Université de Montréal

The effect of oxygen and parenteral nutrition on the redox potential and bronchopulmonary dysplasia in extremely preterm infants

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RÉSUMÉ

Introduction: Le supplément d'oxygène et la nutrition parentérale (NP) sont les deux sources majeures de stress oxydant chez le nouveau-né. Lors de la détoxification des oxydants, le potentiel redox du glutathion s'oxyde. Notre hypothèse est que le supplément d'oxygène et la durée de la NP sont associés à un potentiel redox plus oxydé et à une augmentation de la sévérité de la dysplasie bronchopulmonaire (DBP).

Patients et Méthodes: Une étude observationnelle prospective incluant des enfants de moins de 29 semaines d'âge gestationnel. Les concentrations sanguines de GSH et GSSG à jour 6-7 et à 36 semaines d'âge corrigé étaient mesurées par électrophorèse capillaire et le potentiel redox était calculé selon l'équation de Nernst. La sévérité de la DBP correspondait à la définition du NICHD.

Résultats: Une $FiO_2 \geq 25\%$ au $7^{ième}$ jour de vie ainsi que plus de 14 jours de NP sont significativement associés à un potentiel redox plus oxydé et à une DBP plus sévère. Ces relations sont indépendantes de l'âge de gestation et de la gravité de la maladie initiale. La corrélation entre le potentiel redox et la sévérité de la DBP n'est pas significative. La durée de la NP était responsable de 15% de la variation du potentiel redox ainsi que de 42% de la variation de la sévérité de la DPB.

Conclusion: Ces résultats suggèrent que l'oxygène et la NP induisent un stress oxydant et que les stratégies visant une utilisation plus judicieuse de l'oxygène et de la NP devraient diminuer la sévérité de la DBP.

Mots clés: enfants prématurés, oxygène, alimentation parentérale, stress oxydant, potentiel redox**,** dysplasie bronchopulmonaire.

ABSTRACT

Introduction: oxygen supplementation and total parenteral solution (TPN) are two main clinical practices that sustain oxidative stress. Glutathione is a key molecule that detoxifies peroxides resulting in a more oxidized redox potential. We hypothesize that O_2 supplementation and longer TPN duration are associated with both more oxidized redox potential and more severe bronchopulmonary dysplasia (BPD).

Patients and methods: A prospective observational study including infants of less than 29 weeks gestational age. GSH and GSSG from whole blood sampled on day 6-7 and at 36 weeks of corrected age (CA) were measured by capillary electrophoresis and redox potential was calculated using Nernst equation. BPD was classified according to NICHD guidelines.

Results: There was a significant association between $FiO_2 \geq 25\%$ on day 7 of life and TPN duration longer than 14 days and both more oxidized redox potential and more severe BPD. TPN duration explained both 15 % of total variation observed in redox potential and 42 % of total variation in BPD severity. These associations remained significant after adjustment for gestational age and illness severity. The relation between the severity of BPD and the redox potential in blood was not significant. The statistic power ($1-\beta$) to show an effect of redox potential on severity of BPD was 52%.

Conclusion: Both redox potential of glutathione and BPD severity are both associated with early O_2 supplement and TPN. Strategies targeting judicious use of O_2

supplement and either decreasing the duration or using safer formulation of TPN are expected to help reducing BPD.

Keywords: premature infants, oxygen, total parenteral nutrition, oxidative stress, redox potential, and bronchopulmonary dysplasia.

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LIST OF ABBREVIATIONS

Bronchopulmonary dysplasia: BPD

Neonatal Intensive Care Unit: NICU

Disulfide form of glutathione: GSSG

Reduced form of glutathione: GSH

Total Parenteral Nutrition: TPN

Corrected age: CA

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TO MY FAMILY

I THANK YOU FOR YOUR UNCONDITIONNED SUPPORT ALL THE TIME AND UNDER ALL CIRCUMSTANCES

I – INTRODUCTION

I.A. Prematurity

I.A.1. The number of premature infants is in constant increase

The report issued by Canadian Institute of Health Information (CIHI) in May 2011, indicated an increase of 38000 births (which represent 10.2%) in 2009-2010 compared to 2004-2005 (1). This increase of total number of births was associated with a steady increase in preterm birth rate from approximately 6% in the early 1980 (2) to 8% in recent years (1).

I.A.2. Increasing survival of extreme preterm infants and bronchopulmonary dysplasia

Advances in perinatal and neonatal medicine over the last few decades resulted in marked increase in the survival of infants less than 29 weeks of gestational age (3). In a recent study including 355 806 infants with birth weight of 501 to 1500 g and who were born in 2000–2009, mortality rate decreased from 14.3% to 12.4% (4). Survival of these vulnerable infants led to increased incidence of prematurity related diseases. Bronchopulmonary dysplasia (BPD) is currently the most common chronic lung disease in infancy and carries extremely high costs (5).

I.B. Bronchopulmonary dysplasia (BPD)

I.B.1. Clinical definitions of BPD:

The definition of BPD has changed overtime in the literature. The most important definitions will be presented chronologically in the following section:

Original definition for BPD diagnosis: A U.S. National Institutes of Health (NIH) workshop held in 1979 proposed this BPD definition "continued oxygen dependency during the first 28 days plus compatible clinical and radiographic changes" (6).

Traditional (classic) definition: Instead of the original definition, Shennan et al. (7) suggested a more accurate predictor of BPD to be, "the requirement for additional oxygen at a corrected postnatal gestational age of 36 weeks in infants less than 1,500 g of birth weight". This definition appears to also predict pulmonary outcome among infants with the "new" BPD (8).

Severity definition: the joint National Institute of Child Health & Human Development (NICHD)-National Heart, Lung, and Blood Institute (NHLBI) workshop defined mild, moderate and severe BPD according to specific criteria (9) (table I).

In a validation study, the NICHD–NHLBI workshop definition accurately predicted pulmonary outcomes including percent of patients needing treatment with pulmonary medications and rehospitalization for pulmonary causes (10).

Table I. NICHD classification of BPD diagnosis and severity for infants less than 32 weeks of GA:

Treatment with oxygen 21% for at least 28 d plus	
Mild BPD	Breathing room air at 36 weeks PMA or discharge, whichever comes first
Moderate BPD	Need for < 30% oxygen at 36 weeks PMA or discharge, whichever comes first
Severe BPD	Need for ≥ 30% oxygen and/or positive pressure at 36 weeks PMA or discharge, whichever comes first

Physiological definition: all previous definitions has an inherent limitation, that is the need for oxygen is determined by individual physicians or nursing staff rather than on a physiologic assessment. Accordingly, the physiological definition determined BPD at 36 weeks of correct age as follows, First: neonates on positive pressure support or receiving > 30% supplemental oxygen with saturations between 90% and 96% were assigned the outcome BPD and not tested further. Second, those receiving < 30% oxygen or effective oxygen > 30% with saturations > 96% underwent a room-air challenge with continuous observation and oxygen-saturation monitoring. Outcomes of the room-air challenge were "no BPD" (saturations > 90% during weaning and in room air for 30 minutes) or "BPD" (saturation < 90%) (11). The NICHD neonatal network centers' study demonstrated that many babies who,

according to the nursing staff, required oxygen were able to maintain a SaO2 > 90% on room air. In this study, though 560 (35%) had clinical BPD (oxygen use at 36 weeks), only 398 (25%) had physiological BPD (12).

I.B.2. Incidence of BPD

BPD is the most prevalent and one of the most serious long-term sequelae of preterm birth (13). Variation in reported rates is well documented. Among 4213 infants born in 2003 at 24–31 weeks' gestation in 10 different European regions, the rate of BPD (oxygen requirement at 36 weeks' PMA) was anywhere from 10.5% to 21.5% (14). In 2010, the Neonatal Research Network report on neonatal outcomes of extremely preterm infants assessed 9575 infants born at extremely low gestational ages (22–28 weeks) and very low birth weights (401–1500 g) at network centers between January 1, 2003 and December 31, 2007. The incidence of BPD as determined by the severity-based definition was 68% -Including babies with mild BPD- (oxygen therapy for 28 days but use of room air at 36 weeks); it was down to 42% if using the traditional definition and 40% if using the physiologic definition. In the same cohort, the incidence of BPD is largely affected by the gestational age with an incidence of 85% of infants born at 22 weeks' gestation vs. 23% of those born at 28 weeks' (15).

I.B.3. Normal lung development and the effect of timing of lung injury

Human lung development proceeds in five regulated stages: embryonic (3–7 weeks' gestation), pseudoglandular (7–17 weeks'), canalicular (17–27 weeks'), saccular (28–36 weeks') and alveolar and microvascular maturation (36 weeks' gestation to at least 2 years after birth) (16, 17). The lungs of preterm infants born at 24–28 weeks'

gestation are in the late canalicular and transitioning to early saccular stages; therefore cannot support efficient gas exchange. Branching and expansion of air spaces to form saccules and thinning of mesenchyme occur later in gestation, as do the formation of alveoli and the synthesis of surfactant by type II alveolar cells which only commence in late gestation (16, 17). Alveolarization, the final stage of lung development, begins in the near-term lung before birth, but primarily occurs postnatally, during the first 2–3 years of life, and may continue at a slower rate beyond childhood (18). Any injury to the lung at the early stages of development can potentially alter the developmental process, leading to long-term pulmonary sequelae (19).

I.B.4. Pathogenesis of BPD

Different patterns of pulmonary damage to immature lung may occur as a result of many different potentially harmful factors. The pattern of damage depends on the extent, timing and duration of the exposure to such factors (5). “Old” BPD was described in late preterm infants who were exposed to aggressive ventilation and excessive O_2 supplement (20). This pattern of lung injury was associated with prominent interstitial fibrosis, alveolar overdistention alternating with regions of atelectasis, and airway abnormalities such as squamous metaplasia and excessive muscularization. On the other hand, the “new” BPD, occurring in extremely premature infants, shows histological features consistent with developmental arrest and impaired alveolar development including decreased alveolarization and diminished and dysmorphic platelet endothelial cell adhesion molecule staining (21).

