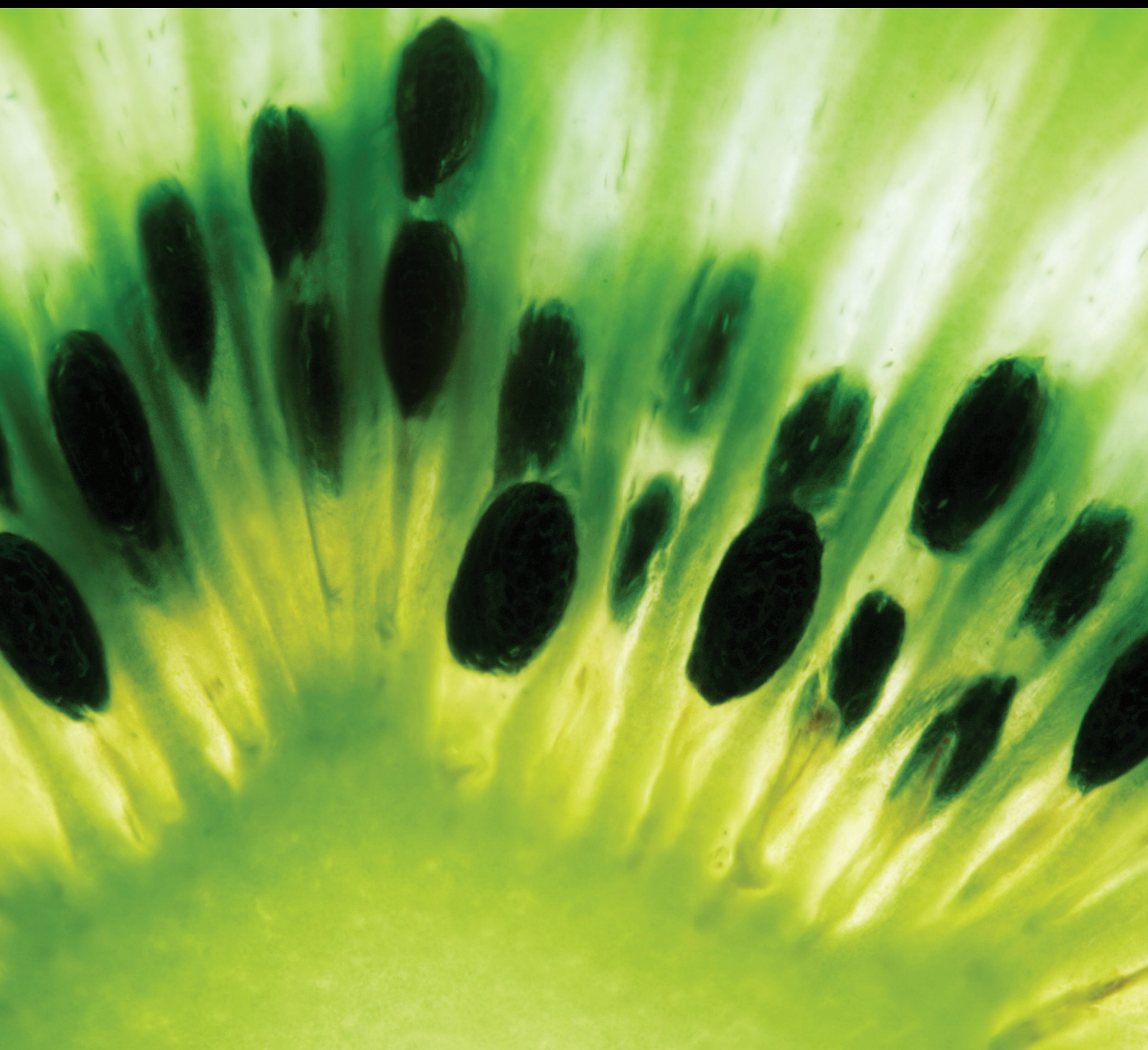
Journal of Nutrition and Metabolism

Nutrition: From Bench to Bedside

Guest Editors: Naim A. Khan, Azeddine Ibrahimi, Aziz Hichami, and Najat Mokhtar



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Editorial Board

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 4295179, 2 pages
http://dx.doi.org/10.1155/2016/4295179

Editorial

Nutrition: From Bench to Bedside

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Received 22 May 2016; Accepted 23 May 2016

The nutritional health in the world is one of the most pressing issues facing different countries today. International organizations like FAO and WHO suggest that there is an improvement of the nutritional situation in Asia and Latin America, though a deterioration in Sub-Saharan Africa does exist. As regards Europe and USA, we are living in the era of "overnutrition." Unfortunately, the situations of famine, hunger, and starvation do exist in some developing countries. Some of the noncommunicable pathologies like obesity are not only due to the excess of fatty food, but intake of unbalanced diet also contributes significantly to their pathogenesis. Nutrient requirements vary as a function of lifestyle. Infants and pregnancy require more attention because these situations are more vulnerable and are at greater risk for malnutrition that might contribute to metabolic memory, responsible for the pathologies like macrosomia, obesity, type 2 diabetes, and hypertension. To cover all of these areas, there is a pressing need to develop a platform for the translational medicine from fundamental to clinical research in the field of nutrition.

This special issue was initially destined to publish the peer-reviewed and selected articles that were presented in the 1st International Congress of Nutrition & Food Science https://www.univ-tlemcen.dz/, held in Tlemcen (Algeria) from 20th to 22nd November 2015; however, the issue was open to all other original and high-quality unpublished articles belonging to the topics of the issue.

The hot topics of the special issue included the following fields: phytotherapy, pediatric nutrition, food security, immunonutrition, pathophysiological complications and nutrition, clinical outcome and nutrition, experimental nutrition and nutrients, and nutritional assessment.

In this issue, we received a large number of articles, but we gave the priority to the high-quality manuscripts during the reviewing process. This issue is comprised of 8 articles which deals with different aspects of nutrition and its importance in health and disease. The phytotherapy has been increasingly considered as safe and an alternative to allopathic medicine which might cause the side effects in the long run. In this issue, two articles deal with plant-derived products in different pathophysiological states. Immune system, particularly the inflammatory state, contributed by high concentrations of proinflammatory cytokines, is the cause of several pathological manifestations. The n-3 polyunsaturated fatty acids (n-3 PUFA) have been proposed to exert beneficial effects during hypertension and dementia. Besides, these agents exert anti-inflammatory effects.

Two articles deal with the immunomodulatory/anti-inflammatory effects of vitamin D3 and n-3 PUFA. There are two articles that contribute to this burning topic. Micronutrition has been proposed as the remedy that exerts immediate beneficial effects as it fulfils the endogenous requirements of the body. Indeed, the deficiency of micronutrients might be the cause of different pathologies. Two articles deal with this subject where one of them sheds light on the role of glutamine and the second one plausibly demonstrates that the deficiency of iron in the primary school children might be associated with their reduced learning capacity and supplementation of iron in their diet improves this parameter.

Acknowledgments

Last, but not the least, we express our sincere thanks to the reviewers who spared their precious time to assess the submitted articles.

Naim A. Khan
Azeddine Ibrahimi
Aziz Hichami
Najat Mokhtar

Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 4280876, 8 pages
http://dx.doi.org/10.1155/2016/4280876

Research Article

Vitamin D3 Suppresses Class II Invariant Chain Peptide Expression on Activated B-Lymphocytes: A Plausible Mechanism for Downregulation of Acute Inflammatory Conditions

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Received 24 December 2015; Revised 17 March 2016; Accepted 18 April 2016

Academic Editor: Azeddine Ibrahimi

Class II invariant chain peptide (CLIP) expression has been demonstrated to play a pivotal role in the regulation of B cell function after nonspecific polyclonal expansion. Several studies have shown vitamin D3 helps regulate the immune response. We hypothesized that activated vitamin D3 suppresses CLIP expression on activated B-cells after nonspecific activation or priming of C57BL/6 mice with CpG. This study showed activated vitamin D3 actively reduced CLIP expression and decreased the number of $CLIP^+$ B-lymphocytes in a dose and formulation dependent fashion. Flow cytometry was used to analyze changes in mean fluorescent intensity (MFI) based on changes in concentration of CLIP on activated B-lymphocytes after treatment with the various formulations of vitamin D3. The human formulation of activated vitamin D (calcitriol) had the most dramatic reduction in CLIP density at an MFI of 257.3 [baseline of 701.1 (P value = 0.01)]. Cholecalciferol and alfacalcidiol had no significant reduction in MFI at 667.7 and 743.0, respectively. Calcitriol seemed to best reduce CLIP overexpression in this ex vivo model. Bioactive vitamin D3 may be an effective compliment to other B cell suppression therapeutics to augment downregulation of nonspecific inflammation associated with many autoimmune disorders. Further study is necessary to confirm these findings.

