to 5 min; hold at 80% B for 1 min. Eluent B was then decreased from 80 to 0% from 6 to 6.2 min and held constant for up to 12 min to permit column equilibration. The injection volume was 3 µL. Mass spectra were acquired from m/z 100-1500 in negative electrospray mode (VCap -3500 V). Extracted ion chromatograms (±0.05 Da mass window) of deprotonated species were used for quantification. Because we have discovered [12,13] the existence of ascorbylperoxide, it is not commercially available. Therefore, L-2-oxo-thiazolidine 4-carboxilic acid (OTC) was used as internal standard [27] and results were reported as OTC equivalent.

Glutathione (GSH and GSSG) was measured by capillary electrophoresis, as previously described [11,20,27]. The redox potential was calculated (25ºC, pH 7) by using the Nernst equation with the assumption that the density of lung is 1 g/mL (needed to calculate the molar concentration of GSH and GSSG). Because the capillary electrophoresis detection threshold is relatively high for GSH and GSSG, respectively 1 and 0.25 µM, the method described by OW Griffith [28] was used to measure the low total glutathione (GSH+GSSG) concentration in plasma.

Caspase-3 was used as marker of apoptosis. The activated caspase-3 was defined by the proportion of cleaved caspase-3 on total caspase-3 (cleaved + non-cleaved) measured by Western blots as previously described [11].

Alveolarization index was obtained by counting the number of intercepts between a standardized straight-line (1 mm) and histological structures. Each datum is the mean value from four different fields of the same lung (200X magnification) as described [10,11].

8-Isoprostane-F2α : Briefly, homogenized lung samples (1 in 9 volumes of buffer (50 mM Tris base, 0.1 mM EDTA, 200 μM indomethacin, pH 7.4)) were centrifuged at 10000 rpm during 10 minutes at room temperature. Sep Pak C18 columns were used to extract isoprostanes from the supernatants according to the method of Powell WS [29]. The levels of 8-Isoprostane-F2α were quantified by a commercial Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA) as previously reported [9].

Statistical analysis

Data are presented as mean ± S.E.M. and were compared by ANOVA with a level of significance set at $p < 0.05$. The homoscedasticity was assessed by the Bartlett's Chi squared test. The five orthogonal comparisons were 1) Sham versus PN(-L) to confirm the safety of the level of ascorbylperoxide that is present in the photo-protected PN as previously shown [11], 2) PN(+L) vs. PN(+L)+GSSG, 3) ascorbylperoxide vs. ascorbylperoxide+GSSG, 4) PN(+L)±GSSG vs. ascorbylperoxide±GSSG to confirm their similarity and 5) the complementary comparison [sham-PN(-L)] vs. [PN(+L) ± GSSG – ascorbylperoxide ± GSSG].

Results

The mean initial body weights of animals (107 ± 2g) were similar between groups. Total glutathione concentration in plasma was higher in PN(+L)+GSSG than in PN(+L) group (0.5 ± 0.2 vs. 14.2 ± 0.4 μM, $p<0.001$).

The Michaelis-Menten kinetics of glutathione peroxidase as shown in **Figure 1A** supports the notion that ascorbylperoxide is detoxified by this enzyme. From Lineweaver-Burk equation (**Figure 1B**), the Km was 126 +/- 6 μM (n = 3 different curves) and Vmax was 38.4+/-2.5 nmol/min/ unit of enzyme (n=3).

In figures 2, 3 and 4, values from PN(-L) group were similar to those from Sham group (no statistic difference). The addition of GSSG in PN(+L) and in solution containing 180 μM ascorbylperoxide did not change statistically the levels of GSH in lungs ($p<0.05$) (**Figure 2A**) but reduced the values of GSSG ($p<0.01$) (**Figure 2B**). These modifications resulted in prevention of oxidation of pulmonary redox potential by PN(+L) and ascorbylperoxide ($p<0.05$) (**Figure 2C**). Similarly, the addition of GSSG has prevented ($p<0.05$) the rise in apoptotic events as suggested by the elevated activation of caspase-3 under PN(+L) and ascorbylperoxide groups (**Figure 3**), and the loss ($p<0.05$) of alveoli number (**Figure 4**). The urinary concentration of ascorbylperoxide (**Figure 5**) was higher in PN(-L) than in Sham group ($p<0.05$) and higher in ascorbylperoxide than in PN(+L) groups ($p<0.01$). The levels of GSH and GSSG were lower ($p<0.05$) in animals infused with ascorbylperoxide in comparison to animals infused with PN(+L), independently of the presence of GSSG in the intravenous solutions. In contrast the redox potential, activation of caspase-3 and the alveoli number

measured in the ascorbylperoxide groups were similar to the values obtained with animals infused with PN(+L). The presence of GSSG in infused solutions had no effect on the urinary concentration of the ascorbylperoxide but this concentration was higher in animals infused with ascorbylperoxide.

There was no significant difference in the pulmonary level of isoprostane-F2α between groups (Sham: 12± 2; PN(-L): 13±1; PN(+L): 15±2; PN(+L)+GSSG: 15±2; ascorbylperoxide: 14±1; ascorbylperoxide+GSSG: 14±1 pg/mg prot.)

Discussion

The main finding of the study is that the addition of GSSG in PN prevents activation of apoptosis and loss of alveoli induced by photo-oxidant molecules generated in PN such as ascorbylperoxide. This finding is of high importance because it provides hope for a possible prevention of BPD.

The mechanism proposed by the results, could be a correction of redox potential of glutathione or an increase in GSH level in lung, even here the 20% increase did not reach the statistical significance. The absence of modification of the marker of lipid peroxidation, isoprostane-F2α, both in animals receiving an intravenous solution contaminated with peroxides than those receiving a PN enriched in GSSG, confirms the non-radical character of the mechanisms involved [9]. The first possibility is linked to the fact that oxidized redox potential is reported to be an inducer of apoptosis [30]. The values obtained in the present study are similar than those reported previously in comparable experiments [9,11]. However, the highest value of about -204 mV is not so high to induce apoptosis. The review from Shaffer *et al* [30] has associated this value to