Alveoli are fewer in number and larger in diameter than normal; the fibrosis, squamous metaplasia and excessive airway muscularization seen in classical BPD are conspicuously absent; airway and microvascular growth are affected (22). In 2001, the 'vascular hypothesis' was described by Abman (23). Vascular endothelial growth factor (VEGF) is involved in angiogenesis. It was found to have impaired signaling and thus thought to play a role in the development of BPD in a preterm baboon model (24) as well as in human infants (25). The work of Van Tuyl et al adds additional insight to the understanding of the epithelial vascular interaction and the importance of the fetal hypoxic state for lung development (26). In this lung explant model, exposure to low oxygen (3% O_2) enhanced both epithelial branching morphogenesis and vascular development compared to 20% O_2, with more complex epithelial and vascular branching. VEGF mRNA was also dramatically increased in explants exposed to 3% O_2. This study lends credence to the current concept that even room air (21% O_2) is likely toxic to the developing lung and that this "relative hyperoxia" inhibits both pulmonary vascular development and epithelial branching morphogenesis (26).

The etiology of BPD continues to be recognized as a multifactorial. Major etiological factors include prematurity, mechanical ventilation and lung trauma, oxygen supplement and sources of oxidative load like total parenteral nutrition (PN) as well as neonatal infection (27-31).

Although the etiology of BPD is multifactorial, the concept of 'pulmonary oxygen toxicity' received additional support in the 1980s. In a series of papers, Saugstad promoted the concept that oxidative stress could result in the pulmonary damage

associated with BPD (27, 32, 33). This concept has continued to be supported, rather than refuted, in recent years. Oxidative stress and its relation to BPD will be discussed in the following sections.

I.C. Oxidative stress

Oxidative stress is classically defined as 'an imbalance between oxidants and antioxidants in favor of the oxidants' (34). Initially, research was concentrated on the chemistry of each oxidant and the damage it can inflict. In the 1980s, the idea that lower concentration of certain oxidants (superoxide and hydrogen peroxide) could induce cell division caused a scientific controversy (35). This idea of considering certain oxidants as an important component of normal biology and that they can act as signaling molecules is now established (36-39). The contemporary definition of oxidative stress has been refined to account for these two different mechanistic pathways of oxidative damage namely the macromolecular damage (caused mainly by radical oxidants) and the disruption of redox signaling (caused mainly by nonradical oxidants). This new definition considers oxidative stress as 'an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage' (39, 40). Biological systems was shown to generate more nonradical oxidants than free radicals than radical oxidants (41). This can explain why free radical macromolecular damage can correlate with the pathological insult and be largely irrelevant to the disease process that is mainly caused by the nonradical oxidant burden (40).

I.D. Oxidants

Oxidants are electron-accepting molecules in chemical reactions in which electrons are transferred from one molecule to another. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions (34). They include both free radical as well as non-radical oxidants. Molecules or ions formed by the incomplete reduction of oxygen are called reactive oxygen species (ROS). These reactive oxygen intermediates include singlet oxygen; superoxide; peroxides and hydroxyl radical. Free radical oxidants include the superoxide anion radical, hydroxyl radical and nitric oxide while nonradical oxidants include hydrogen peroxide, hydroperoxy fatty acids, aldehydes, quinines as well as peroxynitrites (40). Although these oxidant molecules can be derived from detoxification of xenobiotics, the main producer remains the oxidative phosphorylation in mitochondrion (42). Oxygen is easily reduced in water through the various protein complexes of mitochondrion. During these processes, about 1 % of O_2 will be partially reduced, with generation of the radical oxidant superoxide anion (O_2·-) (43). Superoxide dismutase (SOD) converts two superoxide anions into hydrogen peroxide (H_2O_2), nonradical oxidant, and oxygen (Figure I) (44) (45). The two electron, nonradical oxidant, H_2O_2 is produced at 1 to 4% of the rate O_2 consumption and therefore represent a major oxidant. H_2O_2 can be reduced by two enzymes, glutathione peroxidase (GPx) (46) or catalase into O_2 and H_2O (47). If not, H_2O_2 can react with Fe_2^+ forming the hydroxyl radical (·OH) via the Fenton reaction. This hydroxyl radical (·OH) is among the most reactive of molecules, leading to the oxidation of proteins, lipids and DNAs (Figure I).

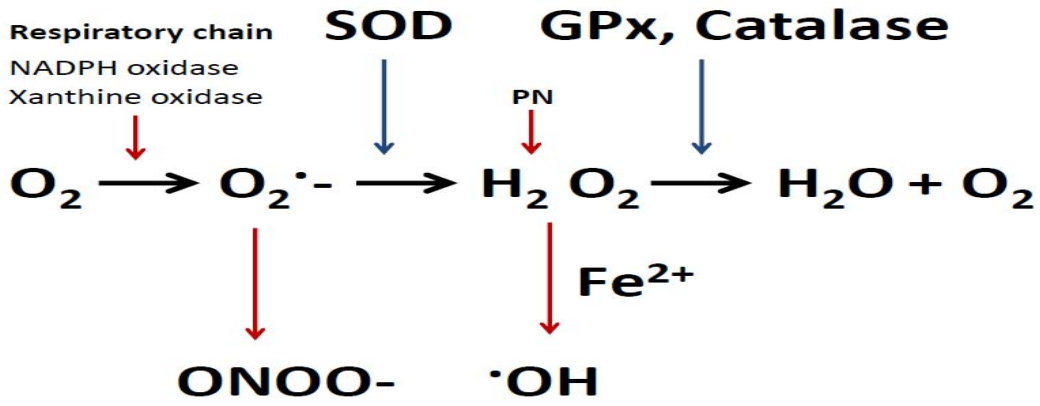

Figure I: Reactive oxygen species formation and the role of different antioxidant enzymes.

I.D.1. Oxygen as an oxidant in preterm infants

Birth is a major transition from relative hypoxic intrauterine environment where arterial pressure of O_2 (PaO_2) is around 25 mm Hg to oxygen rich extra-uterine environment with PaO_2 around 75 mm Hg. This means that even 21% O_2 is considered supra-physiologic for preterm infants and is associated with marked increase in total reactive oxygen species (ROS) burden (48). Many preterm infant will initially have respiratory distress caused by the immaturity of their lung surfactant system (known as hyaline membrane diseases), leading to the need of FiO_2 more than 21%. Greater oxygen load increases the concentration of dissolved oxygen available for oxidative phosphorylation in the mitochondria. Under conditions of hyperoxia, there is a marked increase in rate of ROS with increasing O_2 tension. Under FiO_2 of 95% there is a 10 fold increase of ROS comparing to ambient O_2 (21%) (49).

I.D.2. Parenteral nutrition as an oxidant in preterm infants

Parenteral nutrition allows administration of amino acids, dextrose, vitamins, electrolytes and lipids. Almost all preterm infants will need parenteral nutrition when enteral nutrition is not possible due to gastrointestinal immaturity, gastrointestinal pathology - like necrotizing enterocolitis - or with deterioration of general condition - like sever septicemia with ileus -. Interaction between reducers such as polyunsaturated fatty acids, several amino acids and vitamin C and a strong oxidant as dissolved oxygen generates oxidant molecules. These molecules include aldehydes and hydroperoxides derived from lipid peroxidation and hydrogen peroxide (H_2O_2) from ascorbic acid (50-52). All these molecules are detoxified by the glutathione system. In addition of the generation of these oxidants, these reactions contribute to the loss of fatty acids, amino acids and antioxidant vitamin such as ascorbate. PN contains a photo-sensitive compound, riboflavin, this means that these reactions can be catalyzed by ambient light (51-53). Photoprotection of PN solutions was found to decreases the infused load as well as the urinary excretion of peroxides in premature infants (54). Adequate photoprotection of parenteral nutrition has also been reported to reduce the incidence of BPD in premature infants (55, 56).

I.E. Antioxidant defenses

I.E.1. Definition and summary of different types of antioxidants

'All respiring organisms are caught in the cruel blind in that oxygen which support their lives is a toxic substance in whose presence they survive only by virtue of an elaborate system of defenses' Irwin Fridovitch (born 1929)

Antioxidant can be defined as 'any substance that when present at low concentration compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate' (57). This definition was recently reviewed to include a wider range of antioxidant activity including the repair of any damage caused by oxidants, so the revised definition indicates that antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule" (58). Antioxidants include both non-enzymatic as well as enzymatic compounds (Figure II). This diversity of antioxidants matches the diversity of oxidant molecules (34, 59).

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase are the main components of enzymatic antioxidants (60). Extracellular SOD is produced by type II alveolar cells, airway epithelial cells, macrophages and endothelial cells (61). It is found in high concentration in human lung comparing to plasma and other organs (62). Intracellular SOD includes CuZn-SOD located in the cytoplasm and peroxisome as well as Mn-SOD located in the mitochondria (63). SOD plays a very important role by reducing the free radical anion superoxide in oxygen and hydrogen peroxide that will be reduced in water by catalase or GPx (Figure I). Catalase is present only in the peroxisome and, to a lesser extent, in mitochondria. In this major source of oxidants, GPx is more important than catalase for the reduction of hydrogen peroxide (60).

Non enzymatic antioxidants include both fat soluble compounds like vitamin E (α - tocopherol) and β – carotene (precursor to vitamin A) as well as water soluble compounds like vitamin C, glutathione (GSH) and ceruloplasmin.

Vitamin E is incorporated into cell membranes where it protects them from the oxidative damage by scavenging free radicals resulting oxidized and inactive Vitamin E (64). Vitamin C (water soluble vitamin) is a strong reducing agent. It participates in a variety of oxido-reductive reactions and allows the recycling of the oxidized form of vitamin E into its reduced and active form (65).

The oxidized form of vitamin C can be returned to the reducing form by the action of the glutathione system (66). In addition to the recycling of vitamins C and E, glutathione works as a co-factor of several enzymes, especially glutathione peroxidase and glutathione-S-transferase. As glutathione is considered a key molecule in the antioxidant defenses of the organism, the next section is devoted to the glutathione.