1. Introduction

B cell engagement by mitogens has been shown to result in the activation of B cell dependent inflammatory pathways. This interaction helps to "jump start" the innate immune response [1]. Emerging evidence suggests that vitamin D may also play a pivotal role in the successful downregulation of this aspect of the immune response as the host defense system transitions from innate to adaptive immunity via inactivation of nonspecifically activated, $CLIP^+$ B cells [2–4]. In addition, bioactive vitamin D3 has been shown to inhibit mitogen-induced IgM production by B-lymphocytes as the affected host shifts from a nonspecific to specific immune response. This has been demonstrated to occur in a time-dependent fashion [2].

Furthermore, it has been previously established that mitogens bind to toll-like receptors on B cells, leading to nonspecific B cell activation associated with increased expression of class II-associated invariant chain peptide (CLIP) and polyclonal expansion of B cells into immunoglobulin (IgM) secreting (plasma) cells [1]. Class II-associated invariant chain peptide (CLIP), a polypeptide involved in the formation and transport of major histocompatibility complex (MHC) class II protein, also known as CD74, binds to the MHC class II groove and shields the epitope binding site until the MHC receptor is fully assembled [1, 5]. The purpose of

CLIP is to prevent self-peptide fragments from binding to the receptor prior to MHC II localization within the endosome/lysosome after activation by foreign antigen (Ag) [1, 6]. Under normal conditions, in this special endosome called the major histocompatibility complex (MHC) II compartment, cathepsin S cleaves the invariant chain, leaving a shorter CLIP bound to the MHC II complex [7].

In presence of antigen peptide fragments, human leukocyte antigen- (HLA-) DM, an intracellular protein involved in foreign peptide presentation by MHC class II, interacts with the MHC II complex, leading to the release of CLIP and allowing the antigenic peptides to be bound via one of its epitopes [1, 8]. This is a key step in activation of the innate immune system. MHC II complexes with bound antigen and is subsequently transported to the cell membrane for presentation by antigen presenting cells (APCs), such as macrophages, dendritic cells, and B-lymphocytes [1, 8]. The antigen peptide-MHC class II complexes are then transported to the plasma membrane of the APCs, where they are recognized by T and B-lymphocytes as the host defense system activates its adaptive immune system [1, 5].

In some patients, there appears to be a failure to downregulate this initial inflammatory process [1, 9, 10]. This failure to turn off the acute immune response may lead to postinflammatory, persistent systemic inflammatory disorders, such as rheumatoid arthritis and hay fever, or a local inflammatory condition like psoriasis [1, 11].

As activated vitamin D3 has been demonstrated to be a powerful modulator of the immune system, it may play a significant role in the downregulation of autoimmune inflammatory disorders via suppression of nonspecifically activated immune cells [3, 4, 12–14]. In other words, vitamin D may be potentially useful in the amelioration of the proinflammatory pathway. In fact, vitamin D has previously been shown to reduce polyclonal B cell expansion. Chen et al. evaluated the effects of activated vitamin D3 on B cells and demonstrated its inhibition of ongoing proliferation of activated B cells [3]. This suggests that it may play a role in the attenuation of some chronic inflammatory conditions, which provides a rationale for its use as a component of B cell depletion therapy [3, 15, 16].

Consequently, the correction of vitamin D deficiency to optimal or therapeutic levels may play a significant role in the reduction of postinfectious, chronic inflammatory conditions and other B cell-mediated autoimmune disorders [17]. However, the best formulation or preparation of vitamin D3 which provides the most therapeutically appropriate benefit remains a subject of debate. In this study, we evaluated the effect of vitamin D3 on activated mice splenic B cell using various formulations and concentrations on the intensity of CLIP expression after CpG-mitogen stimulation. We hypothesize that bioactive vitamin D downregulates the density of CLIP expression as well as reducing polyclonally activated splenic B cells, thereby reducing the proinflammatory response.

2. Materials and Methods

Thirty C57Black/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA) and divided into 5 groups of 6. All animal experiments were conducted according to the guidelines for animal use approved by the Texas A&M University Health Sciences. The average weight at the time of the experiments for the mice was 25 grams. All procedures involving the mice were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University Health Sciences.

15G4 monoclonal antibodies (mAb) were used in these experiments, a mAb directed against mouse MHC-CLIP (I-A^b complex), only when CLIP is in the groove of mouse MHC class II I-A^b molecules (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Phycoerythrin- (PE-) conjugated monoclonal anti-mouse B220 was also used and obtained from BD PharMingen (San Diego, CA, USA). Mouse anti-human CLIP (clone CerCLIP) was obtained from BD Biosciences (San Jose, CA, USA). A TLR-9 binding ligand, also known as a toll-ligand, CpG-oligodinucleotide (CpG-ODN) (Invivogen, San Diego, CA), was used to prime the mice.

Thirty C57BL/6 mice were injected with CpG-ODN using ~5 μg/mouse, weighing ~25 g, equivalent to 5 μM of peptide, and then separated into 5 groups containing 6 mice each. Forty-eight hrs after initial TLR stimulation with CpG-ODN, the mice were sacrificed and their spleens were harvested and passed through a nylon mesh to recover single cell suspensions. Each group of mixed, resting B cells and CpG-ODN activated splenocytes were further subdivided into three groups and then treated with different vitamin D formulations of vitamin D3 (human calcitriol, exogenously synthesized calcitriol, cholecalciferol, and alfacalcidiol) at the following concentrations: 0.1 mg/dL, 1 mg/dL, and 10 mg/dL. DMSO was used as a control. The isolated cell populations were then allowed to incubate for 15 hrs before mAb staining with anti-mouse MHC-CLIP and anti-mouse B220. The cell preparation was analyzed using flow cytometry to determine the density of CLIP expression on the surface of the B cell populations by measuring the mean fluorescent intensity [MFI] (Beckman Coulter Excel or Coulter FC500 flow cytometer Beckman Coulter, Fullerton, CA, USA) of each treatment group.

One-sample t-test, 2-sample t-test, and ANOVA were used to analyze the data using MiniTab 17.0 software, State College, PA. One-sample t-test and 2-sample t-test were performed using MiniTab 17.0 software. ANOVA was used to create a generalized linear model using SAS 9.3, Cary, NC. A P value of < 0.05 was considered to be statistically significant.

3. Results

The human activated vitamin D3 (calcitriol) formulation at 0.1 ng/mL (Figure 1) had the most dramatic reduction in the MFI of CLIP on activated B cells at 257.3 compared with a baseline MFI of 701.1 (P value = 0.01). Cholecalciferol and alfacalcidiol had no significant reduction in MFI at 667.7 and 743.0, respectively, fifteen hrs after administration. The effect appeared to be dose dependent with a less dramatic effect at higher doses (MFI of 257.3 versus 482.3 and 443.3) at 0.1, 1.0 (Figure 2), and 10 mg/dL (Figure 3), respectively. TLR-9