the differentiation state of the cells rather than apoptosis. In developing organ, the redox potential must continually fluctuate to allow cells to pass from a state to another between proliferation (low redox value), differentiation and apoptosis (high redox value). In the present study, the redox values obtained in PN(+L) and ascorbylperoxide groups correspond more to differentiation state rather than apoptosis. The discrepancy between redox potential value and activation of apoptosis / loss of alveoli has been previously reported as two parallel effects caused by the ascorbylperoxide [11]. This previous study suggested that apoptosis is induced following activation of death receptors rather than by oxidation of redox potential. However, in the pathology of BPD, the observation of modification of redox potential is important. The histopathology of BPD is associated with a low alveolar development that can result from an exaggerated apoptosis as shown here, or (and) from reduction of pulmonary development that can be caused by a shift in redox potential to a value favouring differentiation. For instance, the association between high oxygen supplement and BPD [1,15] can be explained by the induction of a higher redox potential of glutathione, measured in blood, by the high oxygen concentration inspired by the infant [31,32]. We could extrapolate that high level of oxygen induces a slowing of alveolar development. Hence, the correction of pulmonary redox potential by addition of GSSG in PN could prevent the deleterious effects of oxygen supplement. In theory, increasing glutathione in plasma in order to improve availability of cysteine in tissue would help the infants to improve their antioxidant defenses based on GSH, especially if they are exposed to an oxygen supplementation. High oxygen stimulates the capacity of glutathione synthesis [33] as shown in premature infants [19]. Brown *et al* have previously reported [25] a protective impact of glutathione against the toxicity induced by oxygen. They have demonstrated

that infusion in preterm rabbits of 1 mM GSH (100 fold the concentration of GSSG used here) prevented the toxicity of 95% oxygen on lung compliance, edema and cell viability. By this experiment on 24 hours, the authors shown that the infusion of GSH had re-established the normal glutathione value in lungs (similar to the values presented here in figure 2), which was lower in animals exposed to 95% O_2. Although these results support the notion that glutathione enrichment warns the toxicity of high levels of oxygen, they are not enough to prove that it may prevent deleterious effect of high oxygen supplementation on BPD development. Further studies are needed to assess the impact of adding GSSG in PN on the development of BPD in circumstance of the double exposure to PN and to oxygen support.

The second possibility to explain the positive effect of GSSG addition in infused solutions is, according to the hypothesis, the fact that a better level of GSH favours a higher *in vivo* activity of glutathione peroxidase. Figure 1 shows that ascorbylperoxide is an excellent substrate for this enzyme. Results support the notion that a better availability of GSH allows a greater detoxification of ascorbylperoxide, and of other peroxides generated in PN. The low glutathione level in premature infants could explain their vulnerability to the ascorbylperoxide generated in the PN.

The similarity of the protective effects of GSSG into PN (+L) and ascorbylperoxide groups, suggests that the amount of used GSSG was sufficient to detoxify ascorbylperoxide to a concentration five times greater than that measured in the PN (+L). This high concentration of ascorbylperoxide could be observed in PN solutions containing higher concentration of multivitamin preparation [34]; photo-protection of

such solution could not sufficiently reduce the concentration of ascorbylperoxide in order to observe a lower incidence of BPD.

As was previously demonstrated [11,27], the urinary concentrations of ascorbylperoxide reflect the amount of the infused molecules. These data confirm that animals from ascorbylperoxide groups have received a five time higher amount of ascorbylperoxide than animals infused with PN(+L). The similarity of results observed between PN(-L) and Sham groups could suggest that the photo-protection of PN is a possible procedure to reduce the ascorbylperoxyde to a safe level. However, in clinical situation, an adequate photo-protection is very hard to realize. Between its compounding in pharmacy service to the bedside, PN should never see the light, which is almost impossible in clinical routine.

The rational use of GSSG rather than GSH was to improve the animal's glutathione status while interfering as little as possible with the other compounds of the PN. We can expect that the glutathione will interact with the redox status of other antioxidants. These molecules are the ascorbate and, in a lesser extend, cysteine. By reducing the dissolved oxygen to generate H_2O_2 [4], ascorbate (AA) becomes dehydroascorbate (DHA). Because the standard reduction potential of the couple GSSG;2GSH (-0.23 V) is lower than those of DHA;AA (+0.06 V), GSH has the potential to reduce the DHA in ascorbate, perpetuating the generation of peroxides as oxygen remains available. Hence, all the GSH would become GSSG. If our choice was to use GSH rather that GSSG, the concentration used would be 20 μM, 50 times less than ascorbate (1.1 mM). By its relatively low concentration, GSH would be vulnerable. Its transformation in GSSG implies a radical intermediate (GS•). The high ratio of the other components of the PN

on GSH (at least 4 orders of magnitude) increases the possibility that GS• reacts with other components of PN, with the final consequence of a loss of free glutathione.

On the other hand, the high concentration of H_2O_2 generated in PN [3,9] may certainly interact with the thiol function of cysteine (CSH). This fact may explain the 90% loss of thiol functions in PN (exclusively from cysteine) in the 24 hours duration of PN (changed daily) [35]. The standard reduction potential of the couple CSSC;2CSH (CSSC being cystine) (-0.22 V) [36] is close but higher than that for GSSG;2GSH (-0.23V). Therefore, there remains a possibility that GSH may reduce CSSC into CSH, but not a reduction of GSSG by the CSH, even less at pH 5.5 of the PN solution. The most suitable possibility is a thiol exchange between GSSG and CSH to produce GSSC [36]. Despite these possible interactions, the objective was to improve glutathione level in plasma, and that has been reached.

Another conceivable side impact of the addition of GSSG into the PN is a modification of the redox status of cysteine/cystine or GSH/GSSG in plasma. The ratio cysteine (10-25 μM) / cystine (50-150 μM) in plasma is in favour of cystine, at least in adults [37,38]. This ratio will definitely be more influenced by cysteine/cystine from the PN (initially 3.14 mM of cysteine before its oxidation into cystine) than by the infusion of 10 μM GSSG. On the contrary, Brown *et al* [25] reported that the ratio of GSH / GSSG in plasma of preterm rabbits was in favor of the reduced form (GSH). Of course, by infusing, in continuous, GSSG directly in blood stream, we expect a modification of this ratio. Therefore, even with the favorable results obtained here with the addition of GSSG in PN, further studies are required to investigate the possible metabolic impact of this modification in newborn animals, and more in premature infants. The search for the

minimal efficient dose of GSSG to add into PN solution may help to lowering this potential impact.

Several differences exist between our animal model and extreme premature infants. However, the comparisons of some of the following factors suggest that our observations in animals could occur in human infants. For instance, 1) if GSH plays a key role, its level in premature newborn is low; 2) if a 4 days infusion of PN(+L) is sufficient to induce a loss of alveoli in animals, the median duration of PN is 20 (25^{th}-75^{th}: 11-31; n=104) days (Sainte-Justine Hospital experience) in premies, 3) if the lung development of guinea pig newborn is mature at birth, the immaturity of lungs of premature infants can only worsen the situation. The toxicity associated to the ascorbylperoxide generated into the PN(+L) seems independent of the developmental stage of the lung. Indeed, the cell death (apoptosis) observed here in animals infused with PN(+L) has also been reported by others in isolated cells in culture. Nasef N *et al* [4] showed the loss of viability of lung epithelial cells derived from preterm rats in presence of a solution of PN (primene + dextrose + multivitamins or ascorbate) devoid of photo-protection. They have associated this toxicity to the presence of hydroperoxides generated by ascorbate. Zaniolo K *et al* [39] had similar results (cell death) with human skin or lung fibroblasts as well as with mice embryonic fibroblasts cells exposed to a solution of parenteral multivitamin preparation devoid of light protection. For both studies, the cellular death was prevented by the photo-protection.