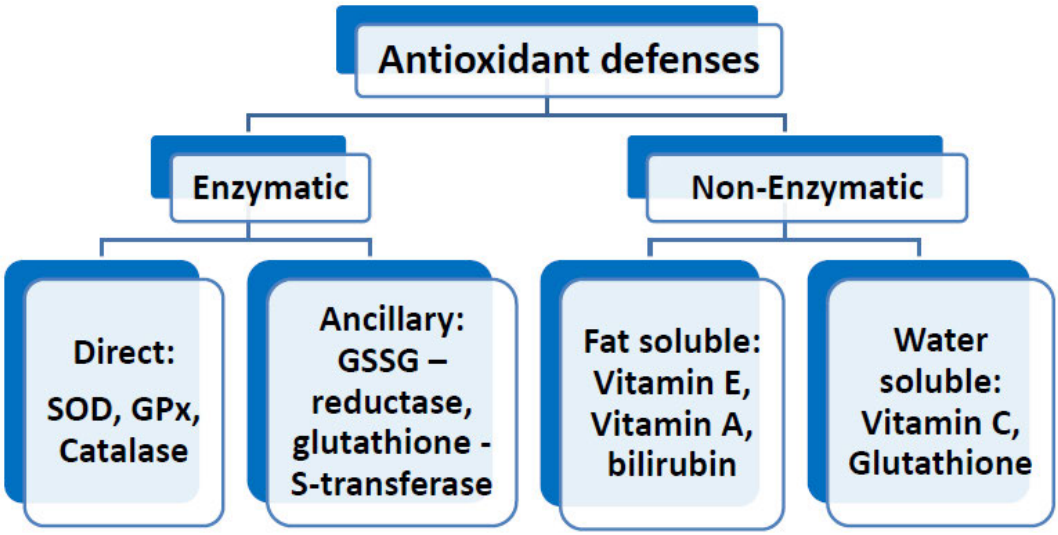

Figure II: summary of antioxidant compounds and enzymes.

I.E.2.Glutathione (GSH) as a main antioxidant

Glutathione (γ-glutamylcysteinylglycine) has a key role in maintaining the redox environment as antioxidant (67, 68). GSH is the most prevalent cellular thiol and the most abundant low molecular-weight peptide present in cells. It is produced in large quantities in all cells and distributed into subcellular compartments: the cytosolic (1-11 mM) in which GSH is synthesized, the nucleic (3-15 mM) where it maintains a functional redox state of DNA, and mitochondrial (5-11 mM) where the oxidative stress is high (38). By its abundance, glutathione is recognized as the main buffer of the cellular redox environment (38).

Reactive oxygen species such as peroxides are reduced by glutathione (GSH) resulting in an oxidized glutathione (GSSG). This results in a more oxidized redox environment in cells (Figure III).

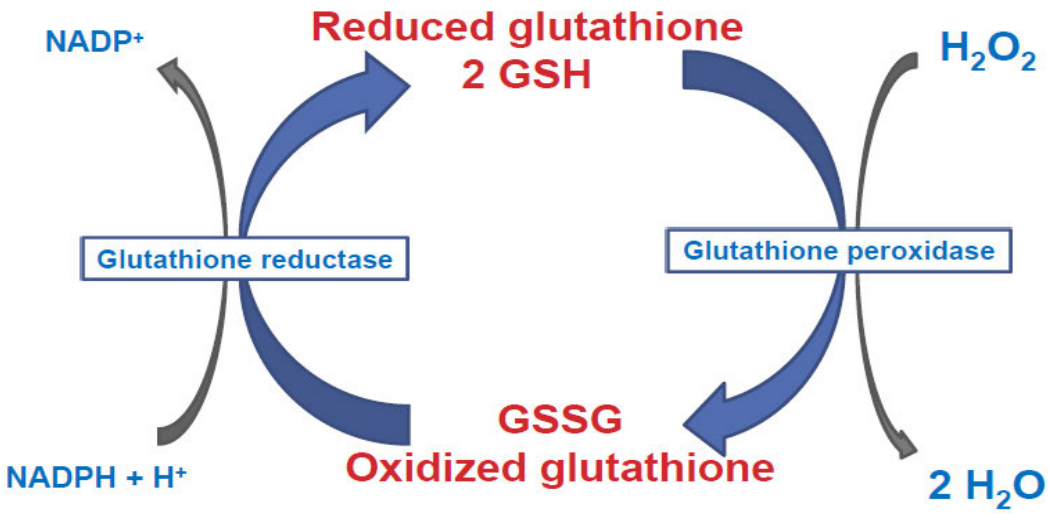

Figure III: Peroxides are reduced using the reduced glutathione leading to the formation of oxidized glutathione resulting in a more oxidized cell redox potential.

The redox potential of glutathione is dependent of the concentration of GSH and GSSG according to the Nernst equation: $\Delta E = \Delta E^{\circ} \cdot (RT/nF) \cdot \log ([GSH]^2/[GSSG])$ (38). Changes in the redox potential of glutathione can lead to important changes in redox signaling and cellular status as it will be described in the next section.

I.E.3. Redox potential as physiologic regulators

The redox potential acts as a switch for a number of metabolic pathways, inducing cellular proliferation, differentiation or apoptosis (38).

During organ development, cells must pass through the various cell cycle stages in order to allow for continued remodeling. This process is essential to proper lung development (69-71). The proliferation phase is accompanied by a higher metabolic rate leading to increased generation of ROS. These ROS in turn favor a shift of the redox potential toward a more oxidized status, inducing the differentiation phase (Figure IV). The more oxidized status of proliferating cells may 1) induce apoptosis, which favors tissue remodeling, or 2) activate redox-sensitive factors inducing the transcription of genes that encode enzymes involved in glutathione synthesis and GSSG recycling (glutathione reductase). This last event will shift the redox potential toward reduced state, this will lead to the start of a new cell cycle (38, 58).

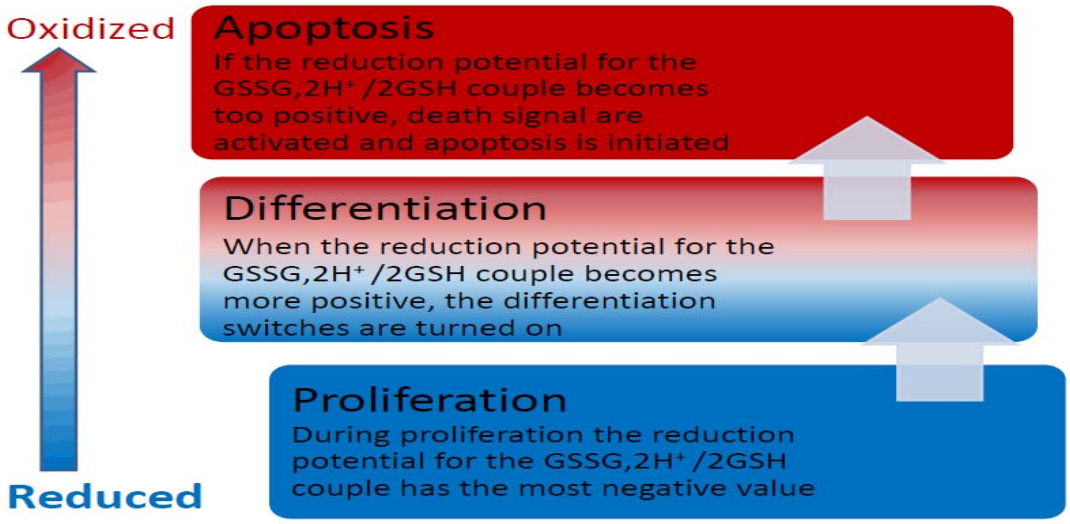

Figure IV: Alteration of redox potential moves cells through different biological stages.

I.F. Why preterm infant is more susceptible to oxidative stress

Fetal development occurs in a hypoxic environment. At birth the oxidative load is sharply increased. At the same time, oxygen demands increase abruptly. Compared to full term infants who easily adapts to this transition, preterm infants' transition from the intra- to extra-uterine environment carries many risks. Among the reasons why the preterm infant is more likely to experience oxidative injury than more mature newborns are the following (Figure V):

1- Enzymatic defenses against oxidative stress are still poorly developed. Frank and Groseclose measured antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase) in lungs during prenatal, newborn, and postnatal periods of rat development and demonstrated a sharp increase (110 % to 200%) just prior to term birth and a continued rise after birth (72, 73). These findings suggest

that the fetal lung is programmed to increase these enzymes as term approaches in preparation for the increased oxidation associated with atmospheric air breathing, with its relatively high O_2 concentration (72, 74). Available human data suggested that human fetal lung has the same programmed increase of antioxidant enzymes as gestation progresses (75).

2- Non enzymatic antioxidant defenses (vitamin E, vitamin C, glutathione, etc.) are also deficient as they are mainly transferred from the maternal to fetal circulation in the later part of the third trimester (76-78).

3- The preterm infant is often exposed to high load of oxidant molecules as oxygen and total parenteral nutrition (79); and

4- The fetus and premature infant are also susceptible to inflammation and infection that may lead to increased oxidative stress (32).

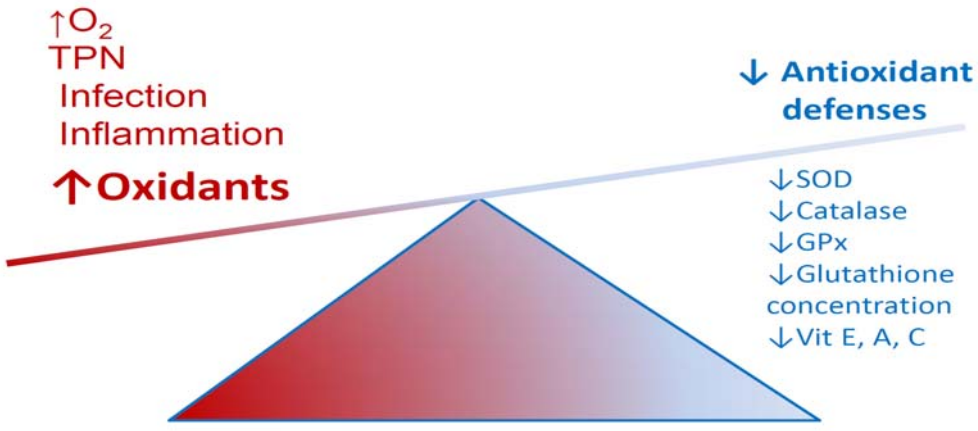

Figure V: Causes of increased risk of oxidative stress in preterm infants.

I.G. Association between oxidative stress and BPD

Northway et al. coined the term “bronchopulmonary dysplasia”, in 1967, to describe findings of pulmonary complications following respiratory therapy for hyaline membrane disease in preterm infants. Northway et al. believed the critical factor to be exposure to an inspired oxygen concentration > 80% for longer than 150 hours (20). This was quickly related to free radicals injury and the inability of preterm infants to increase their antioxidant capacity (80). Some years later, Saugstade et al. suggested that it is not only hyperoxia, but also oxidative stress per se, that might be a contributing factor in the development of BPD (27).

Even with recent advances in neonatal medicine including prenatal steroids, surfactant replacement therapy and noninvasive ventilation; multiple studies have shown that babies who develop new BPD also have elevated markers of oxidative stress in the first days or weeks of life before the diagnosis of BPD. These markers included more oxidized redox potential , a clinical marker of oxidant stress or markers that reflect both protein and lipid peroxidation (81-84). These finding despite not proving a casual relation between oxidative stress and BPD, they still highlight the pertinence of the question to what extent does oxidative stress contribute to the development of BPD.