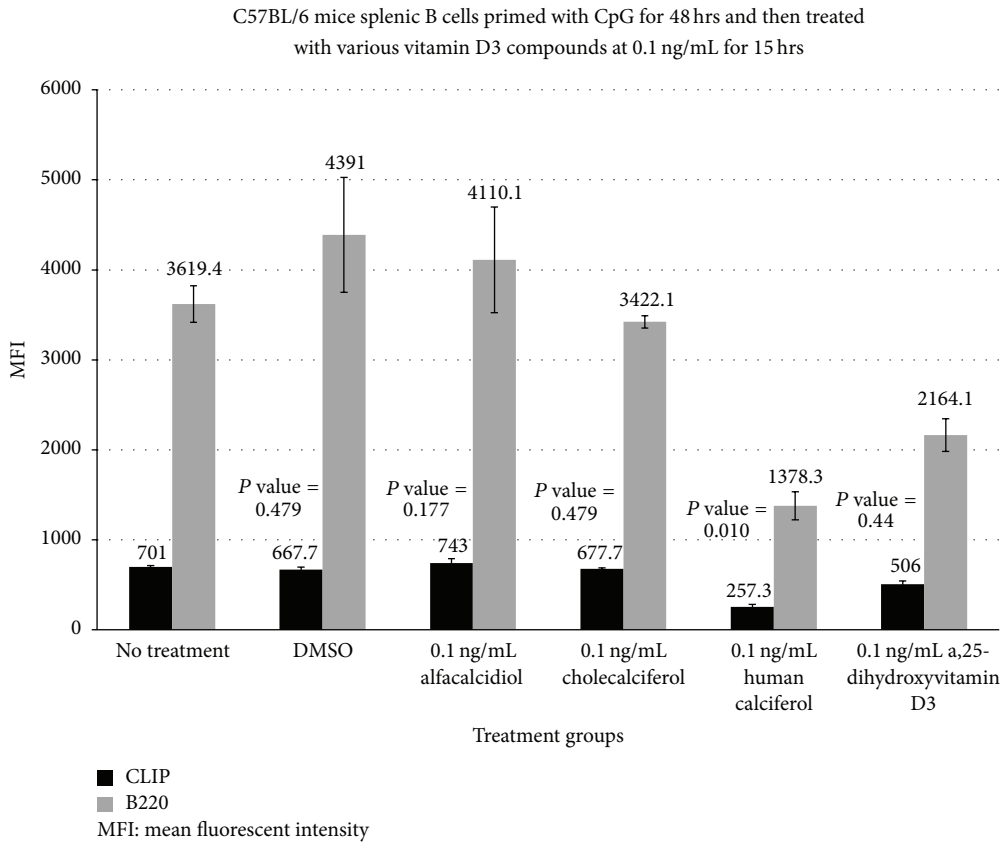

FIGURE 1: This bar graph shows the effects of various vitamin D3 compounds at a low concentration of 0.1 ng/mL on the mean fluorescent intensity (MFI) of CLIP$^+$ B cells after TLR-engagement. This figure demonstrates the changes in MFI of CLIP and CLIP$^+$ B220 subsets of B cells after CpG-induced TLR-9 activation. These results suggest that the active formulation of vitamin D3 may lead to a statistically significant reduction in CLIP expression on polyclonally activated splenic B cells. Administration of the human calcitriol of vitamin D3 ($P = 0.01$) resulted in the greatest overall reduction in the MFI (257 versus a baseline of 701.1) for CLIP expression on stimulated B cells.

activation with CpG-ODN caused enhanced ectopic CLIP expression on activated B-lymphocytes at baseline.

In this study, CpG-ODN stimulation of mouse splenocytes resulted in a time-dependent increase in exogenous CLIP expression along with MHC class II complexes on B cells, as determined by immunofluorescent staining with an anti-mouse CLIP/class II-specific antibody versus anti-mouse B220 (Figure 1, bar graph). Upon analysis of the variations in percent of CLIP$^+$ B cells over time with the changes in geometric mean fluorescent intensity (MFI), we observed that the relative number of CLIP molecules per cell decreased in response to administration of activated vitamin D3 [human and exogenous 1,25(OH)^{2}D3] and incubation over the 15-hour time period, as well as the percentage of CLIP$^+$ B cells over total number of B cells decreased in response to activated vitamin D3.

4. Discussion

In these experiments, C57BL/6 mice were injected with CpG-ODN and incubated for 48 hrs to stimulate polyclonal B cell expansion. The cells were then treated with bioactive and precursor formulation of vitamin D3 at varying concentrations to determine if treatment of this nature had any effect on the density of CLIP expression. CpG stimulation induced an approximately 8-fold increase in the number of CLIP$^+$ B cells from baseline, resting B cells. A significant decrease in ectopic CLIP expression on the surface of activated B cells was observed fifteen hours after treatment with activated vitamin D3 (Figures 4–6) [1, 16].

To rule out the possibility that ectopic CLIP resulted solely from coincident, increased levels of nascent MHC class II on the activated B cells, we counterstained activated B cells with an MHC class II anti-human HLA-DR antibody [1]. The increase in cell surface CLIP levels in response to CpG-ODN did not correspond with the TLR-dependent changes in MHC class II, suggesting that TLR-mediated ectopic CLIP expression is not merely the consequences of randomly increasing levels of cell surface MHC class II. Actually, cell surface CLIP is considered to be an indicator of immaturity in antigen presenting cells until they are activated by peptide antigen specific MHC receptor engagement, at which point cell surface CLIP expression decreases [1, 18–20].

As B cell-specific antigen receptor (BCR) engagement results in signals that increase the acidity in the lysosomes,

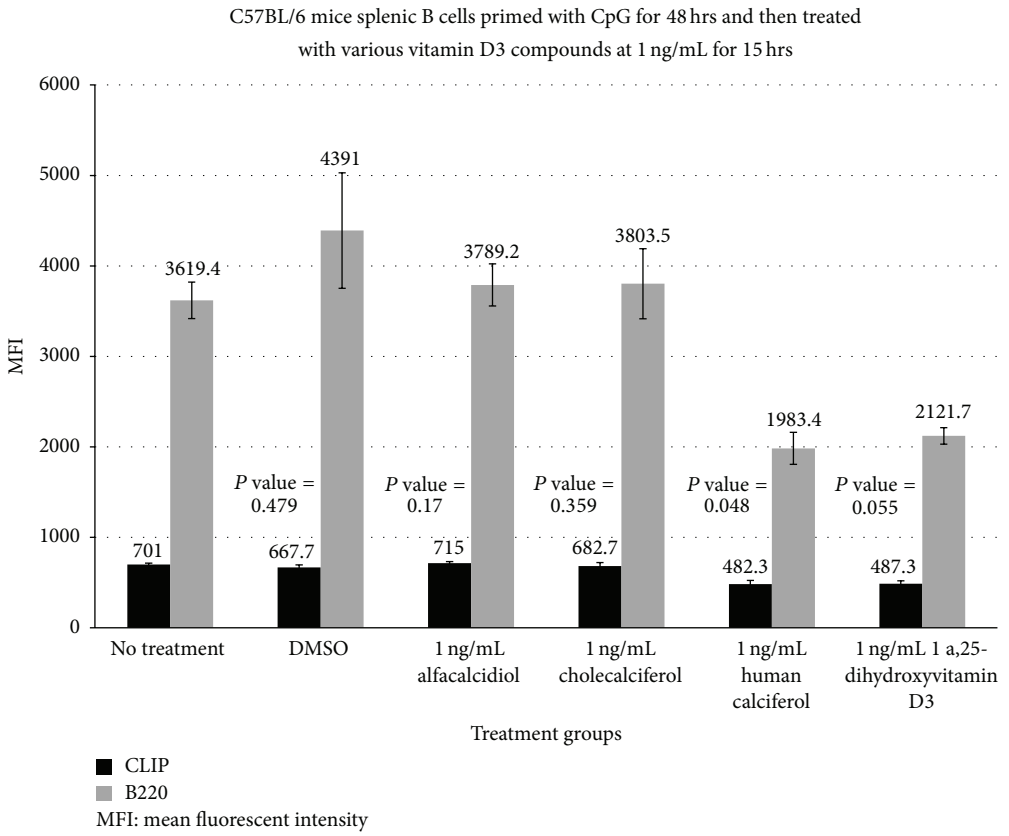

Figure 2: Bar graph showing the effects of 1 ng/mL of various vitamin D3 compounds on the distribution of TLR-activated CLIP$^+$ and B220 subsets of B cells. This figure demonstrates the changes in MFI of CLIP and CLIP$^+$ B220 subsets of B cells 15 hrs after administration of different compounds of vitamin D3 versus no treatment or DMSO (control). These results suggest that the active formulation of vitamin D3 may lead to a statistically significant reduction in CLIP expression on polyclonally activated splenic B cells. Administration of the human calcitriol of vitamin D3 ($P = 0.048$) resulted in the greatest overall reduction in the MFI (482.3 versus a baseline of 701.1) for CLIP expression on stimulated B cells. Administration of the active form of vitamin D3 again resulted in statistically significant reduction in CLIP expression on CpG-activated B cells.

the BCR subsequently works in conjunction with 1, 25-dihydroxyvitamin D3 to suppress the expression of CLIP on activated B cells [3, 5, 6]. We directly evaluated the effects of TLR stimulation on CLIP expression versus B2220 presence on the cell surface. We used anti-Ig stimulation as a known activator for B220 receptor signaling and compared levels of ectopic CLIP and percentage of CLIP$^+$ B cells after TLR-9 dependent B cell activation versus stimulation through the B cell antigen receptor [1]. As predicted, we observed significantly less ectopic CLIP per cell by measuring geometric mean fluorescent intensity (MFI) in cell populations in which there was no TLR stimulation (resting B cells).