In conclusion, addition of GSSG in PN prevents the deleterious effect of ascorbylperoxide, generated in PN, on lung alveolarization of newborn animals born at term. Results suggest that the mechanism is by an improving of the GSH status in lung

that favours detoxification of this peroxide. Even if caution is required before to extrapolate our finding to infants, the study brings hope in the fight against BPD.

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Figure 1

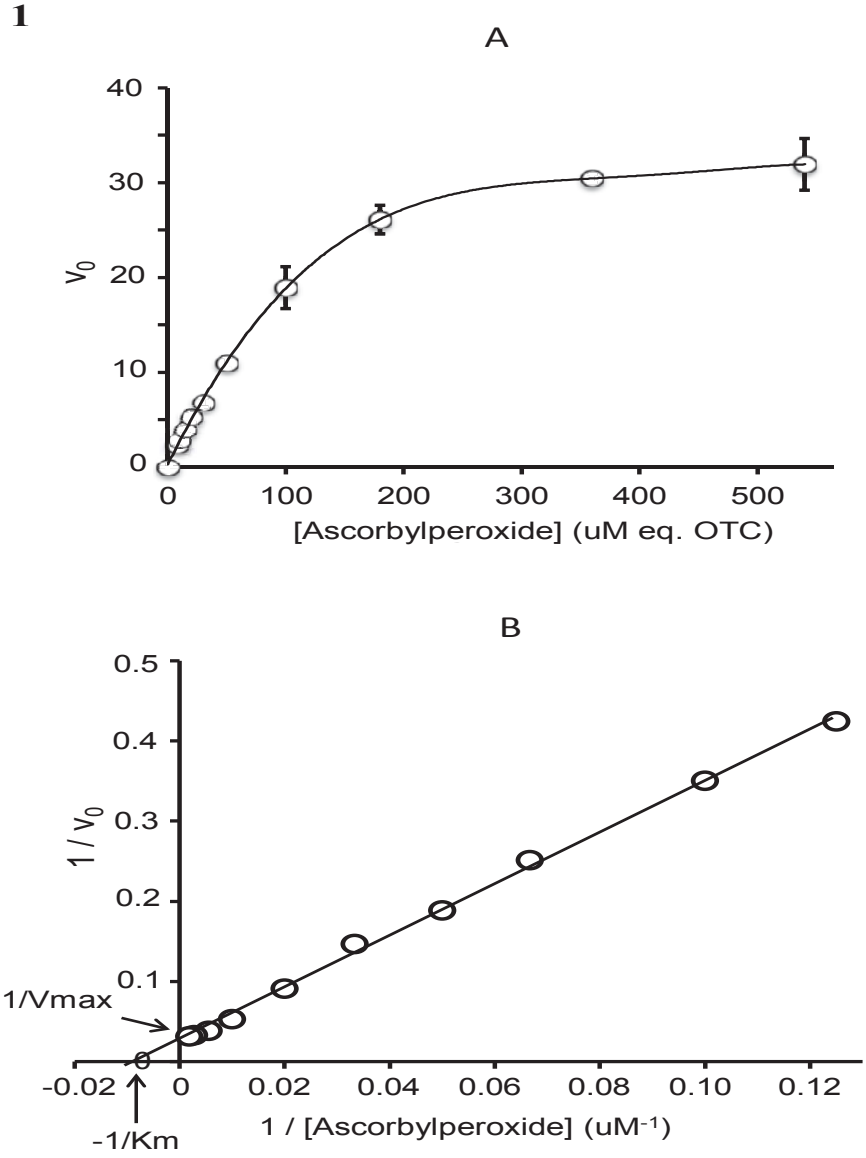

A
40
30
20
10
0
v_0
0
100
200
300
400
500
[Ascorbylperoxide] (uM eq. OTC)
B
0.5
0.4
0.3
0.2
0.1
1 / v_0
1/Vmax
-0.02
0
0.02
0.04
0.06
0.08
0.10
0.12
-1/Km
1 / [Ascorbylperoxide] (uM^{-1})