In a recent work comparing markers of oxidant stress in premature neonates receiving PN with different combinations of multivitamins and Intralipids, Chessex et al. were able to demonstrate that preterm infants less than 28 weeks of gestational age who exhibited the more oxidized redox potential on day 7 of life had more sever BPD at

36 weeks CA (81). This was the first report of redox potential of glutathione in extremely preterm infants in the NICU settings. The cell status, proliferation – differentiation – apoptosis, varies in function of the redox value (38). A more oxidized redox potential value may predispose to a loss of alveoli by apoptosis (85, 86). As preterm infants are often exposed oxygen and total parenteral nutrition as two sources of oxidative stress we sought to document the relation between these two major oxidants and the redox potential as well as the development of BPD.

II - HYPOTHESIS AND OBJECTIVES

II.A. Hypothesis:

We hypothesize that oxidative stress, caused by oxygen supplement and TPN, early in life induces the development of BPD, and that the clinical practices maintaining this oxidative stress contribute to the severity of the pathology.

II.B. Objectives:

1- To investigate the relation between O_2 supplementation and the glutathione redox status measured in whole blood all in relation with the development and severity of BPD in preterm infants less than 29 weeks of gestational age.

2- To investigate the relation between PN duration and the glutathione redox status measured in whole blood all in relation with the development and severity of BPD in preterm infants less than 29 weeks of gestational age.

III - METHODS AND IV - RESULTS

Methods and results are described in the following article that will be submitted soon to the scientific journal Pediatric Research

The effect of oxygen supplementation and total parenteral nutrition duration on the redox potential of glutathione and the severity of bronchopulmonary dysplasia in extremely preterm infants

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Short running title: Oxygen and TPN in relation to oxidative stress and bronchopulmonary dysplasia

List of abbreviations

Bronchopulmonary dysplasia: BPD

Neonatal Intensive Care Unit: NICU

Total Parenteral Nutrition: TPN

Corrected age: CA

Abstract:

Introduction: oxygen supplementation and total parenteral solution (TPN) are two main clinical practices that sustain oxidative stress. Glutathione is a key molecule that detoxifies peroxides resulting in a more oxidized redox potential. We hypothesize that O_2 supplementation and longer TPN duration are associated with both more oxidized redox potential and more severe bronchopulmonary dysplasia (BPD).

Patients and methods: A prospective observational study including infants of less than 29 weeks gestational age. GSH and GSSG from whole blood sampled on day 6-7 and at 36 weeks of corrected age (CA) were measured by capillary electrophoresis and redox potential was calculated using Nernst equation. BPD was classified according to NICHD guidelines.

Results: There was a significant association between $FiO_2 \geq 25\%$ on day 7 of life and TPN duration longer than 14 days and both more oxidized redox potential and more severe BPD. TPN duration explained both 15 % of total variation observed in redox potential and 42 % of total variation in BPD severity. These associations remained significant after adjustment for gestational age and illness severity. The relation between the severity of BPD and the redox potential in blood was not significant. The statistic power ($1-\beta$) to show an effect of redox potential on severity of BPD was 52%.

Conclusion: Both redox potential of glutathione and BPD severity are both associated with early O_2 supplement and TPN. Strategies targeting judicious use of O_2

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Introduction:

Advances in perinatal and neonatal medicine over the last few decades resulted in marked increase in survival after extremely premature birth (1). Survival of these vulnerable infants led to increased incidence of prematurity related diseases. Currently, bronchopulmonary dysplasia (BPD) has been proposed the most common chronic lung disease in infancy and carries extremely high costs (2). The etiology of bronchopulmonary dysplasia appears to be multi-factorial but the oxidative stress seems a common point (3-10). Clinical practices that can sustain a major oxidative stress are oxygen supplementation and TPN.

Birth is a major transition from relative hypoxic intrauterine environment where arterial pressure of O_2 (PaO_2) is around 25 mm Hg to oxygen rich extra-uterine environment with PaO_2 around 75 mm Hg. This means that even 21% O_2 is considered supra-physiologic for preterm infants and is associated with marked increase in total reactive oxygen species burden (11). Furthermore, many preterm infants will need supplemental oxygen due to hyaline membrane disease. Under conditions of hyperoxia, there is marked increase in the rate of reactive oxygen species production with increasing O2 tension (12). In human as well as in animal model, oxygen supplement seemed to play an important role in the development of bronchopulmonary dysplasia (3, 13-22).

Parenteral nutrition allows administration of amino acids, dextrose, vitamins, electrolytes and lipids. Almost all preterm infants will need parenteral nutrition whenever enteral nutrition is not possible. Interaction between reducers such as polyunsaturated fatty acids, several amino acids and vitamin C and a strong oxidant

as dissolved oxygen generates oxidant molecules. These molecules include aldehydes and hydroperoxides derived from lipid peroxidation and hydrogen peroxide (H_2O_2) from ascorbic acid (23-25). While the role of TPN in the development of bronchopulmonary dysplasia is poorly understood in preterm infants (26, 27) , the low alveoli level observed in PBD (28, 29) is reproduced in animals model infused with total parenteral nutrition (TPN) (30, 31). This was explained by higher rate of apoptotic events (30).

Using oxidative stress markers, Chessex P. et al have recently reported a significant relation between the severity of BPD and the redox potential of glutathione measured in whole blood at one week (9). Infants with the more severe stage of BPD had the more oxidized redox. The intracellular redox environment is known to be a major regulator of cellular cycle, from proliferation toward apoptosis; an oxidized status favors apoptosis (32, 33). Chessex P. et al suppose that glutathione redox potential value observed in whole blood reflects that of the lung. However, newborn animal model of light exposed TPN did not support this statement. Indeed, in these animals, light exposed TPN induced a more oxidized redox potential in blood whereas lung redox potential was more reduced (31). Therefore measurement of the redox potential in the whole blood may not reflect that in the lung tissue.

We hypothesize that oxidative stress early in life induces the development of BPD, and that the clinical practices maintaining this oxidative stress including O_2 supplement and TPN contribute to the severity of the pathology. Therefore, the aim of the study was to assess the relations between the two mains sources of oxidant,

oxygen supplement and TPN, and redox potential of glutathione, all in relation with the severity of BPD in infants less than 29 weeks of gestational age.

Patients and methods:

This prospective observational study included all infants less than 29 weeks admitted to the neonatal intensive care unit (NICU) before 24 hours of life between August 2010 and July 2011. Only infants with congenital malformations were excluded. A written informed consent was obtained before enrolling subject to have their redox potential of glutathione measured on day 6-7 and at 36 weeks of CA (group C). To ensure that the studied group C represents the whole studies population, clinical data collection included all infants admitted to the NICU during that period (group A) as shown in figure 1 and both groups were compared. The study protocol was approved by the Research Ethics Board of the CHU Sainte-Justine with registration number 2792.

Local NICU practices

Oxygen supplementation guidelines:

Oxygen saturation is continuously measured in all infants. Target O_2 saturation was set between 85 to 92 % for infants who need supplemental O_2.

TPN and enteral nutrition guidelines:

Total parenteral nutrition is prescribed in the first day of life at 80 ml/kg/day and increased 10-20 ml/kg/day till to achieve 150 ± 10 ml/kg/day. Amino acids (TrophAmine®) are started on day 1 of life at 2.5 g/kg/d then increased 0.5 g/kg/day

to achieve 3.5g/kg/day. Lipides (Intalipid® 20%) are started at 1g/kg/day and increase by 0.5 g/kg/day to 2.5 mg/kg/day. Enteral feeding is started with minimal enteral feeding of 20 ml/kg/day as soon as the medical condition of the infants stabilizes. If mother chooses to breast feed her infant a maximum delay of 3 days was permitted to have the first maternal breast milk given to the baby otherwise formula milk was started. This minimal enteral feeding is kept for 4 days and then a progressive daily increase of 20 ml/kg/day was prescribed if infant is tolerating well. According to these guidelines TPN duration of 14 days could be considered as baseline minimal duration of TPN for this group.

Bronchopulmonary dysplasia

BPD was defined according to the joint U.S. National Institute of Child Health & Human Development (NICHD)-National Heart, Lung, and Blood Institute (NHLBI) workshop (34). This definition allows the evaluation of BPD severity. Mild BPD was defined as the need for supplemental oxygen at 28 days after birth but not at 36 weeks' CA; moderate BPD, the need for supplemental oxygen at 28 days and at a fraction of inspired oxygen (FiO_2) < 30% at 36 weeks' CA; and severe BPD, the need for supplemental oxygen at 28 days and, at 36 weeks' CA, the need for positive pressure and/or $FiO_2 \geq 30\%$. In this study, BPD-0 refers to no-BPD, BPD-1 refers to mild BPD, BPD-2 refers to moderate BPD and BPD-3 refers to severe BPD.

Redox potential of glutathione (mV)

Within 4 min of sampling, aliquots of whole blood (EDTA tube) were homogenized in freshly prepared metaphosphoric acid (5% w/v), centrifuged at 10000 rpm for one

minute. Pellet and supernatant were separated and stored at −80 °C. Concentrations of GSH and GSSG were determined in supernatant fraction after the samples were thawed on ice and diluted one in four in water. The sample was injected at 0.5 psi for 10 sec on a fused-silica capillary (75 μm×50 cm) at 28°C (P/ACE MDQ system from Beckman Coulter). The electrophoresis separation was carried out at 18 kV, for 10 min, using boric acid 75mM+ Bis-TRIS 25 mM, pH 8.4 as buffer; GSH and GSSG were detected at 200 nm (31). Between separations, the capillary was pressure-rinsed at 20 psi with 0.1 M NaOH (5 min), water (2 min) and running buffer (5 min). Standard curves of GSH (0 to 100 μM) and GSSG (0 to 10 μM) were used for quantification. The redox potential was calculated from the Nernst equation (25 °C, pH 7.0) (32).