Similarly, the percentage of CLIP$^+$ B cells after vitamin D3 treatment was reduced significantly relative to the percentage of CLIP$^+$ B cells after initial TLR-9 activation (Figures 4–6). DMSO (control), cholecalciferol, and alfacalcidiol treatment resulted in no relevant decrease in CLIP expression as measured by changes in relative MFI. These results indicate that activated vitamin D3 versus its precursor cholecalciferol and alfacalcidiol formulations resulted in a statistically significant decreased level of expression of surface CLIP on activated B cells. Therefore, unabated TLR stimulation by CpG-ODN or other mitogens, which is not followed by active vitamin D receptor engagement, may significantly increase the percentage of cell surface, CLIP-positive B cells, and the relative amount of CLIP expressed per cell leading to ongoing nonspecific inflammation [1, 17, 21, 22].

Our data support the fact that nonspecifically activated B cells, which bear increased levels of ectopic CLIP, are important for promoting nonspecific, proinflammatory immune activation. Treatment with an anti-inflammatory agent, such as the activated form of vitamin D3, might decrease the density of CLIP on activated B cells [22, 23]. This may cause or help facilitate the transition of the host defense system from nonspecific to specific adaptive immunity.

As mentioned earlier, accumulating research data suggest that 1,25(OH)^{2}D3-mediated signaling is important in the regulation of protective inflammatory responses against harmful pathogens [23, 24]. In addition to inhibiting expression of nonspecific immunoglobulin (IgM) by B cells, activated vitamin D3 has been shown to enhance production of anti-inflammatory mediators as well as suppressing the expression

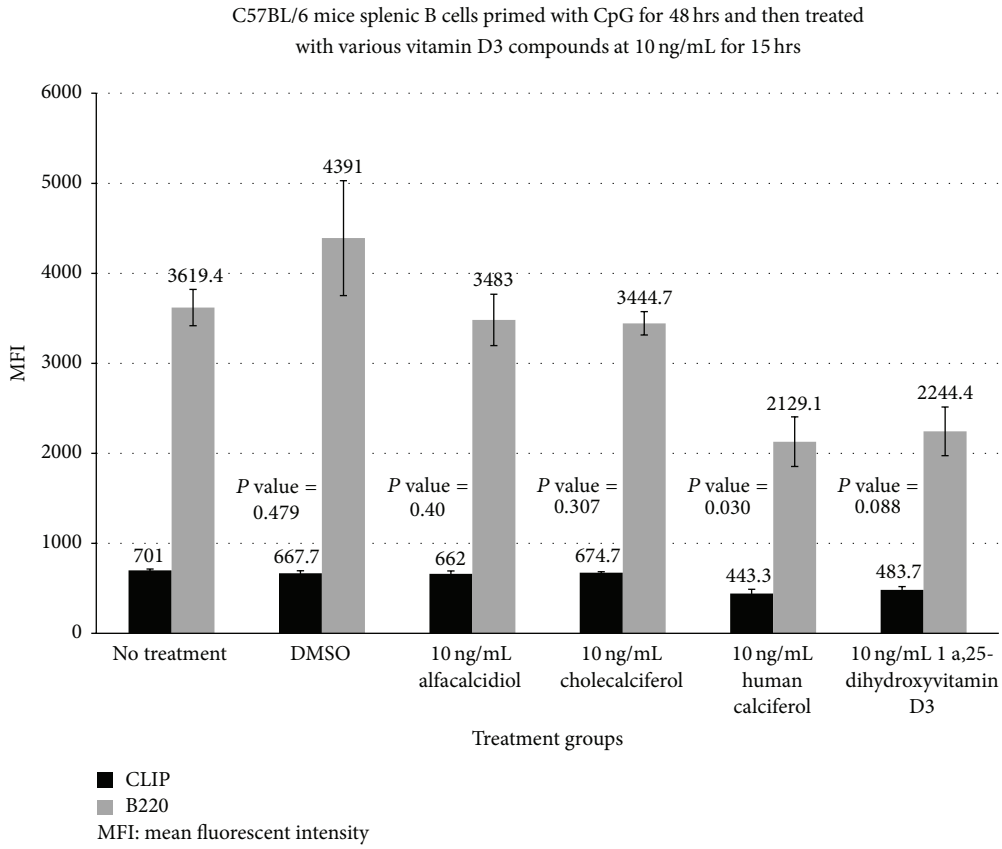

Figure 3: This bar graph also shows the effects of different vitamin D3 compounds at a concentration of 10 ng/mL on the MFI of CLIP expression and $CLIP^+$ B220 subsets of B cells after TLR-activation. This graph compares the changes in MFI of CLIP and $CLIP^+$ B220 subsets of B cells 15 hrs after administration of different compounds of vitamin D3 versus no treatment or DMSO (control). These results suggest that exogenously produced bioactive $1,25(OH)^2D3$ may be needed in higher doses of activated vitamin D3 in order to reduce the level of CLIP expression on polyclonally activated B cells ($P = 0.055$). Administration of human calcitriol again resulted in most significant reduction in CLIP expression on polyclonally activated B-lymphocytes.

of IL-2 receptors on B cell blasts [3]. In order to optimally regulate immune function, research suggests that serum levels of cholecalciferol must be greater than 30 ng/mL in order to undergo local, paracrine conversion to calcitriol [24–28]. The biofeedback of locally produced activated vitamin D3 creates a condition whereby antigen-responsive B cells are able to simultaneously engage in the induction of adaptive immune responses to specific antigenic stimuli. Therefore, activated vitamin D3 at appropriate levels may serve as an important contributor to anti-inflammatory processes, as it may help to dampen both acute and chronic, nonspecific inflammatory response pathways and prevent or enhance amelioration of some autoimmune conditions [29–32].

In this study, we observed that the relative number of CLIP molecules per cell decreased in response to administration and incubation with activated vitamin D3 [human calcitriol and exogenous 1, 25 $(OH)^2$ vitamin D3] over the treatment period. Furthermore, the MFI for the total number of $CLIP^+$ B220 cells was noted to decrease in response to activated vitamin D3. This suggests that calcitriol may directly, or indirectly, play a vital role in augmenting humoral immunity as well as advances in B cell depletion therapies for certain autoimmune conditions. Based on our preliminary study, plus several others which suggest that dampening chronic immune activation responses using innovative therapeutics may be beneficial, vitamin D3 may be a useful adjunct to use with other B cell depletion therapies for the various autoimmune diseases and disorders characterized by chronic inflammation. However, the mechanism by which this B cell depletion takes place is still being elucidated and is an area of ongoing research [33–40]. Furthermore, additional study will be necessary to determine the most salient aspects of this potential intervention before specifically testing this type of therapy to treat any specific autoimmune-related medical condition.

5. Conclusion

B cell depletion therapy has been suspected to be a useful modality in dampening of chronic inflammatory conditions. As activated vitamin D was demonstrated to suppress CLIP expression on the surface of polyclonally activated splenic B cells after nonspecific priming with mitogen, it may be a useful adjunct to further explore and study to enhance the

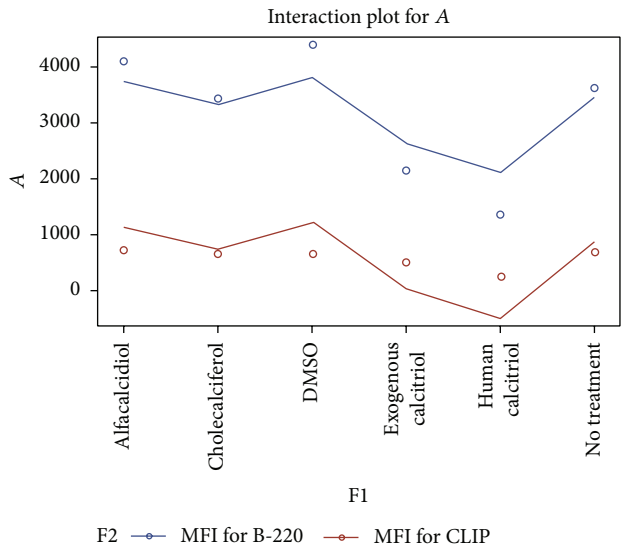

Figure 4: This figure compares the impact of the various vitamin D compounds at 0.1 mg/dL on the density of cell surface CLIP expression and B220 subsets of B cells 15 hrs after treatment using the vitamin D3 compounds versus nontreatment or DMSO. These results suggest that the active formulation of vitamin D3 once again leads to a statistically significant reduction in CLIP expression on polyclonally activated splenic B cells even at a higher concentration of 10 mg/dL. Administration of the human calcitriol of vitamin D3 ($P = 0.0181$) resulted in the greatest overall reduction in the MFI (257.3 versus a baseline of 701.1 and 667.7 for the control) of CLIP expression on mitogen stimulated B cells.