Figure 2

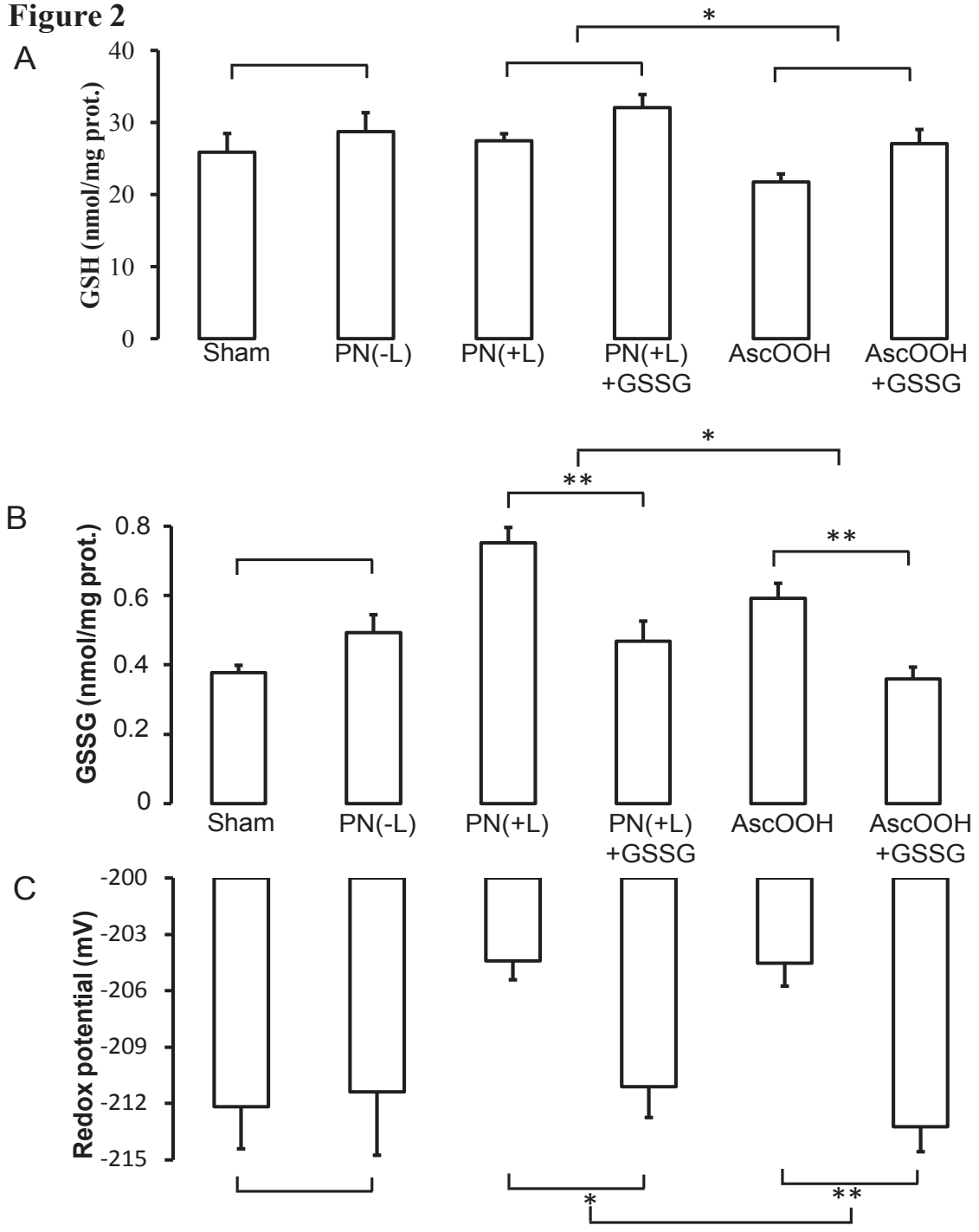

A
GSH (nmol/mg prot.)
40
30
20
10
0
Sham
PN(-L)
PN(+L)
PN(+L) +GSSG
AscOOH
AscOOH +GSSG
*
B
GSSG (nmol/mg prot.)
0.8
0.6
0.4
0.2
0
Sham
PN(-L)
PN(+L)
PN(+L) +GSSG
AscOOH
AscOOH +GSSG
*
**
**
C
Redox potential (mV)
-200
-203
-206
-209
-212
-215
*
**

Figure 3

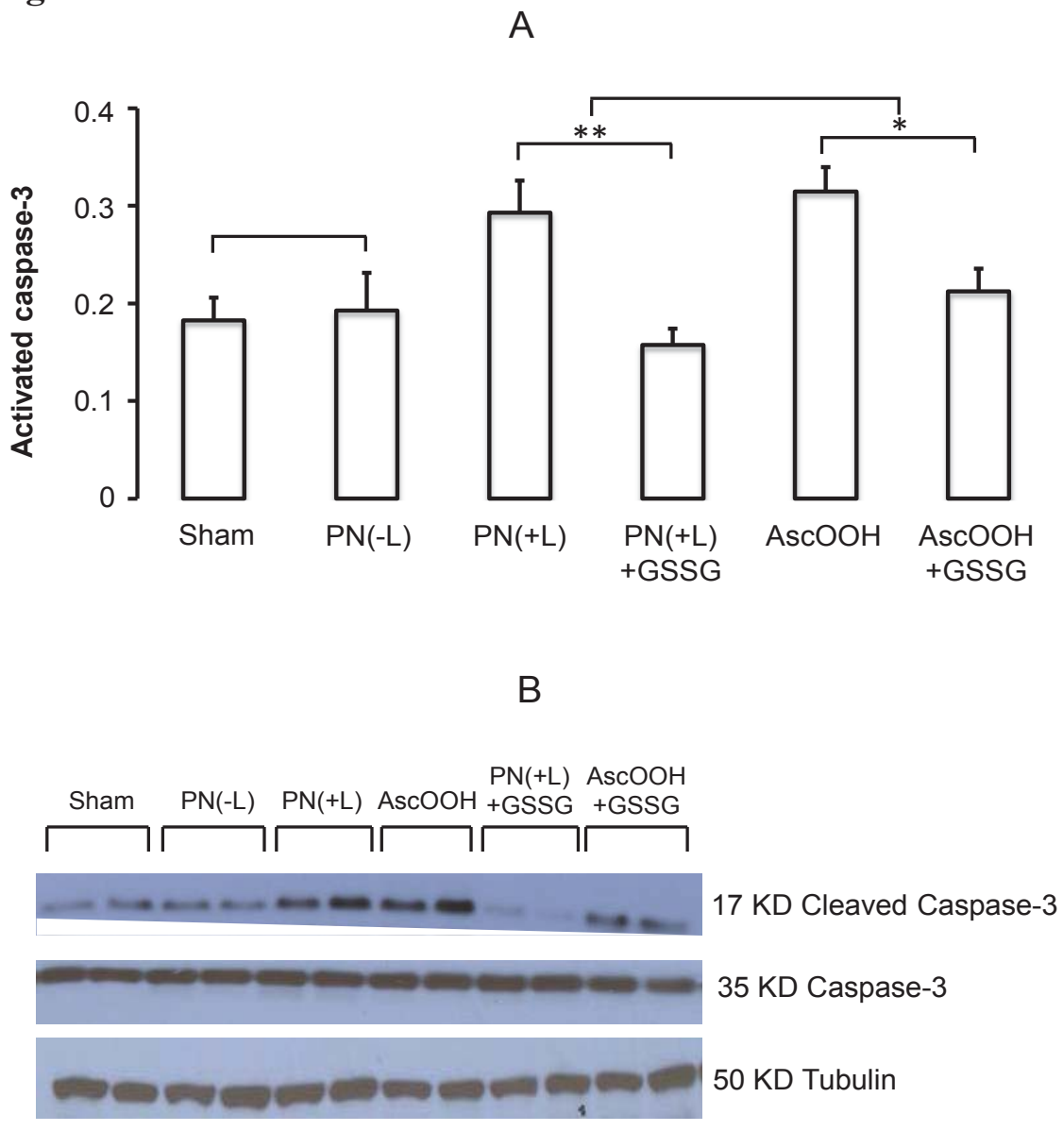

A
Activated caspase-3
0.4
0.3
0.2
0.1
0
**
*
Sham
PN(-L)
PN(+L)
PN(+L) +GSSG
AscOOH
AscOOH +GSSG
B
Sham
PN(-L)
PN(+L)
AscOOH
PN(+L) +GSSG
AscOOH +GSSG
17 KD Cleaved Caspase-3
35 KD Caspase-3
50 KD Tubulin