Statistical analysis

Data were summarized as proportions, means with standard error of the mean (SEM), median with 25^{th} and 75^{th} percentiles or as frequency distribution. The comparisons were performed by ANOVA or factorial ANOVA after verification of homoscedasticity by the Bartlett's χ^2 test. In case of significant heterogeneity of variance, data were logarithmically transformed to meet the homoscedasticity. Because no mathematical transformation satisfied the Bartlett's χ^2 test for the comparison of FiO_2 values observed at 7 days of life in function of severity of BPD, the Chi-squared test has been used to compare the liaison between the number of infants with $FiO_2 \geq 25\%$ and the severity of BPD. All comparisons were orthogonal and the significance was set at $p < 0.05$. The ratio of the sum of squares (from ANOVA table) for a factor (i.e. severity of BPD, duration of TPN or FiO_2) on the sum of squares for the total

variation (x 100) is reported as the percentage of total variation explained by this factor.

Results:

General characteristics

Clinical baseline characteristics of group C did not differ from those of group A (**Table 1)**.

The description of parameters measured during this study is shown in **figure 2** as distributions of frequencies.

Oxygen and redox potential of glutathione

The impact of FiO_2 on days 7 and 28 as well as at 36 weeks CA on redox potential of glutathione is presented in **figure 3**. The factorial ANOVA reveals that $FiO_2 \geq 25\%$ is associated with a more oxidized redox potential ($F_{(1,111)} = 14.2$, $p<0.01$) independently of the postnatal age since the interaction was not significant ($F_{(2,111)} = 0.2$). There was no effect of postnatal age ($F_{(2,111)} = 0.4$). The FiO_2 explained 11% of the total variation in redox potential.

Oxygen and severity of BPD

The relation between FiO_2 at 28 days of age and severity of BPD was not analyzed because $FiO_2 \geq 22\%$ at 28 days of age is part of definition of BPD. However, analysis of the relation between FiO_2 at 7 days and severity of BPD could be informative. The value of FiO_2 in function of the severity of BPD has not been evaluated by ANOVA since even after mathematical transformation, the heterogeneity of variances remained highly significant (Bartlett's $\chi^2 = 55.1$, $p<0.01$). Therefore, data were

analyzed as non-parametric data using χ^2 test. The relation between the severity of BPD and the number of infants with a $FiO_2 \geq 25\%$ at day 7 of age (**Table 2**) was highly significant with a $\chi^2 = 23.7$, $p<0.01$.

TPN and redox potential of Glutathione

The redox potential measured in blood at 36 weeks CA was also influenced by the duration of TPN (**Figure 4**). The ANOVA shows that redox potential in blood at 36 weeks CA was more oxidized if the infants received TPN for more than 14 days ($F_{(1,31)} = 5.5$, $p<0.05$). This effect of TPN duration explained 15 % of total variation observed in the redox potential.

TPN and severity of BPD

The severity of BPD was also influenced by the number of days that the neonates remained on TPN (**Figure 5**). Data from all neonates (22 to 28 weeks of gestation) **(Figure 5-A)** demonstrated that the number of days on TPN was higher in neonates with BPD-2 ($F_{(1,84)} = 19.9$, $p<0.01$) and more with BPD-3($F_{(1,84)} = 29.5$, $p<0.01$). There was no difference between BPD-0 and 1 for the number of days on TPN ($F_{(1,84)} = 1.1$). Forty-two percent of total variation in BPD was explained by the variation in TPN duration. This relation between TPN and severity of BPD was independent of the gestation age at birth since the same TPN effect is reproduces in groups born at 22-24 weeks **(Figure 5-B)**, 25-26 weeks **(Figure 5-C)** and 27-28 weeks of gestation **(Figure 5-D)**. The duration of TPN could be the intermediate by which the severity of infant disease may cause BPD. In a logistic regression model both the duration of

TPN and the SNAPII score were both independent and significant factors in the development of BPD with a $P<0.01$ for both factors.

Whole blood redox potential of glutathione and BPD

The last aim was to assess the relation between redox potential of glutathione, measured on day 6-7 and at 36 weeks CA, and severity of BPD. The factorial ANOVA of data presented in **figure 6** reveals a less oxidized redox potential at 36 weeks CA than at 6-7 days of age ($F_{(1,71)} = 5.1$, $p<0.05$). The factor "postnatal age" explained 7% of total variation observed in redox potential. The relation between the severity of BPD and the redox potential was not significant ($F_{(2,84)} = 1.3$). The statistic power (1-β) to show an effect of redox potential on severity of BPD was 52%. There was no interaction between parameters ($F_{(2,84)} = 0.3$).

Discussion

This study indicates that a supplement of oxygen ($FiO_2 \geq 25\%$) or a TPN duration of more than two weeks is associated both more oxidized redox potential in blood and a greater severity of BPD. However, the link between redox potential measured in blood at 6-7 days of age or at 36 weeks CA and the severity of BPD has not been demonstrated.

A similar impact of FiO_2 has previously been reported, with similar redox values, to occur at one week of age (9). The present study shows that the oxygen effect is independent of the postnatal age; suggesting that an oxygen supplement is always toxic. Although the oxygen supplement at 28 days and at 36 weeks CA is a part of BPD definition, it is surprising that $FiO_2 \geq 25\%$ at so early in life, at one week of age,

can be associated with the severity of BPD as evaluated at 36 week CA. The incidence of infants with $FiO_2 \geq 25\%$ increases in function of the severity of BPD: 9% of infants who do not develop BPD or who develop a mild BPD; 38% for moderate BPD; 65% for severe BPD (from Table 2). This suggests that for most infants who developed a severe BPD, lung injury could occur in the early days or weeks of life.

The second source of oxidant load is TPN. The redox potential at 36 weeks CA shifted toward a more oxidized status after 2 weeks on TPN, a same duration observed for the association with the severity of BPD. Because more premature or sicker is the infant, the longer will be the need for TPN, the gestational age or the illness severity could be a confounding parameter. However, the analyses done on grouped data according to gestational age (figure B to D) and the independent effect of both TPN and SNAPII score suggest that the impact of TPN on BPD is independent of gestational age at birth and severity of illness as estimated by SNAPII score. The fact that two weeks on TPN seem enough to induce BPD supports the concern raised with oxygen supplementation that lung injury that correlates with the severity of BPD could occur in the early days and weeks of life.

Both high FiO_2 and long TPN exposure induced oxidation of redox potential in blood and are associated with moderate to severe BPD. However, redox potentials in blood measured on 6-7 days or at 36 weeks CA were not associated with the severity of BPD. However, the relatively low statistical power (52%) prevents us from confirming that the effect does not exist.

With a population similar to ours in number and in prematurity, Chessex et al (9) showed this relation between the redox at one week of age and the severity of BPD. The main difference between both studies resides in the formulation of TPN used. The Chessex' study compared three groups of neonates receiving similar nutrients but that were administered in different modes. All TPN were administered in a binary mode that separates the lipid emulsion (LIP) and the amino acids - dextrose (AA). In one group, multivitamin preparation (MV) was added to the AA moiety of TPN whereas for the two other groups MV were mixed with LIP. The last two groups were separated according to light protection of the solution. The animals infused with TPN in which MV was mixed with LIP presented a greater alveoli number than the animals infused with TPN in which MV was mixed with AA (31). Moreover, the impact of $FiO_2 \geq 25\%$ on the redox potential was observed only in the group receiving TPN in which MV was in AA. The different TPN modes used by Chessex et al (9) were largely different from that used in this study (MV in AA). These different modes have different effects on the alveolarization as much as on the redox potential.

In regards of our hypothesis we can conclude that indeed, the clinical practices (oxygen supplement and TPN) affect the oxidative stress in premature neonates and may contribute to the severity of BPD. However, our results did not confirm that redox potential is the mechanism by which oxidative stress contributes to the severity of BPD. The relation between redox status in blood and in lung is not proved (31). The discrepancy between the impacts of TPN on total variation observed for redox potential in blood (15%) and for BPD (42%) suggests that the effect of TPN is

mediated through another mechanism than the direct effect on redox potential. Photo-protection of TPN reduces peroxide concentration in the intravenous solution (35) it was also shown to reduce the incidence of BPD (36) or chronic lung diseases (8) in premature infants. Peroxides may be suspected to have a direct effect on lung development. On the other hand, photo-protection of TPN was found to allow enhancing advancement of minimal enteral nutrition preterm infants leading to decrease duration of TPN (37). With data from the present study, the beneficial impact of photo-protection may be related to a shorter duration of TPN for these infants. The beneficial impact of adequate photo-protection (8, 36, 37) suggests that a toxic element is generated during photo oxidation of nutrients.

In conclusion, results from this study indicate that early O_2 supplement as well as the TPN as currently compounded, are toxic for the lung of preterm infants. The exact mechanisms remain to be discovered. We speculate that strategies targeting judicious use of O_2 supplement and either decreasing the duration or using safer formulation of TPN are expected to help reducing BPD incidence and severity. It is of prime importance to find a new way of compounding parenteral nutrition to provide a nutritional solution devoid of toxic elements; a TPN that allows the best development of the infants without adverse effects.

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	Group A (n = 116)	Group C (n = 51)
Gestational age, mean ± SEM (weeks)	$26^{3/7} \pm {}^{1/7}$	$26^{4/7} \pm {}^{1/7}$
Birth weight, mean ± SEM (gram)	867 ± 21	846 ± 23
Apgar score at 5 minutes[a]	6 (5 - 7)	6 (5 - 8)
SNAP - II score, mean ± SEM	19 ± 1	19 ± 2
Sex		
Female, n (%)	49 (42%)	21 (41%)
Male, n (%)	67 (58%)	30 (59%)
IUGR, n (%)	24 (21%)	8 (16%)
Vaginal delivery, n (%)	41 (35%)	17 (33%)
Antenatal steroid		
Complete course, n (%)	60 (52%)	26 (51%)
Incomplete course, n (%)	44 (38%)	23 (45%)
Suspected chorioamnionoitis, n (%)	16 (14%)	8 (16%)
Maternal preeclampsia, n (%)	22 (19%)	7 (14%)
Maternal diabetes, n (%)	16 (14%)	7 (14%)

Table 1: baseline clinical characteristics of group A and group C showed no statistically significant differences.