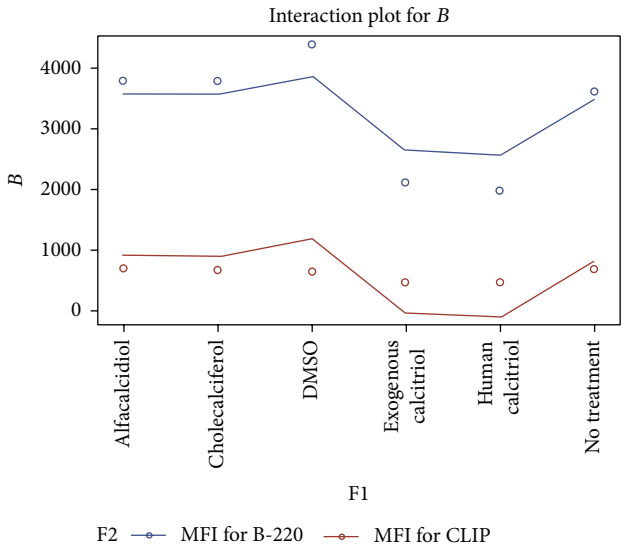

Figure 5: This figure compares the impact of the various vitamin D compounds at 1.0 mg/dL on the density of cell surface CLIP expression and B220 subsets of B cells 15 hrs after treatment using the vitamin D3 compounds versus nontreatment or DMSO. These results suggest that the active formulation of vitamin D3 once again leads to a statistically significant reduction in CLIP expression on polyclonally activated splenic B cells even at a higher concentration of 10 mg/dL. Administration of the human calcitriol of vitamin D3 ($P = 0.0181$) resulted in the greatest overall reduction in the MFI (482.3 versus a baseline of 701.1 and 667.7 for the control) of CLIP expression on mitogen stimulated B cells.

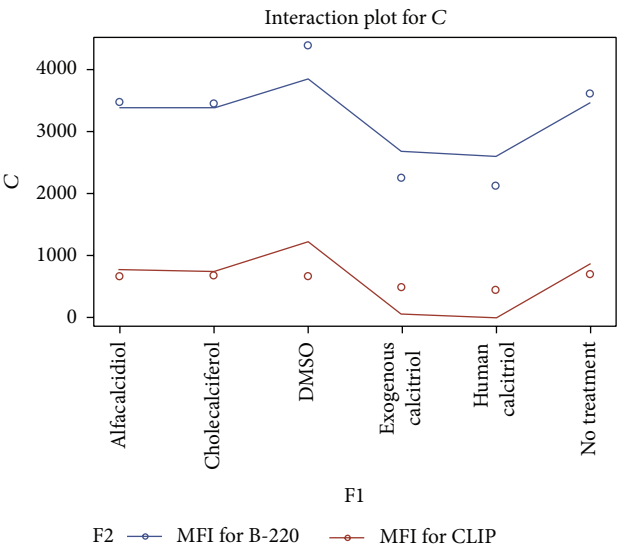

Figure 6: This linear graph demonstrates the impact of the various vitamin D compounds at 10.0 mg/dL on the density of cell surface CLIP expression and B220 subsets of B cells 15 hrs after treatment using the vitamin D3 compounds versus nontreatment or DMSO. These results suggest that the active formulation of vitamin D3 once again leads to a statistically significant reduction in CLIP expression on polyclonally activated splenic B cells even at a higher concentration of 10 mg/dL. Administration of the human calcitriol of vitamin D3 ($P = 0.0181$) resulted in the greatest overall reduction in the MFI (443.3) versus a baseline of 701.1 for no treatment and 667.7 for the control of CLIP expression on mitogen stimulated B cells.

success of B cell depletion. Our study suggest that bioactive vitamin D3 may be helpful in the amelioration of some aspect of inflammation, as increased CLIP expression on polyclonally activated B-lymphocytes has been linked to acute nonspecific inflammatory processes. Plasma levels of vitamin D3 in the range of ≥30 to 40 ng/mL may help augment this immune downregulation process and/or facilitate the reversal of some acute and chronic autoimmune disorders through suppression of some aspects of the proinflammatory pathway [41–44]. As activated vitamin D3 has been demonstrated to be a powerful modulator of the entire immune system, it may play a potentially significant role in the downregulation of nonspecifically activated, Ig-M secreting B cell activation through suppression of CLIP expression on polyclonal B-lymphocytes. This may be used to compliment other mechanisms and strategies through which B-lymphocyte depletion therapies may help to diminish chronic immune activation. Nevertheless, further study is necessary to confirm the results of our preliminary findings.

Disclosure

This paper's contents are solely the responsibility of the authors and do not necessarily represent the official views of NIMHD or NIH.

Competing Interests

None of the authors have any competing interests with respect to this paper.

Acknowledgments

Special thanks are due to Dr. Ernest Alema-Mensah and Dr. Muhammad Mubasher, Morehouse School of Medicine R-CENTER, for their assistance with the statistical analysis. This project described was supported by the National Institute on Minority Health and Health Disparities (NIMHD), Grant no. 8 U54 MD007588, a component of the National Institutes of Health (NIH).

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 3124960, 10 pages
http://dx.doi.org/10.1155/2016/3124960

Review Article

Role of T-Cell Polarization and Inflammation and Their Modulation by n-3 Fatty Acids in Gestational Diabetes and Macrosomia

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Received 24 December 2015; Revised 10 April 2016; Accepted 5 May 2016

Academic Editor: Michael B. Zemel

Th (T helper) cells are differentiated into either Th1 or Th2 phenotype. It is generally considered that Th1 phenotype is proinflammatory, whereas Th2 phenotype exerts anti-inflammatory or protective effects. Gestational diabetes mellitus (GDM) has been associated with a decreased Th1 phenotype, whereas macrosomia is marked with high expression of Th1 cytokines. Besides, these two pathological situations are marked with high concentrations of inflammatory mediators like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), known to play a pivotal role in insulin resistance. Dietary n-3 polyunsaturated fatty acids (n-3 PUFAs) may exert a beneficial effect by shifting Th1/Th2 balance to a Th2 phenotype and increasing insulin sensitivity. In this paper, we shed light on the role of T-cell malfunction that leads to an inflammatory and pathophysiological state, related to insulin resistance in GDM and macrosomia. We will also discuss the nutritional management of these pathologies by dietary n-3 polyunsaturated fatty acids (PUFAs).

1. Introduction

Maternal diabetes during pregnancy, also called gestational diabetes mellitus (GDM), is an important risk factor for foetal overgrowth, termed macrosomia, which is influenced by maternal hyperglycemia and endocrine status through placental circulation [1].

In humans, macrosomia has generally been defined as a birth weight greater than or equal to the 90th percentile birth weight for gestational age, that is, infants who weigh >4000 g at delivery, regardless of gestational age or sex [2–4]. Infants born to diabetic mothers are at an increased risk for hypoglycaemia, respiratory distress syndrome, hyperbilirubinemia, and hypertrophic cardiomyopathy [3]. It seems that maternal hyperglycaemia leads to foetal hyperglycaemia, which stimulates foetal pancreatic islet cells and, consequently, induces foetal hyperinsulinaemia. Moreover, there exists a correlationship between maternal and foetal plasma cholesterol levels in 5-6-month-old human foetuses [5, 6]. It is noteworthy that several alterations in the metabolism of carbohydrates and lipids, observed in newborn babies of diabetic mothers, also persist postnatally [7–9].

2. *In Utero* Programming Is Responsible for Alterations Observed in Adulthood

It is possible that foetal hyperinsulinaemia may be an endogenous teratogen factor during critical periods of foetal development, leading to permanent structural or functional changes and consequent programming of "metabolic memory." Hyperinsulinemia *in utero* may affect the induction and activity of various hepatic enzymes associated with fat and carbohydrate metabolism [10]. This phenomenon may be accompanied, in placenta, by modifications in the expression of the transcriptional factors such as sterol

regulatory element binding protein-1c (SREBP-1c), known to induce expression of genes involved in lipogenesis [11].