Figure 4

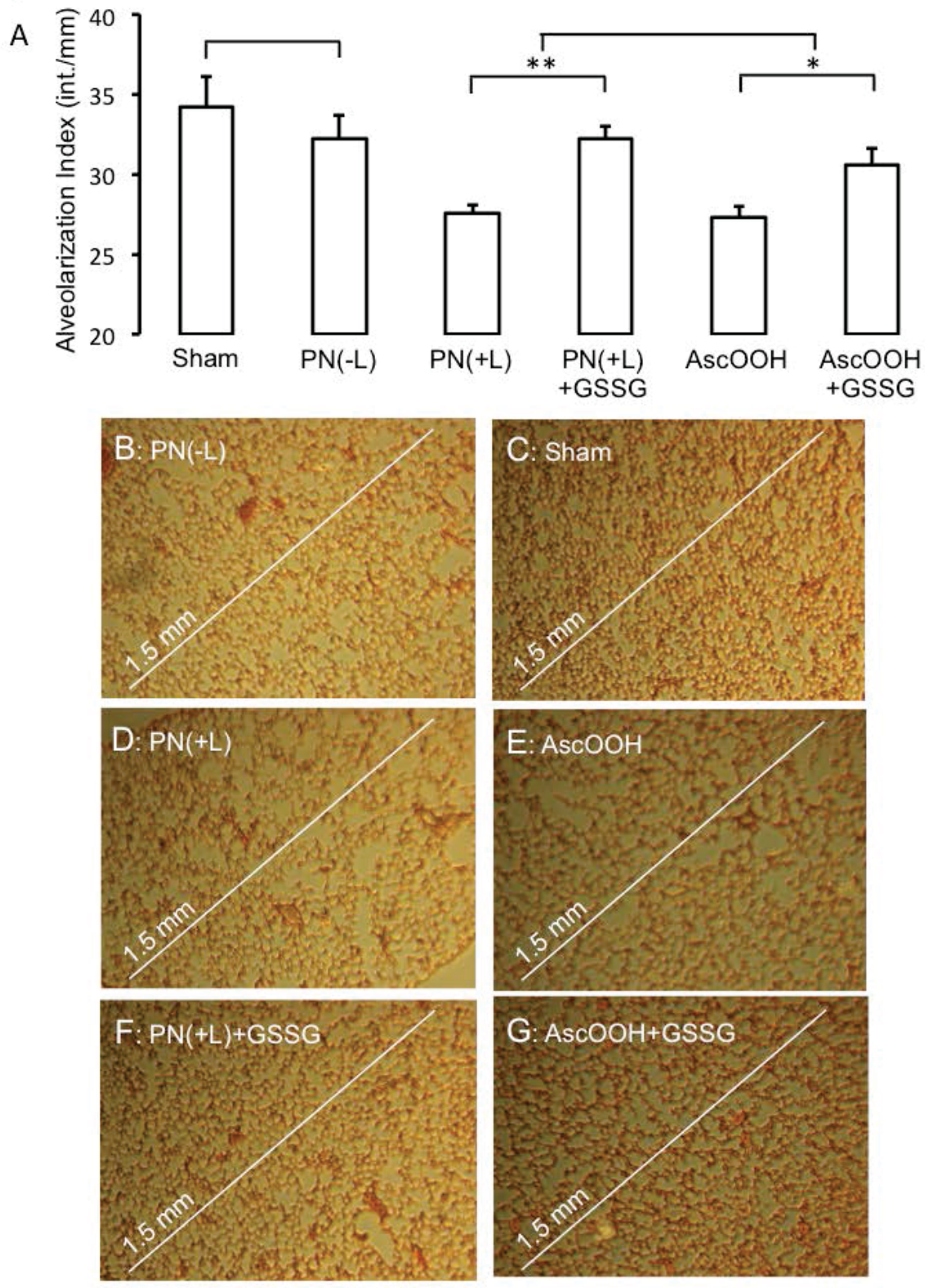
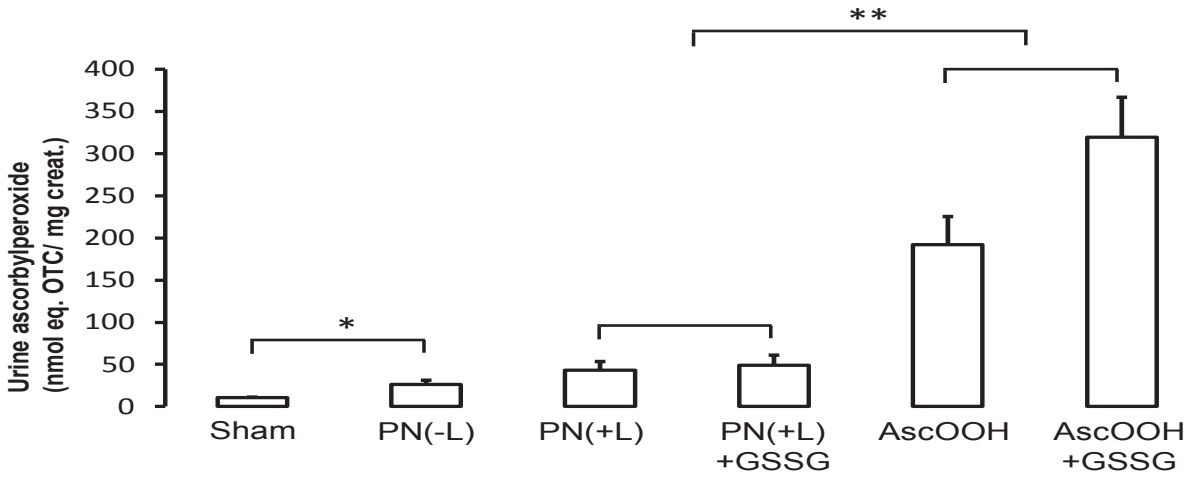

A
Alveolarization Index (int./mm)
40
35
30
25
20
**
*
Sham
PN(-L)
PN(+L)
PN(+L) +GSSG
AscOOH
AscOOH +GSSG
B: PN(-L)
C: Sham
D: PN(+L)
E: AscOOH
F: PN(+L)+GSSG
G: AscOOH+GSSG
1.5 mm

Figure 5


**
*
400
350
300
250
200
150
100
50
0
Urine ascorbylperoxide
(nmol eq. OTC/ mg creat.)
Sham
PN(-L)
PN(+L)
PN(+L)
+GSSG
AscOOH
AscOOH
+GSSG

Legends for Figures

Figure 1: Enzymatic kinetics of glutathione peroxidase in function of increasing concentration of ascorbylperoxide.

Panel A: Initial velocity of the reaction (v_0) in function of increasing concentration of ascorbylperoxyde. The kinetic correspond to the Michaelis-Menten model. Data are expressed as mean ± S.E.M., n=3. For some mean data, the S.E.M is smaller than the symbol. Panel B: Lineweaver-Burk representation of mean data from the panel A. The linear equation $1/ v_0 = 3.246 (1/[S]) + 0.026 (1/v_0)$ was highly significant ($r^2 = 0.998$). From three different experiments, the apparent Km was 126 ± 6 μM whereas the Vmax was 38.4 ± 2.5 μmol NADP generated / minute / U glutathione peroxidase.

Figure 2: GSH, GSSG and redox potential of glutathione in lungs.