[a] presented as median (25^{th} – 75^{th} percentiles)

	$FiO_2 < 25\%$	$FiO_2 \geq 25\%$
Grade of BPD- 0,1*	29	3
Grade of BPD-2	10	6
Grade of BPD-3	15	28

Table 2: Number of infants with $FiO_2 < 25\%$ and $\geq 25\%$ at day 7 of age in function of the severity of BPD. (*BPD 0 and 1 grouped together due to very few infants with $FiO_2 \geq 25\%$)

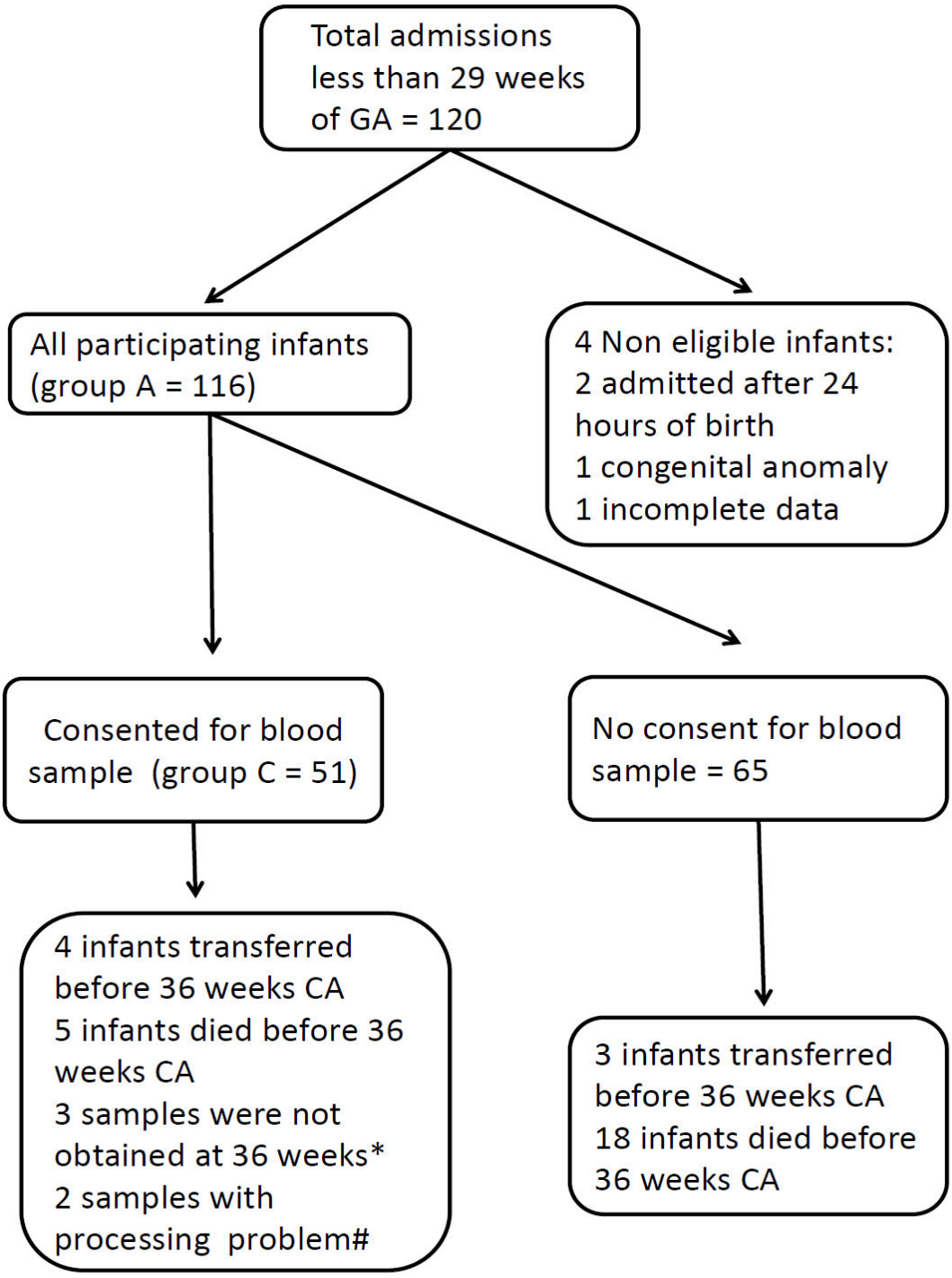

Figure 1. Participant flow.

* These 3 infants did not have clinically indicated samples at 36 weeks of corrected age. # 2 samples were stored in -20 instead of – 80 so results were excluded.

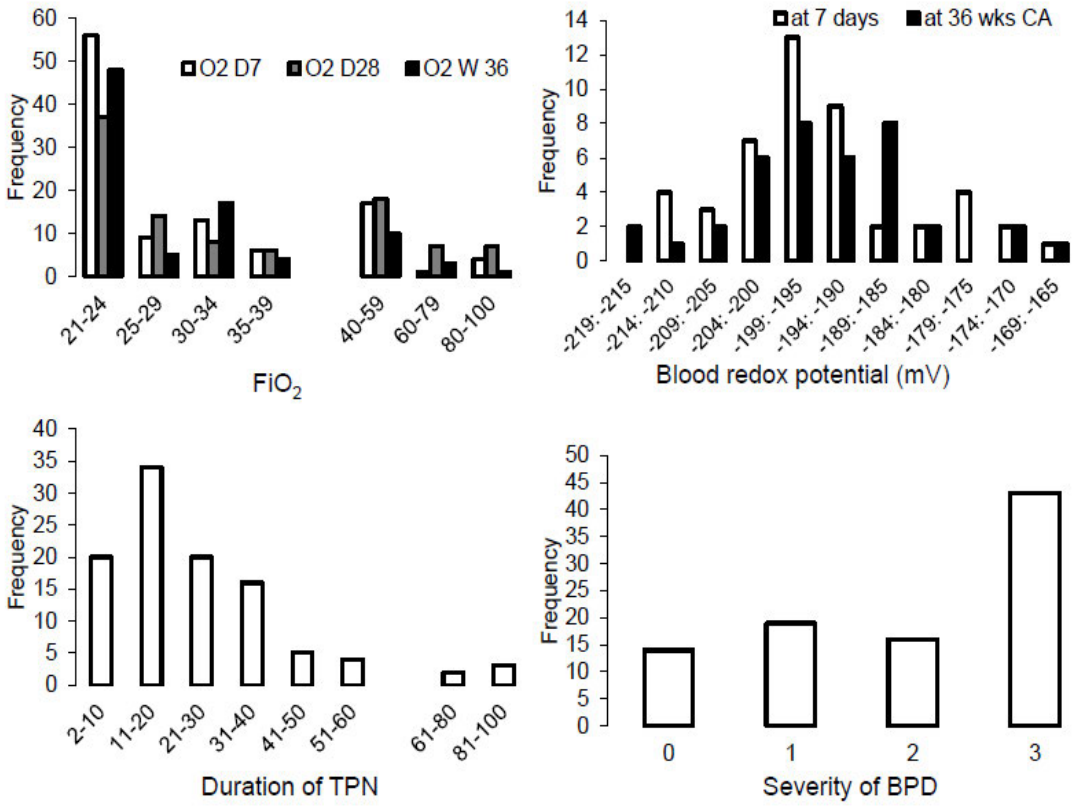

Figure 2: Distributions of frequencies of the parameters measured during this study (group A).

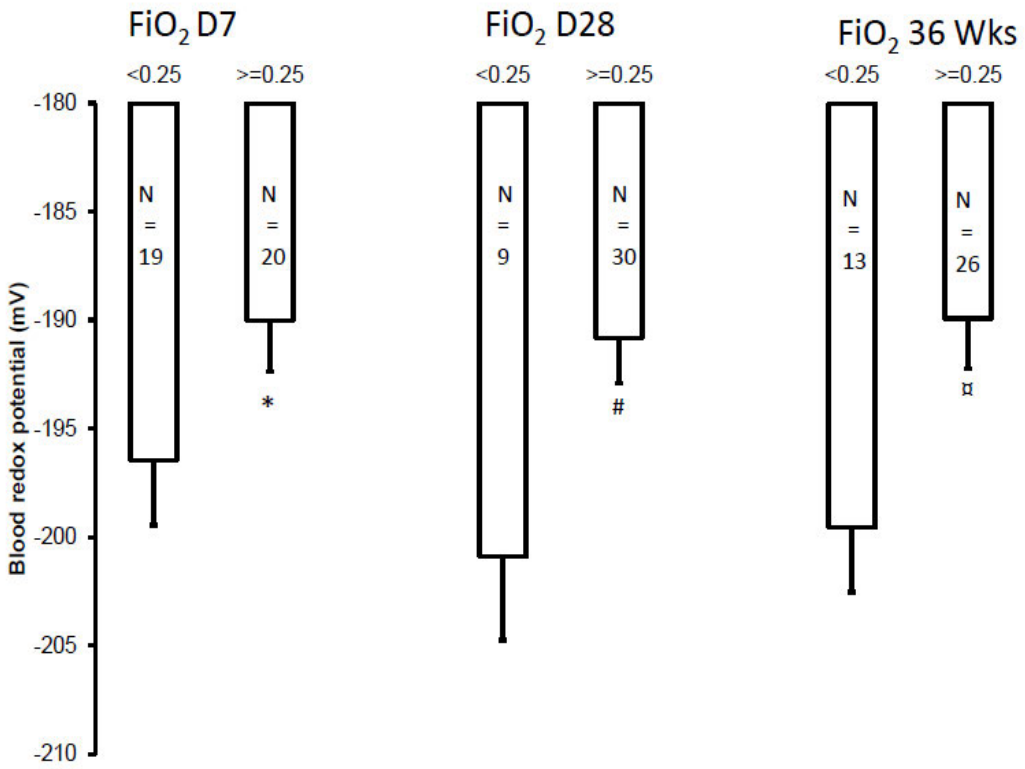

Figure 3: The impact of FiO_2 on days 7 and 28 days as well as at 36 weeks CA on redox potential of glutathione. The redox potential of glutathione is more oxidized in infant with $FiO_2 \geq 25\%$ on days 7, 28 and at 36 weeks of corrected age.