In 1995, Barker [12] proposed that disproportionate foetal growth induced by foetal malnutrition, which could happen either in the middle or in a later period of the gestation, programmed coronary diseases in adulthood. The *in utero* programming seems to create a kind of "metabolic memory" as the physiological abnormalities, observed during the gestational period, are responsible for the induction of diseases associated with the metabolic syndrome such as type 2 diabetes (T2D) and obesity in adulthood. Indeed, in human GDM, there exists a correlationship between GDM and 6-month-old foetal plasma cholesterol levels and other abnormal lipid parameters, including high concentrations of triglycerides (TAG), apoB100, very-low-density lipoprotein (VLDL), and low-density lipoprotein (LDL), which often persist in macrosomia [2].

Leptin, an adipocyte-derived hormone, by decreasing food intake and increasing energy expenditure, stabilizes body adiposity [13]. A transient increase in leptin during neonatal life, called "neonatal leptin surge," has been shown to exert a neurotrophic effect and the development of energy-regulation circuits in mouse hypothalamus [14]. Furthermore, experimental premature leptin surge "from day 5.5 to day 10.5 of life" in mice pups led to decreased hypothalamic leptin sensitivity and accelerated weight gain when pups were fed a high-fat diet [15]. In healthy women, both maternal and foetal leptin concentrations were correlated with infant size at birth. Reference [16] reported that leptin levels were always higher in overweight than in normal weight newborns, and plasma leptin level was correlated with birth weight. Hence, it is conceivable that foetal leptin plays a role in *in utero* programming.

In utero nutritional environment may induce epigenetic alterations in the foetus. Epigenetic regulation is mediated by methylation and acetylation of histones. As far as foetal nutrition is concerned, it has been reported that dietary methyl-group intake (choline, methionine, and folate) during critical periods of development can alter DNA and histone methylation which may result in lifelong changes in gene expression [17]. Hence, these epigenetic mechanisms might contribute to the development of macrosomia and its related adulthood pathologies such as obesity and T2D [18].

Growth factors might be implicated in GDM and in the pathology of macrosomia *via* materno-foeto-placental axis. We have conducted a clinical study in which we have determined circulating growth factors and the expressions of their genes in placenta in GDM mothers and their macrosomic babies. We observed that serum concentrations of IGF-I, IGF-BP3, EGF, FGF-2, and PDGF-B were higher in GDM dames and their macrosomic babies as compared to their respective controls. Besides, the placental expression of the mRNA of growth factors (FGF-2 or PDGF-B) and growth factor receptors, that is, IGF-IR, EGFR, and PDGFR-beta, was upregulated in GDM women compared to controls [19].

3. Cell-Mediated Immunity in Diabetic Pregnancy and Macrosomia

The immune system is composed of two major subdivisions, the innate (phagocytic cells and NK cells) and adaptive immune responses. Though the interactions between innate and adaptive immunity are complex, the innate mechanisms control both the initiation and the type of adaptive response (Th1/Th2). It has been shown that the abnormalities in humoral and cell-mediated immunity in T1D females may persist during pregnancy and, hence, may complicate immune-foetal interaction [20].

About 10% of all GDM women develop T2D after delivery. Though pancreatic autoimmunity does not seem to represent a typical marker of GDM [21], high prevalence of autoantibodies, such as anti-GAD65 and anti-IA2 antibodies, has been observed in GDM subjects [22]. Maternal transmission of these autoantibodies did not affect diabetic risk in the offspring. On the contrary, it has been reported that foetal exposure to maternal T2D protects offspring, during the first 2 decades of life, from the development of islet autoimmunity and diabetes in the later life [23]. This is explained by the fact that the immune systems of children receiving autoantibodies from their diabetic mothers are primed during foetal life [24]. Such priming is demonstrated by an increase in MHC class II positive lymphocytes in infants of diabetic mothers compared to controls [25].

With regard to T-cell activation, only a few studies are available on the subject [20]. In humans, fully activated T-cells are detected in the cord blood of infants and mothers with T1D, but not in infants from normal mothers [20]. Interleukin-2 (IL-2) is a potent T-cell mitogen. Badr et al. [26] have reported a decrease in T-cell proliferation, associated with a decrease of plasma levels of IL-2 in the offspring of diabetic mothers, as compared to those of control mothers. Also, in rat model, probably because of their priming, *ex vivo* T-cell proliferation is significantly lower in diabetic pregnant rats and their macrosomic offspring, as compared to control animals [27]. This phenomenon may trigger a decrease in the number of circulating and thymus homing T-cells [26].

In addition, the number of T- and B-cells in the neonates of diabetic mothers was significantly decreased compared to the neonates of healthy mothers [28]. Lapolla et al. [29] reported an increase in the number of lymphocytes but a decrease in natural killer (NK) subset in children from GDM mothers. Another alteration in lymphocyte subset pattern is observed in GDM mothers, who had high number of $CD8^+$ cells, expressing TCR gamma/delta, and low number of $CD3^+$ cells, expressing TCR alpha/beta. Also, infants born to GDM women had higher $CD8^+$ gamma/delta cells than control babies [21]. This immunological imbalance may correlate with a greater risk for developing T1D, later in life [21].

T helper (Th) dichotomy in GDM and macrosomia has not yet been well explored. On the basis of production of cytokines, Th cells can be classified into two principal populations, Th1 and Th2 (Figure 1). Th1 cells support cell-mediated immunity and, as a consequence, promote inflammation, cytotoxicity, and delayed-type hypersensitivity, whereas Th2 cells support humoral immunity and downregulate

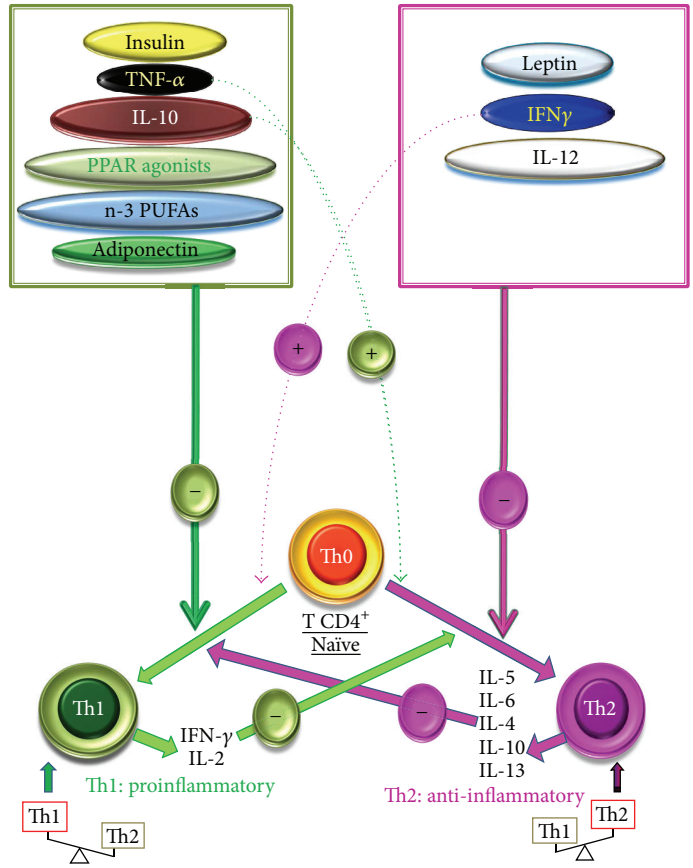

FIGURE 1: Differentiation of Th0 cells into Th1 and Th2 cells and their modulation. The secretion of their respective cytokines identifies the cells. Th0 cells, which principally secrete IL-2 along with other some cytokines, are differentiated either into Th1, under the action of IL-12 and IFN-γ, released by the macrophages and NK cells (natural killer), respectively, or into Th2 phenotype by the action of IL-4 produced by the mastocytes. The IFN-γ and IL-10 exert an inhibitory effect on the differentiation of Th1 and Th2 phenotypes, respectively. Insulin, PPAR agonists, and n-3 PUFAs promote the differentiation into Th2 phenotype. Leptin promotes the differentiation into Th1 cells. (+) inducing effect; (−) inhibitory effect.

the inflammatory actions of Th1 cells [30]. Th1 cells secrete IL-2, IFN-α, and TNF-β while Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 [31]. Analysis of T-cell markers in placenta showed an increase in T-cell infiltration that expresses GATA3, a marker of Th2 phenotype, in placenta of GDM women [32].