Sham: animals with a closed catheter, animals were fed with regular food for guinea pig; PN(-L): animals fed exclusively with parenteral nutrition (PN) protected from light (-L); PN(+L): PN without photo-protection; AscOOH: animals infused with solution containing 180 μM ascorbylperoxide (AscOOH); +GSSG: 10 μM GSSG were added to the intravenous solutions. Panel A: The GSH level was lower in the ascorbylperoxide group than in the PN(+L) group ($p<0.05$) independently of the presence of GSSG in the intravenous solutions. Panel B: the addition of GSSG in intravenous solutions has prevented ($p<0.01$) the increase of GSSG in lungs of animals infused with PN(+L) or ascorbylperoxide. GSSG was higher ($p<0.05$) in PN(+L) than in ascorbylperoxide. Panel C: The presence of GSSG in intravenous solutions have prevented ($p<0.05$) the oxidation

of redox potential of glutathione by PN(+L) and ascorbylperoxide. Mean ± S.E.M., n=4-8 per group; *: $p<0.05$; **: $p<0.01$.

Figure 3: Activation of caspase-3 in lungs.

Sham: animals with a closed catheter, animals were fed with regular food for guinea pig; PN(-L): animals fed exclusively with parenteral nutrition (PN) protected from light (-L); TPN(+L): PN without photo-protection; AscOOH: animals infused with solution containing 180 μM ascorbylperoxide (AscOOH); +GSSG: 10 μM GSSG were added to the intravenous solutions. Panel A: The presence of GSSG in intravenous solutions have prevented ($p<0.05$) the activation of caspase-3 (marker of apoptosis) by PN(+L) and ascorbylperoxide. Panel B: Representative examples (n=2 per group) of Western blot obtained for the cleaved and non-cleaved caspase-3, relatively to tubulin. Mean ± S.E.M., n=4-7 par group; *: $p<0.05$; **: $p<0.01$.

Figure 4: Alveolarization index.

Sham: animals with a closed catheter, animals were fed with regular food for guinea pig; PN(-L): animals fed exclusively with parenteral nutrition (PN) protected from light (-L); TPN(+L): PN without photo-protection; AscOOH: animals infused with solution containing 180 μM ascorbylperoxide (AscOOH); +GSSG: 10 μM GSSG were added to the intravenous solutions. Panel A: The presence of GSSG in intravenous solutions have prevented ($p<0.05$) the loss of alveoli by PN(+L) and ascorbylperoxide. Panels B-G: Representative examples of histological pictures of lungs of animals from PN(-L) group (B), Sham group (C), PN(+L) group (D), 180 μM ascorbylperoxide (E), PN(+L)+GSSG group (F) and 180 μM ascorbylperoxide+ GSSG group (G). The alveolarization index is

based on the number of histological structures crossing the calibrated (1.5 mm) line. Mean ± S.E.M., n=4-7 per group; *: $p<0.05$; **: $p<0.01$.

Figure 5: Urinary concentrations of ascorbylperoxide.

Sham: animals with a closed catheter, animals were fed with regular food for guinea pig; PN(-L): animals fed exclusively with parenteral nutrition (PN) protected from light (-L); TPN(+L): PN without photo-protection; AscOOH: animals infused with solution containing 180 μM ascorbylperoxide (AscOOH); +GSSG: 10 μM GSSG were added to the intravenous solutions. The concentration of ascorbylperoxide was higher in the PN(-L) than in the sham group ($p<0.05$), higher in the ascorbylperoxide groups than in the PN(+L) groups ($p<0.01$). The presence of GSSG in intravenous solutions did not affect the urinary levels of ascorbylperoxide.

Mean ± S.E.M., n=4-6 per group; *: $p<0.05$; **: $p<0.01$.

4. General discussion

PN is a necessary form of nutrition for neonates with an impaired or immature gastrointestinal tract. However, laboratory animal studies and human investigations have shown that PN was associated with the development of BPD. Due to limited knowledge in the literature regarding PN and the incidence of BPD, no approach has yet been developed to address the mechanism with certainty. Nevertheless, through the various studies performed in our laboratory, we have discovered a by-product of vitamin C, an oxidant called ascorbylperoxide, which we think may be the main cause of BPD. The mechanisms whereby ascorbylperoxide, as a contaminant of PN, might lead to BPD are as yet unclear. An understanding thereof may help to optimize nutritional support of preterm infants and decrease the risk of BPD and subsequent chronic lung disease. Previous studies from our laboratory and other teams have shown that photoprotection of PN decreases the risk of BPD. This was shown by the complete protection of PN from light which in turn decreased the generation of peroxides contaminating PN, especially ascorbylperoxide and hydrogen peroxides. Recently, special attention paid to minimizing PN photodegradation by-products has shown a significant direct effect on preventing BPD, and it is becoming increasingly clear that light protection of PN can avoid specific metabolic complications in neonatal patients.

Unfortunately, it is difficult to obtain such photoprotection in the clinical setting. Because only a few minutes of light exposure is sufficient to generate significant amounts of oxidant molecules in PN solutions, photoprotection must be initiated from the time of PN preparation (without any exposure to light) all the way to the patient's bedside where the bag and delivery tubes must be fully covered.

Mohamed et al. have recently shown that early exposure to PN was associated with protracted oxidative stress until 36 weeks' PMA, along with BPD. [167] This study showed that more than two weeks on PN was sufficient to observe an increasing in oxidized redox potential of glutathione at 36 weeks' PMA. Oxidized redox potential can be considered a measure of oxidative stress. This threshold in duration of PN on redox potential was the same as that for the induction of BPD. [167] Thus our general hypothesis was that PN as administered in neonatal units is a major source of oxidative stress that contributes to the development of BPD.

The oxidative stress seems to derive mainly from the interaction between vitamin C and dissolved oxygen in the PN solution, which generates the oxidized form of vitamin C, DHA, as well as H_2O_2. The inorganic peroxides constitute approximately 80% of all peroxides generated in PN [170]. The reaction is accelerated by the photo-excited riboflavin that transfers energy from ambient light to the reaction. Photo-excited riboflavin also potentiates the reaction of the free radical species H_2O_2 with DHA, to form a new compound, ascorbylperoxide. [169, 172] We hypothesized that this oxidative stress, especially the modification of redox potential, might interfere with lung development.

Lung maturation must pass through the cell cycle stages of proliferation, differentiation, and apoptosis in order to continue lung remodeling. Theses stages are dependent on the redox potential. The shift from a reduced state to an oxidized state induces proliferation, differentiation and apoptosis. During the proliferation phase, when the redox value is highly reduced, cells have a high metabolic rate leading to increased generation of ROS. These ROS shift the redox potential toward an oxidized state, inducing the differentiation phase; apoptosis occurs when the cell is in an even more oxidized state. [195] So we think that the peroxides contaminating the PN solution increase the redox potential to an oxidized

state, leading to apoptosis. Apoptosis decreases the number of the alveolar cells, which is the hallmark characteristic of BPD.