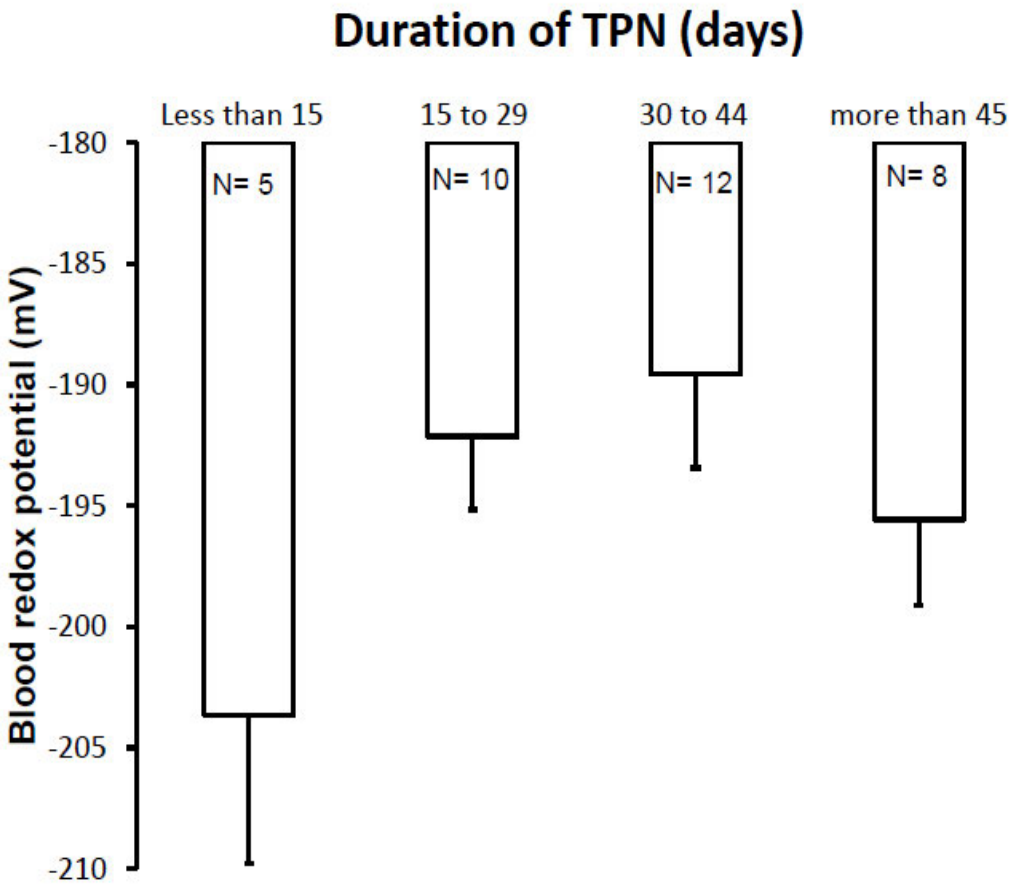

Figure 4: The relation between the duration of TPN and the redox potential of glutathione at 36 weeks CA. The redox potential of glutathione is more oxidized if the infants received TPN for more than 14 days.

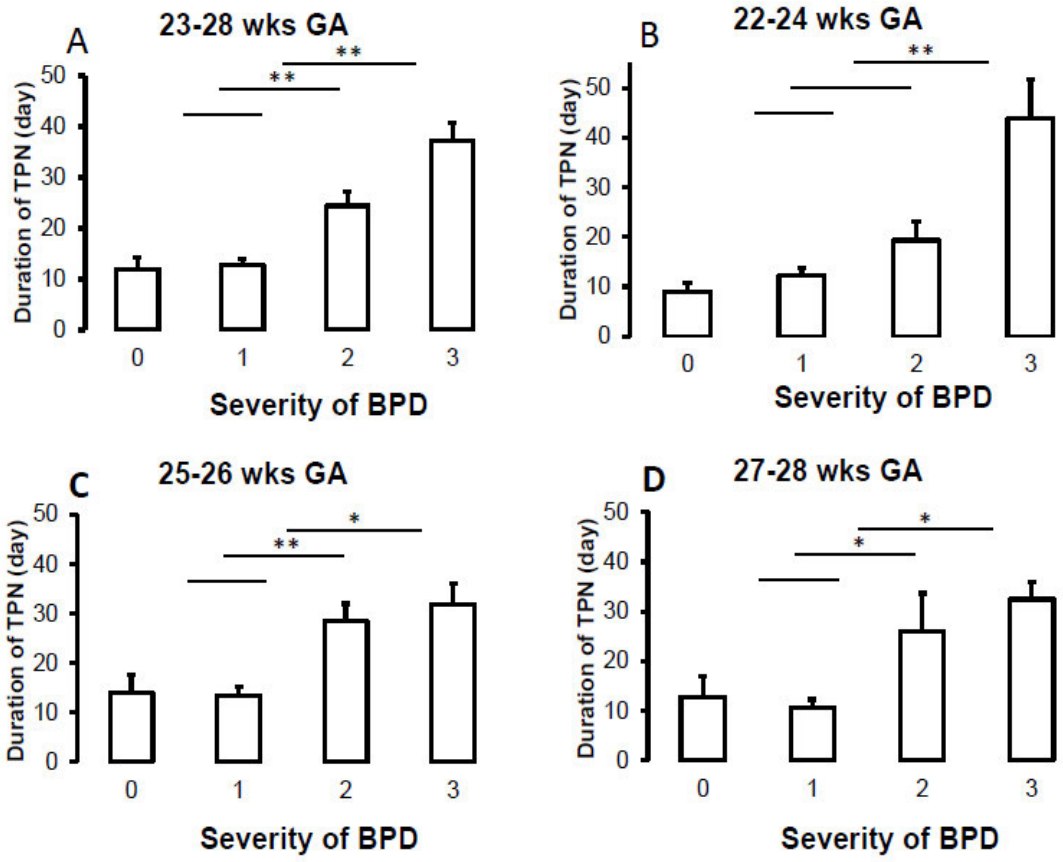

Figure 5: A. In the group A, Increased duration of TPN is associated with increased severity of BPD. This relation remained significant in different subgroups of gestational age at birth (B. 22-24 weeks, C. 25-26 weeks, D. 27-28 weeks).

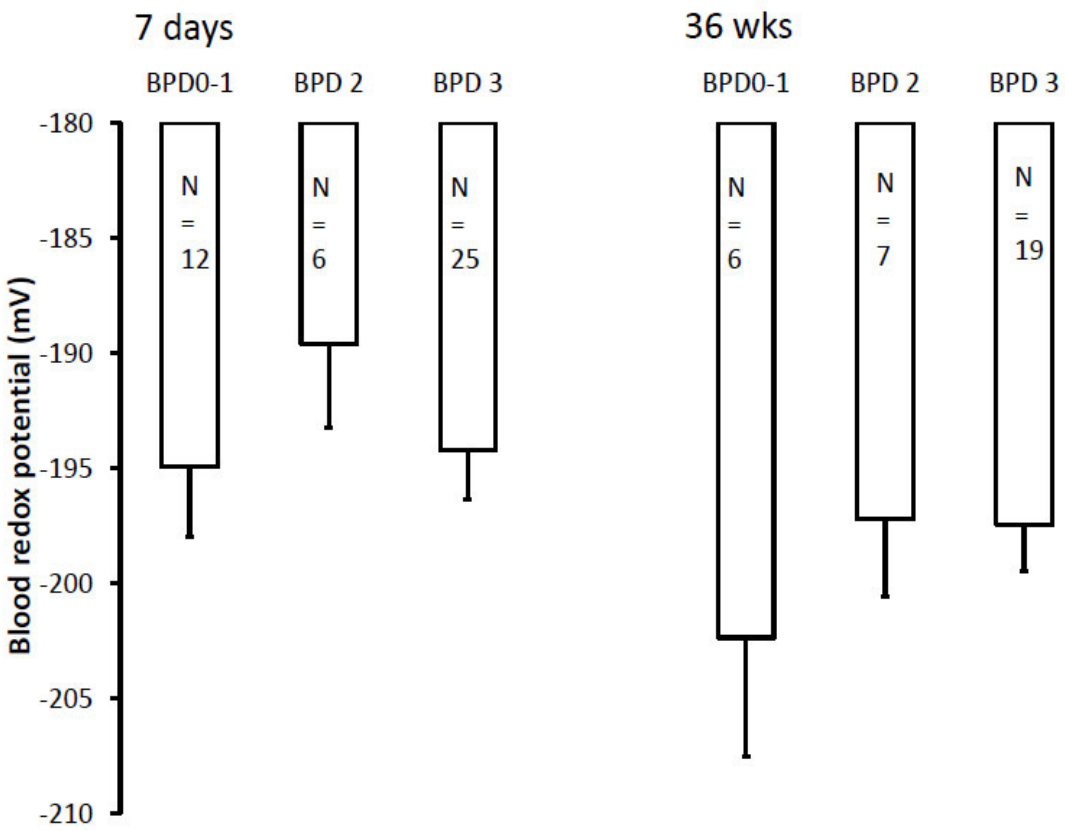

Figure 6: The relation between the severity of BPD and the redox potential on day 6-7 and at 36 weeks CA was not significant.

V - DISCUSSION

This study tested the hypothesis that oxidative stress, caused by oxygen supplement and TPN, early in life contributes to the severity of BPD. Our data demonstrated that indeed both $FiO_2 \geq 25\%$ as well as TPN for > 14 days are associated with more oxidized redox potential and more severe BPD. In our cohort there were no significant correlation between the redox potential and the severity of BPD. Here I will discuss some important points that could not be discussed in the manuscript due to editorial limitations:

V.A. Exposure to oxidants early in life and protracted oxidative stress:

In our cohort infants who were exposed to $FiO_2 \geq 25\%$ on day 7 of life had significantly more oxidized redox potential, even after few weeks, at 36 weeks of CA. This reveals a protracted oxidative stress that lasts for several weeks. Similar prolonged oxidative stress was reported in a randomized controlled study by Vento, M. et al who demonstrated a decreased GSH/GSSG ratio after 4 weeks of life in infants who were resuscitated in 100% oxygen for few minutes versus those who were resuscitated in room air (87). Others have described this prolonged oxidative stress after short oxidant event has stopped in myocardial infarction model (88).This phenomenon needs to be explored to define the mechanism of such prolonged effect.

V.B. Why choosing the redox potential as an oxidative stress marker?

Choosing the marker of oxidative stress is an important part of any work discussing this subject. As discussed in the introduction (page 9) oxidative stress includes two different mechanistic pathways of oxidative damage namely the macromolecular damage (caused mainly by radical oxidants) and the disruption of redox signaling

(caused mainly by nonradical oxidants) (45). Biological systems generate more non radical oxidants than free radicals (41). In the presence of free radical scavenging enzymes like SOD, radical scavenging chemicals like vitamins C and E, and very high protein concentration in biological systems; free radical chain reactions are nearly completely prevented. The nonradical oxidants (like H_2O_2) however represent a major oxidant burden. They contribute to disease pathology by disruption of redox signaling and control mechanisms (40). We have chosen the use the redox potential as surrogate of oxidative stress due to the major role of glutathione in detoxifying nonradical oxidants (through glutathione oxidase enzyme reaction) and its role as a major determinant of cell redox status which controls the cell cycle and modifies different enzymatic activities (38-40, 45, 58).