Concerning experimental models of GDM, we would like to mention that we have developed a model by administrating streptozotocin (STZ) to Wistar female rats [2, 9]. The rate of success in obtaining macrosomic pups was 75%. We confirmed in this model a decrease in Th1 cells, as observed in human GDM [33]. Furthermore, in GDM rat, the decrease in circulating IFN-γ was accompanied with an increase in IL-10 (Th2 marker) levels, as compared to control rats [34, 35]. This upregulation of Th2 phenotype in pregnancy is normalized after the delivery [34]. In fact, the shift from the Th1 phenotype to the Th2, during pregnancy, has been shown to encourage vigorous production of antibodies which not only combat infections during pregnancy, but also offer passive immunity to foetus [36]. A low Th1 profile in diabetic pregnant rats, associated with successful pregnancy, may also result from the elevated levels of reproductive hormones like human chorionic gonadotrophin (hCG) hormone, whose administration is known to diminish the production of the Th1 cytokines [37].

In rats, the upregulated Th1 profile in macrosomic animals may be due to difference in physiological status between GDM dames and their offspring [38]. A study conducted on Tunisian women with GDM and their macrosomic babies corroborates these experimental observations [39]. Indeed, the comparison of Th1/Th2 ratio showed an increase in the Th2 phenotype in GDM mothers, whereas an increase in Th1 phenotype was observed in macrosomic infants [39].

The regulatory T (T-reg) cells represent a specialized population of T-cells ($CD4^+CD25^+$), known for their properties as potent suppressors of inflammatory responses and for their ability to mediate immune tolerance. T-reg cells induce immune tolerance throughout the production of two immunosuppressive cytokines: TGF-β and IL-10 [40]. Both in humans [41] and in mice [42], T-regs cells increase very early in pregnancy, a period which coincides with an intense vascular activity [43]. The importance of T-reg cells in

the success of pregnancy was demonstrated by Aluvihare et al. [44] who reported that adoptive transfer of T-lymphocytes depleted of T-regs cells into pregnant T-cell-deficient mice led to the rejection of allogeneic foetal units. Furthermore, spontaneous abortion cases and patients with recurrent miscarriage are associated with lower systemic T-reg cells compared to normal pregnancies [45]. In Kuwaiti women, high number of T-cells expressing the activation-associated HLA antigen ($CD4^+$HLA-DR), memory T-cells, and T-reg cells have been observed during GDM [46, 47].

The frequency of T-reg cells is significantly higher in children born to T1D mothers than in those born to GDM or normal women [48, 49]. Indeed, in the case of T1D, the maternal autoimmunity and the transplacental passage of auto-GAD antibodies may influence the generation and expansion of foetal T-reg cells, which may suppress the GAD65-specific T-cell responses [48]. Besides, the T-reg cells of children born to T1D mothers exhibit a more pronounced memory phenotype (increased CCR4 expression and downregulation of CD62L), suggesting an early activation of the foetal immune system, as a consequence of maternal autoimmunity [48]. It seems that the suppressive activity of T-reg cells was significantly reduced in GDM patients when compared to healthy pregnancy [50].

It is noteworthy that obesity-induced insulin resistance is associated with the development of a specialized T-reg population in visceral adipose tissue, called "VAT resident T-reg" [51]. Visceral adipose inflammation and insulin resistance have been associated with a dramatic reduction in VAT T-reg cells in several animal models of obesity. T-reg cells, by secreting IL-10, decrease the inflammatory state of adipose tissue and, thereby, improve insulin resistance [52]. Loss-of-function and gain-of-function experiments demonstrated that VAT T-reg cells are indispensable to reducing inflammation and increasing insulin sensitivity [52]. Hence, the implication of VAT resident T-reg cells deserves deep investigations in macrosomia.

4. T-Cells Present a Defect in Calcium Signaling in Diabetic Pregnancy and Macrosomia

During T-cell activation, an increase in intracellular free calcium concentrations, $[Ca^{2+}]$i, is one of the earliest events which is triggered as a result of the hydrolysis of phosphatidylinositol-bisphosphate, catalyzed by the phospholipase C (PLC). Hence, PLC gives rise to inositol trisphosphate, which recruits calcium from endoplasmic reticulum pool, and diacylglycerol which activates the protein kinase C. According to capacitive model of calcium entry, first calcium is released *via* T-cell receptor (TCR) activation from the endoplasmic reticulum (ER) and then it is extruded into the extracellular medium. In turn, the cells refill their intracellular emptied pool by opening calcium channels [49]. Ionomycin opens calcium channels, leading to calcium influx from extracellular medium and thapsigargin (TG) recruits calcium which belongs to endoplasmic reticulum (ER) pool. Interestingly, ionomycin-induced increases in $[Ca^{2+}]$i in T-cells of GDM dames and their macrosomic offspring were greater than those in control rats [27]. In 0% of calcium buffer, TG induces increases in $[Ca^{2+}]$i exclusively from ER pool and no influx occurs in the absence of calcium from the extracellular medium [53]. Hence, both in 100% and in 0% calcium media, TG-induced increases in $[Ca^{2+}]$i in T-cells are higher in GDM dames and macrosomic rats than those in control animals [27], demonstrating that T-cell calcium signaling is altered in these two pathological situations.

5. Proinflammatory Adipokines and Cytokines in GDM and Macrosomia

TNF-α and IL-6 represent the main inflammatory cytokines increased in the insulin-resistant states of obesity and T2D [26, 54]. Increasing evidence suggests that GDM is a proinflammatory state similarly to T2D. Monocyte chemotactic protein-1 (MCP-1) is known to be elevated in inflammatory diseases like arthritis and lupus [55]. The elevation of MCP-1 in the third trimester of GDM suggests an association between inflammation and GDM [56]. Besides, it has been suggested that hyperglycaemia and its related oxidative stress are usually associated with increased proinflammatory cytokines production [57, 58].

Increased concentrations of TNF-α and IL-6 might not only diminish insulin sensitivity by suppressing insulin signal transduction, but also interfere with the anti-inflammatory effect of insulin (Figure 2) [54, 59]. Indeed, insulin exerts its anti-inflammatory effect by decreasing the production of reactive oxygen species (ROS) from mononuclear cells and nuclear NF-κB translocation [54]. Furthermore, insulin decreases the concentration of MCP-1, PAI-1, and EGR-1 [60]. The *in vivo* administration of insulin not only decreases the severity of T2D, but also diminishes the levels of MCP-1 and C-reactive protein (CRP), the two indicators of the inflammatory state [61]. IL-6 promotes insulin resistance in liver cells [62] and negatively regulates insulin signaling and glucose metabolism in adipocytes [63]. TNF-α inhibits tyrosine phosphorylation of insulin receptor and, thereby, insulin signaling [64]. It has been suggested that the increase in TNF-α and IL-6 in diabetic conditions might be a result of oxidative stress and inflammatory changes caused by hyperglycaemia [65]. IL-6 and TNF-α are mainly produced by adipose tissues. Indeed, during insulin-resistant state, adipocytes secrete MCP-1 which favors the infiltration of macrophages that, consequently, produce IL-6 and TNF-α in high quantities (Figure 2) [66, 67]. We have reported that TNF-α and IL-6 are increased in GDM women [39]. During pregnancy, IL-6 secretion has been proposed to aggravate insulin resistance and participates in the pathogenesis of GDM [59].