In fact, PN solutions are contaminated with a number of molecules having the ability to disturb the redox status of the lung, such as ascorbylperoxide [119, 196] and H_2O_2. [170, 197] Glutathione is considered a key element of antioxidant defenses and an important regulator of the redox system. All of these molecules, including AscOOH and H_2O_2, are detoxified by the glutathione system. On the other hand, glutathione levels are low in preterm infants, so these molecules can overcome the glutathione system, allowing the redox potential to shift toward an oxidized state. This fact is supported by studies in animals and premature infants. In animals, specifically in newborn guinea pigs, the infusion of PN without light protection for 4 days showed: 1) a decreased level of glutathione [198]; 2) a more oxidized glutathione redox potential [199]; and 3) a lower alveolar count as compared to animals infused with a fully photo-protected solution. [199, 119] In premature infants (26 ± 1 weeks' gestation), the blood glutathione redox potential measured on postnatal day 7 was correlated with the severity of BPD; a more oxidized status was measured in the most severe cases. [200] According to our hypothesis, peroxides generated in PN induce the development of BPD in preterm infants. The proposed mechanisms are oxidation of the redox potential of glutathione by AscOOH and/or H_2O_2; this oxidized state induces overstimulation of apoptotic mechanisms, followed by loss of alveolar tissue. We therefore evaluated the impact of AscOOH with and without H_2O_2 on the metabolism of glutathione. This was achieved by infusing newborn guinea pigs with solutions containing increasing concentrations of AscOOH with and without H_2O_2, and measuring lung concentrations of GSH and GSSG, redox potential, and level of apoptosis. The results were reported in the first article.

AscOOH induced: 1) oxidation of the redox potential; 2) activation of caspase-3, which is a step of no return in apoptosis; and 3) a lower alveolarization index. The second part of the hypothesis was that contamination of PN with H_2O_2 exacerbates the oxidative stress and consequently influences the impact of ascorbylperoxide. Indeed, infusion of 350 μM H_2O_2 (without AscOOH) induced an increasing of the redox potential that reached a plateau. The mean redox value measured in animals infused with solutions containing H_2O_2 was similar to that observed in animals infused with solutions containing the two highest concentrations of AscOOH (without H_2O_2). This observation suggests that the redox potential might not be the main trigger of apoptosis. Indeed, although animals infused with H_2O_2 had the most oxidized redox potential, independently of the presence of AscOOH, the activation of caspase-3 induced by AscOOH was 15% lower in H_2O_2 groups. Nonetheless, the activation of caspase-3 induced by AscOOH in the presence of H_2O_2 remained sufficient to induce a decrease in alveolar number. The effect of AscOOH on the alveolarization index was similar to the values previously reported in studies investigating the effect of PN. [199]

The observations that the redox potential does not seem to play a key role in the activation of caspase-3 and that H_2O_2 reduces the activation of caspase-3 suggest that the impact of ascorbylperoxide on caspase-3 is through the death receptors. In the pathway from these receptors to apoptosis, caspase-8 is first activated and then caspase-3 is activated. The fact that caspase-8 is sensitive to inhibition by H_2O_2 [201] could explain the negative impact of H_2O_2 on activation of caspase-3 in our animal model. This pathway remains to be investigated.

The main finding of this research is that AscOOH, a by-product of peroxidation of DHA generated in the solution of PN, is toxic to the lung in our animal model. Similarly, Zaniolo

K et al [202] reported that incubation of isolated cells in culture with the parenteral multivitamin Multi-12, as used here, induced cell death; photoprotection of the parenteral solution prevented this toxic effect. After a four-day infusion in animals, AscOOH induced a main feature of BPD, namely a lower alveoli number, independently of the presence of H_2O_2.

According to our hypothesis, higher levels of GSH favour higher *in vivo* activity of glutathione peroxidase. Our results showed that AscOOH was an excellent substrate for this enzyme. Results support the notion that greater availability of GSH allows for greater detoxification of AscOOH and other peroxides generated in PN. In parallel, Mohamed et al. has shown that preterm infants have a limited capacity for AscOOH detoxification, as shown by the accumulation of AscOOH in the first week following birth. A urinary AscOOH level on postnatal day 7 was shown in this study to be a good predictor of BPD severity in preterm infants less than 29 weeks of gestation [203].

Because GSH is the main contributor to the cellular redox environment and the main antioxidant for detoxification of peroxides via glutathione peroxidase, the low GSH level observed in premature infants could be a key factor in BPD development. Thus we believe that a possible strategy to prevent BPD development or reduce the severity of this disease may be the preservation or increase of intracellular concentrations of GSH. Our hypothesis is based on the fact that the low GSH levels observed in preterm infants are not caused by immaturity of the enzymatic process. GSH synthesis is very active, even in newborns of 26 weeks' gestation [154]. The low intracellular availability of cysteine limits glutathione synthesis [152]. The immaturity of the cellular cysteine transport system explains the

failure of intravenous enrichment of cysteine or N-acetylcysteine to improve GSH levels and prevent the development of BPD [204].

Gamma glutamyl transpeptidase plays an important role in supplying cells with needed cysteine for the synthesis of GSH [205]. This omnipresent enzyme transfers the γ-glutamyl moiety of GSH present in plasma (from liver) onto a second amino acid, forming two dipeptides (glutamyl-amino acid and cysteinylglycine). These dipeptides are absorbed by the cell and hydrolysed into free amino acids. Free cysteine availability in the cell is a limiting factor for *de novo* synthesis of GSH [148].

The capabilities of γ-glutamyl transpeptidase and other synthesizing enzymes are well developed in preterm infants [154]. GSH is present in the bloodstream and emanates from the liver. This organ has a high capacity for synthesizing GSH through the transformation of methionine to cysteine. However, the first enzyme of this transformation, MAT, is inhibited by peroxides from PN, as shown in animal models, and the last enzymatic step, cystathionase, is immature in premature infants. The inhibition of MAT by PN has been associated with low GSH levels in the blood [206], liver [206], and lung [207].

In order to prevent the development of BPD in premature infants, we have to reduce the oxidative stress or help newborns detoxify peroxides from PN. This can be done by preventing GSH loss or by increasing intracellular levels of GSH. On the other hand, decreasing or limiting peroxides that contaminate PN is critical but difficult to achieve.

Thus we hypothesized that the addition of GSH to PN solutions, bypassing the liver for GSH production, should increase the cellular capacity to detoxify AscOOH and consequently prevent the loss of alveoli associated with PN. We chose to add GSSG rather GSH to PN. The rational for this choice was based on: 1) stability of the molecule in PN solution, as explained in the second article of the thesis and demonstrated in the third one;

and 2) similar affinity of γ-glutamyl transpeptidase for both forms of glutathione [148, 208]. The results were spectacular: by correcting the low plasma glutathione (from 0.5 μM to 14 μM), we prevented the oxidation of the redox potential, the activation of caspase-3, and the loss of alveoli in animals on PN. This demonstration suggests that individuals with low glutathione such as premature infants, are susceptible to the toxicity of peroxides, especially AscOOH, that are infused with PN. In fact, administration of PN induces a pernicious cycle, because peroxides from PN also inhibit the generation of glutathione by the liver. We have reported that, at least in newborn guinea pigs, the mechanism of PN inhibition of hepatic MAT is not explained solely by the classical and reversible oxidation of its thiol functions by peroxides present in solution [206]. The prevention of peroxide formation in PN or improving the redox potential in the liver would therefore not be enough to eliminate metabolic complications associated with this mode of nutrition. Our interpretation of these results leads me to believe that the inhibition of MAT in animals infused with PN is caused not only by peroxides but also by other compounds such as aldehydes from the peroxidation of endogenous lipids in the parenteral lipid emulsion. Our results highlight the fact that the addition of GSSG is good for the lung, whereas the hepatic metabolism is still disrupted. We must therefore also work to reduce the generation of undesirable molecules in PN.