V.C. If BPD is related to oxidative stress, why studies using different antioxidants had very limited impact on BPD?

Understanding the nature, quantitative importance and mechanism of action of oxidants as well as antioxidants is very important milestone if one would like to target the right intervention aiming to decrease or prevent oxidative stress and its complications.

An important example is the use of CuZn superoxide dismutase in mechanically ventilated preterm infants to reduce the risk of BPD (89-91). A recent Cochrane review concluded that the use of superoxide dismutase to prevent chronic lung disease of prematurity is not recommended (92). As discussed in the introduction section SOD plays a very important role by reducing the free radical anion

superoxide in oxygen and hydrogen peroxide that will be reduced in water by catalase or GPx. That means in these studies all infants in the intervention group had very effective defense against radical oxidants with the SOD but were exposed to higher load of nonradical oxidants (hydrogen peroxide) produced by the SOD. Knowing that these preterm infants have deficient glutathione oxidase and catalase systems it will be expected that they will experience a more oxidized redox potential and will still be prone to BPD.

The trials to supplement TPN with either cysteine or N-acetycysteine to help establishing good glutathione reserve and ameliorate the antioxidant defenses and prevent BPD are another example. In a recent Cochrane review including 6 studies, the effect of N-acetylcysteine supplementation on death and bronchopulmonary dysplasia at 36 weeks corrected age was available in 391 infants (194 in the treatment group and 197 in the placebo group). N-acetylcysteine supplementation did not significantly affect the risk of death, the risk of bronchopulmonary dysplasia or the risk of combined outcome death or bronchopulmonary dysplasia (RR 1.04, 95% CI 0.85 - 1.27) (93). The study by Lavoie JC et al demonstrated that it is not the availability of cysteine but the cysteine uptake that appears to be the limiting step explaining the reported low levels of glutathione in premature infants. In this study cysteine uptake, which was found to be immature in this population, was shown to be responsible for 78% of the variation in glutathione content (94). In the light of these finding we cannot presume that cysteine supplement will automatically lead to increase intracellular glutathione. Considering these finding the results of the Cochrane review will not be surprising

V.D. The impact of the used BPD definition on the results of this study:

Our study objective was to study the effect of two major oxidants frequently used in the NICU with both the redox potential of glutathione as a marker of oxidative stress and the severity of BPD. The severity of BPD was defined using the NICHD classification (9). With this definition we could not find a significant relation between the redox potential measured on day 6-7 or at 36 weeks and the severity of BPD. Will this finding remain the same if our study was looking to the development of BPD as a dichotomous outcome according to the classic definition (7, 8) which is "the requirement for additional oxygen at a corrected postnatal gestational age of 36 weeks in infants less than 1,500 g of birth weight"? The answer is no. In this case there is a significant association between more oxidized redox potential and BPD (Figure VI).

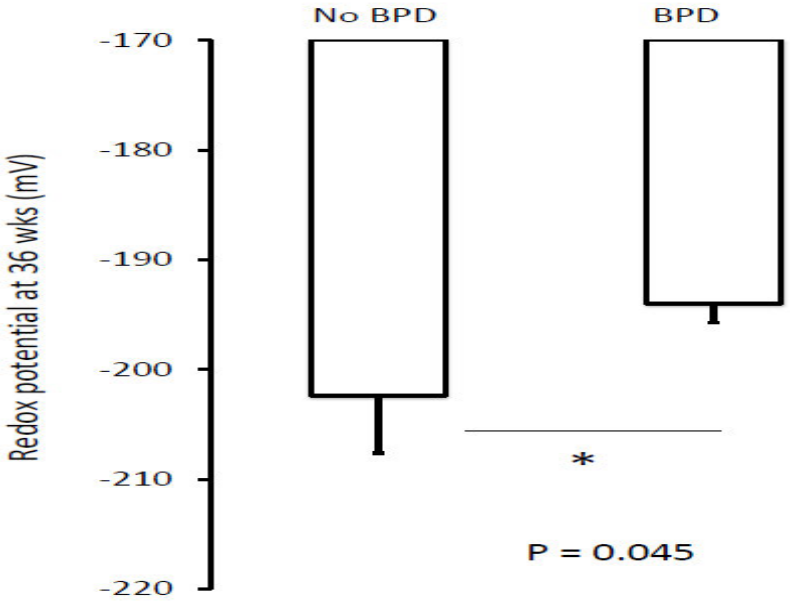

Figure VI: When using the classic definition of BPD there is a significant correlation between more oxidized redox potential and BPD.

This relation between more oxidized redox potential and BPD could be viewed as a cause-effect association if we consider that the more oxidative stress resulted in the BPD development as supported by animal model using O_2 supplement or TPN to develop BPD (86, 95, 96). Alternatively, this more oxidized redox in the BPD group could be viewed as a consequence of receiving O_2 supplement. Animal model study could permit to determine whether this is a cause-effect relationship or not.

V.E. The impact of normal development of antioxidant defenses on the results of this study:

Our population included infant between 22 and 28 weeks of gestational age. Animal model (72, 74)as well as human (75) available data suggest that the fetal lung is programmed to increase antioxidant enzymes as term approaches in preparation for the increased oxidation associated with atmospheric air breathing, with its relatively high O_2 concentration.

There are no available data on redox potential measurement in this age group. Redox potential of these infants on day 6-7 could be affected by this chronological maturation raising the question if this group is homogenous. We were not able to perform our analyses according to GA strata due to sample size limitations.

When we tried to test the effect of performing this analysis on a more homogenous subgroup (infants of 26-27 weeks of gestational age), the relation between the redox potential on day 6-7 and the development of BPD approached significance with $P = 0.058$.

It will be of interest to have a larger sample size with more infants in each gestational age to be able to adjust for the effect of GA.

V.F. Strengths and Limitations

V.F.1. An Observational prospective cohort study:

In this work, an observational prospective cohort design was used. This was the most appropriate design in human neonate context as exposure to O_2 and TPN should be guided only by individual infant need in conformity with the current clinical guidelines.

We aimed to study the effect of both O_2 and TPN on the redox potential as a marker of oxidative stress and BPD. In this case the casual inference was enhanced by temporal sequence, prior evidence and biologic plausibility. However, the relation between redox potential and BPD was compromised by incapacity to stratify or adjust by the gestational age (due to sample size limitation) in the case of redox potential on day 6-7 and by the lack of temporal sequence in the case of redox potential at 36 weeks corrected age.

V.F.2. Study population and sample:

We aimed to study the relation between O_2 and TPN, redox potential and BPD in infants less than 29 weeks gestational age. This selection was justified by the high

risk of exposure to both O_2 and TPN with limited antioxidant capacity. To insure that our sample represent the whole studied population we used all admitted infants with that gestational age during the whole study period as a comparator. All the demographic data and initial severity of illness score were similar between our sample and the whole study population.

V.F.3. The effect of level III NICU dynamics on our results:

As a referral center our unit policy is to transfer all stable infants to their local hospitals, this lead to the noticed small number in both no BPD and mild BPD categories. To be able to perform severity of BPD analyses we were obliged to combine these 2 categories (no BPD and mild BPD) together to get a reasonable number in this group. In our clinical setting it will be challenging to have measures of redox potential from enough infant with no or mild BPD and this fact needs to be addressed in further studies.

V.G. Future works

V.G.1. On the same cohort:

Parenteral nutrition is contaminated by peroxides including the ascorbylperoxide which cause an oxidized redox potential in premature infants. Previous works from the laboratory of Dr Jean-Claude Lavoie suggest that ascorbyperoxide is associated with perturbation of alveolar development (52, 85). We collected urine samples from group C on day 3, 5 and 7 of life as well as at 36 weeks of corrected age. We will investigate the relation between the ascorbyperoxide in the urine and the redox

potential as well as the BPD in preterm infants of less than 29 weeks of gestational age.

V.G.2. Future combined animal and human study project:

Our work team with other collaborators obtained a CIHR fund (CIHR 246505) to perform a larger scale study combining the animal model with a clinical study. The objective of this study is to understand the biochemical mechanisms of the relation between the ascorbylperoxide, oxidized redox potential and BPD in order to develop a nutritional alternative that could help in decreasing the incidence and severity of BPD. The specific objectives of this project are:

1- Validate that, in the animal model, the effect of the ascorbyperoxide on the alveolar development could be through perturbation of the glutathione system leading to oxidized redox potential and induction of alveolar apoptosis in the lung. This objective will be achieved by measuring in the newborn guinea pigs infused with increasing doses of ascorbyperoxide, the alveolarization index, apoptosis, glutathione redox state (potential redox, glutathione peroxidase and reductase, activation of transcription factors Nrf2 and NFκB) in relation to the level of ascorbyperoxide in the lung and the urine.

2- Test, in the animal model, 2 suggested alternatives to prevent the alveolar development perturbation by the TPN:

a- The addition of glutathione to the TPN in order to ameliorate the glutathione defense system and prevent the oxidative stress and the alveolar

hypoplasia. This objective will be achieved by measuring the alveolarization index, apoptosis, the glutathione system and the pulmonary and urinary level of ascorbyperoxide in guinea pigs receiving TPN supplemented or not with glutathione.

b- Using vitamin C-2Phosphate as an alternative to vitamin C. This form is stable *in vitro* and will not form the ascorbyperoxide molecule and could help avoiding alveolar hypoplasia by preventing ascorbyperoxide formation. This objective will be achieved by measuring the alveolarization index, apoptosis, the glutathione system and the pulmonary and urinary level of ascorbyperoxide and vitamin C in guinea pigs receiving TPN containing either vitamin C-2Phosphate or vitamin C.

3- Confirm the association between the ascorbylperoxide and BPD in premature infants less than 32 weeks of gestational age using the correlation between the levels of urinary ascorbylperoxide, the redox potential with the severity of BPD. In this study gestational age is considered as co-variable, sex and FiO_2 concentration will be considered as variable and sample size for ANCOVA analysis is estimated at 240 infants.

V - CONCLUSION

In preterm infants less than 29 weeks of gestational age, early exposure to O_2 (≥25% on day 7 of life) and longer duration of TPN (more than 14 days) are associated with more oxidized whole blood redox potential at 36 weeks and more severe BPD. More efforts should be directed towards strategies targeting judicious use of O_2 supplement and either decreasing the duration or using a safer formulation of TPN. There is an urgent need to find a new way of compounding parenteral nutrition to provide a nutritional solution without toxic elements; a TPN that allows the best development of these infants without adverse effects.

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