5. Conclusion

PN as administered in neonatal units seems to be the major source of oxidative stress that contributes to the development of BPD. AscOOH generated in PN appears to be the active component leading to the loss of alveoli with apoptosis. Although AscOOH induces oxidation of the redox potential, the results of the present study suggest that it is a parallel phenomenon to the apoptosis. Indeed, the redox value obtained could be insufficiently oxidized to induce apoptosis. However, this change should not be taken lightly. The oxidation of the redox potential obtained with AscOOH or H_2O_2 could affect the balance between the proliferation and differentiation stages of the cell. A developing organ such as the lung in newborns must pass through different stages (cellular proliferation, differentiation and apoptosis) to promote its remodelling until final maturity. BPD may result from the perturbation of several of these cellular stages. Here, with our experimental model, we have explored the loss of alveoli, a feature of BPD, induced by the AscOOH generated in PN solutions. Nevertheless, because the generation of AscOOH is dependent on light exposure, the present study explains the beneficial effect of photoprotection of PN solutions on chronic lung disease such as neonatal BPD. The results generated by this study add an important piece of information regarding the mechanisms by which PN as presently compounded may induce the development of BPD.

GSH plays a vital role against ROS-induced injury under physiological and disease conditions. The concentrations of GSH and GSSG are very important in the maintenance of the redox potential, pivotal to cell survival and development. Premature infants exposed to high concentrations of oxygen in the first few weeks after birth experience an additional oxidative burden on the GSH system.

In animals born at term, the addition of GSSG in PN prevents the deleterious effect on lung alveolarization of the AscOOH generated in PN solutions. Results suggest that the mechanism may involve an improvement of the GSH status in the lung, favouring peroxide detoxification. Even if caution is required before implementing these findings in infants because of the unknown physiological impact of increasing GSH concentrations, the study brings hope in the fight against BPD.

6. Limitations

PN is a mode of nutrition frequently used in neonates born before 30 weeks of gestation. The investigation of the impact of PN in this population is difficult because of the lack of the biological samples from premature infants and the absence of a control group (premature infants not given PN). The use of newborn animals born at term enabled us to study the impact of PN during the neonatal period, independent of prematurity. Therefore, caution is needed in the extrapolation of data to human premature newborns. Further, the physiological impact of increasing GSH concentrations is unknown. Further research in long- and short-term effects is necessary before introducing these strategies in the clinical setting.

Gender is an important determinant in the incidence of BPD, which is higher in boys than girls [143]. An animal model was used to demonstrate the links between AscOOH and alveolar hypoplasia and to test the safety of PN solutions. The achievement of these objectives could be reached using animals of the same gender, all males. However, because the animals used in our experiments were all males, results of my work do not provide additional information to explain the gender factor. In my opinion, it would be interesting to repeat the experiments with female animals to isolate the effect of gender. With the hypothesis that low levels of glutathione are the main cause of the BPD, and glutathione levels are reportedly lower in male premature infants [143], it makes sense that males have a higher prevalence of BPD. If this hypothesis were proven true, we would obtain the same results with females. On the other hand, if a gender difference remains independently of glutathione, a new avenue of research could be undertaken to explain the impact of gender on health fragility in this age group.

Oxygen supplementation and PN, the main sources of oxidative stress, are both frequently administered to preterm infants. In our experiments, the animals were exposed only to the PN, without hyperoxia. This is another limitation in the extrapolation of findings to premature infants. Moreover, we expect that the effects of the two sources of oxidants are additive. Further studies are needed to investigate the combination of the two.

7. Perspectives and future work

Our results demonstrate that AscOOH generated in PN appears to be the active component leading to apoptosis and alveolar loss [207]. However, AscOOH activation of apoptosis through death receptors remains to be validated. One possible mechanism would be the oxidation of cysteine in death receptor proteins (similar to the way that AscOOH inhibits MAT), i.e. through oxidation of an active thiol receiver. Further studies are needed to investigate the impact of AscOOH on the death receptor activation.

In our research, we infused 10 μM of GSSS and obtained 14 μM glutathione in the plasma. The physiological and maximal value reported as being normal is 10 μM. It will be important to investigate different concentrations of GSH to add to PN solutions, to determine the minimal and effective plasma level of GSH that can prevent the deleterious effects of peroxides in PN. This value would be the concentration to expect during a clinical trial. Moreover, before introducing this practice in a clinical setting, further research is necessary to investigate possible long-term consequences of early-life modification of the glutathione system.

Our results demonstrate that the addition of GSSG to PN prevents the activation of apoptosis and the loss of alveoli induced by photo-oxidant molecules generated in PN, such as ascorbylperoxide [207]. An equally important question is whether the addition of GSSG will provide protection during oxygen supplementation or hyperoxia. In fact, increasing GSH in plasma to improve the availability of cysteine in tissues could help infants improve their GSH-related antioxidant defenses, especially if they are exposed to oxygen supplementation. High oxygen stimulates glutathione synthesis [209], as shown in premature infants [154]. Brown et al have previously reported [210] the protective effect of GSH against the toxicity induced by oxygen. They demonstrated that infusion of 1 mM

GSH in preterm rabbits (100-fold concentration of the GSSG used in our protocol) prevented the toxicity of 95% oxygen on lung compliance, edema, and cell viability. With this experiment, which spanned a period of 24 hours, the authors showed that the infusion of GSH had re-established normal glutathione levels in the lungs (similar to our values), which was lower in animals exposed to 95% O_2. Although these results support the notion that glutathione enrichment wards off the toxicity of high levels of oxygen, they are not enough to prove that it may prevent the deleterious effect of high oxygen supplementation on lung development, namely BPD. Further studies are needed to assess the impact of adding GSSG to PN to prevent the development of BPD in infants exposed to a combination of both PN and oxygen support.

MAT is at the crossroads of several metabolic pathways for example DNA methylation further studies needed to examine the impact of PN on the DNA methylation.

Finally it will be urgent to apply this new nutritive solution for the neonates in order to decrease the incidence of the complications related to oxidative stress for this population by increase the production of GSH.

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