Adipocytes secrete a number of molecules, including adiponectin, leptin, and resistin, that modulate peripheral insulin sensitivity [66]. From the immunological point of view, adiponectin exhibits anti-inflammatory properties [66] and leptin polarizes Th cytokine production toward a proinflammatory (Th1) phenotype (Figure 1) [68]. Since adipocytokines may play an important role in the early defects of T2D [69], women with GDM represent an ideal population

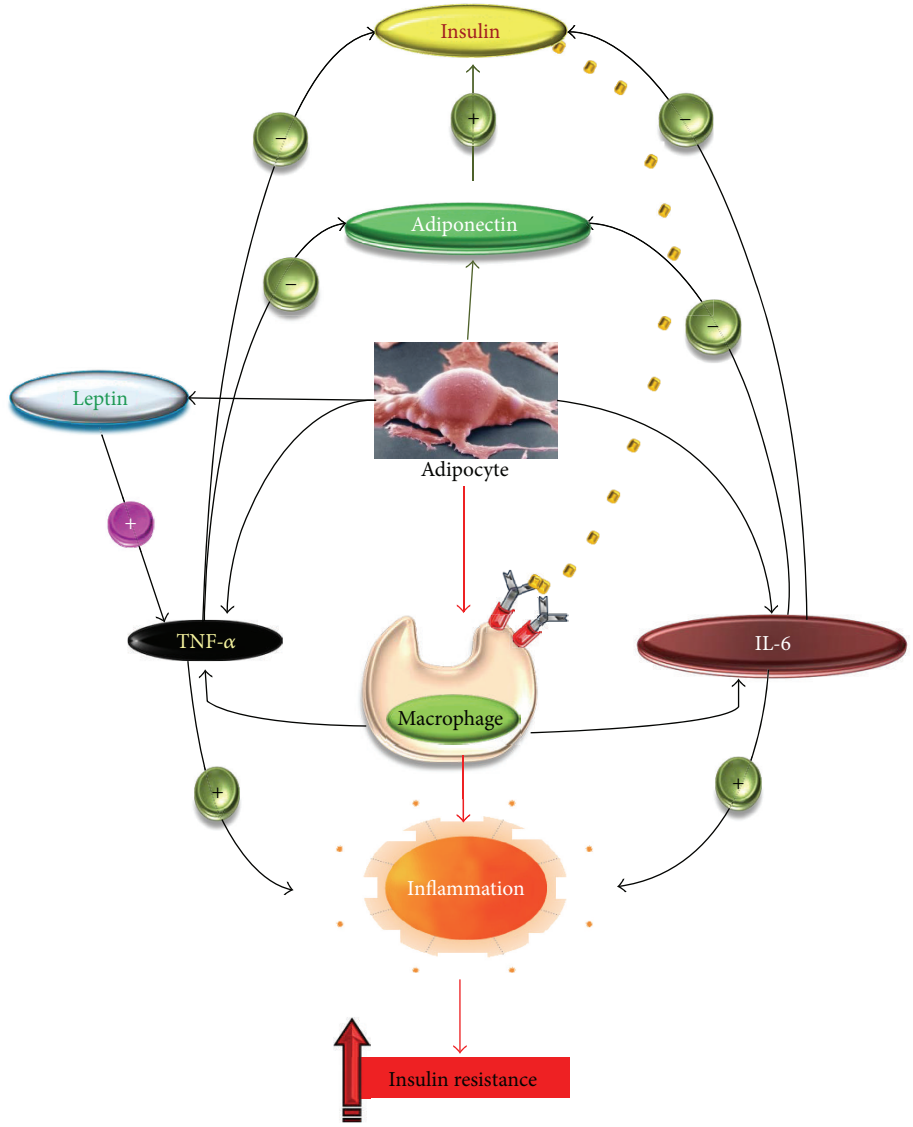

Figure 2: Secretion of cytokines and adipokines and their implications in insulin resistance. The adipocytes secrete adipokines (leptin and adiponectin). Proinflammatory cytokines released by macrophages. Leptin contributes to inflammation by increasing the secretion of TNF-α. Both TNF-α and IL-6 antagonize the action of insulin and decrease the secretion of adiponectin which exerts insulin-sensitizing action. (+) inducing effect; (−) inhibitory effect.

model for studying this interrelationship [70]. Leptin is also produced by placenta and involved in weight regulation and lipid metabolism. Contradictory results have been reported on its secretion in GDM. Depending on studies, elevated [71], constant [72], or decreased [73] levels of leptin have been observed in GDM women. Hence, in contrast to obesity which leads to an inflammatory Th1 state, leptin does not play an important role in GDM, marked with Th2 response, probably because of the hormonal status during pregnancy. Besides hyperglycaemia, chronic foetal hypoxia, detected in GDM, may also increase the inflammatory burden incurred by the foetus [74].

6. n-3 PUFAs Exert Beneficial Effects in Macrosomia

Dietary n-3 PUFAs have been considered as immunosuppressors and, therefore, are used in the management of a number of inflammatory and autoimmune diseases, including rheumatoid arthritis and multiple sclerosis [75, 76], as these pathologies are characterized by the presence of activated T-cells and inflammatory cytokines either at the site of tissue injury [77, 78] or in blood circulation [79, 80]. Fat-1 transgenic mice, known to convert endogenous n-6 PUFAs to n-3 PUFAs, were protected from diabetes, because of low

concentrations of TNF-α and IL-1β [81]. Generally, both in animal models and in humans, n-3 PUFAs decrease TNF-α and IL-6 production [82]. n-3 PUFAs exert their effect on the inflammatory gene expression through the inhibition of intracellular signaling pathways that lead to NF-κB activation [83].

n-3 PUFAs have been shown to suppress mitogen-stimulated proliferation of lymphocytes isolated from lymph nodes [84], spleen [85], and lymphatic duct [86], in mice and human beings [75, 85]. Feeding the n-3 PUFA-diet corrected intracellular calcium homeostasis in T-cells of diabetic pregnant dames and their macrosomic obese rats [27]. We have assessed the Th1/Th2 dichotomy by dietary n-3 PUFAs in diabetic pregnancy and macrosomia. We observed that the n-3 PUFAs-diet upregulated the Th2 profile in GDM rats. In macrosomic offspring, the Th1 phenotype is upregulated and an n-3 PUFAs-diet downregulated this phenomenon [33]. In agreement with our finding, Wallace et al. [76] have also observed that feeding fish oil to mice induced a shift in the IFN-α/IL-4 ratio, by a factor of four, as compared to animals fed the low fat diets. n-3 PUFAs also regulated T-reg functioning. We have investigated the molecular mechanisms by which n-3 PUFAs-diet controls T-reg cell suppressive capacity. We used docosahexaenoic acid (DHA), the end product of α-linolenic acid metabolism in animal tissues, and observed that the exposure of T-reg cells to this fatty acid or its *in vivo* supplementation upregulated TGF-β but downregulated IL-10 in these cells, suggesting that DHA might be orienting the T-reg cell differentiation toward a Th3 phenotype [87]. Th3 phenotypes that infiltrate decidua are known to prevent abortion and contribute to the success of pregnancy [34].

Furthermore, DHA diminished the suppressive activity of T-reg cells on effector T-cell proliferation. It is now becoming clear that the interaction of Foxp3 with other transcription factors (like NAFT or Runx-1) or histone deacetyltransferase and class II histone deacetylase is critical for the repression of the transcription of IL-2 gene by Foxp3 [88]. Hence, we hypothesized that DHA might downregulate T-reg cell activity, by interfering with the critical downstream components of the Foxp3-driven suppressor pathway. Furthermore, DHA reduced the migration of T-reg cells toward chemokines by downregulating the expression of chemokines receptors (CCR-4 and CXCR-4) in these cells [87].

T-reg cell migration and activity have been found to be associated with mitogen-activated protein kinase (MAPK), that is, ERK1/2, activation [89]. Besides ERK1/2, the phosphatidylinositol-3-kinase (PI3K) and Akt/protein kinase B (hence referred to as Akt) play a critical role in the T-cell survival, expansion, and differentiation [90]. ERK1/2 and Akt phosphorylation controls the expression of $p27^{KIP1}$, an inhibitor of cyclinE/cyclinD kinase 2 that regulates cell cycle [91]. We noticed that DHA significantly diminished the MAPK phosphorylation in activated T-reg cells, and this phenomenon was associated with an increase of $p27^{KIP1}$ in T-reg cells. Hence, DHA seems to reinforce the anergic state of T-reg cells [87].

Concerning the molecular mechanism of action of n-3 PUFAs, we have previously shown that dietary n-3 PUFAs are incorporated into plasma membrane phospholipids [92]. Hence, we assume that dietary n-3 PUFAs may exert their beneficial action by modulating cell signaling. We have recently shown that T-cell activation and T-cell calcium signaling are altered in diabetic pregnancy and macrosomia, and dietary fish oils, particularly eicosapentaenoic acid (EPA) and DHA, restore these T-cell abnormalities [93]. During cell activation, a modification in the intracellular pH also plays an important role in the cell cycle progression and, hence, DHA and EPA have been shown to modulate this phenomenon. Dietary n-3 PUFAs, incorporated into plasma membrane, may also give rise to diacylglycerols which, in turn, may modulate cell activation. It has been shown that diacylglycerols, containing EPA and DHA, modulate PKC activation [94], calcium signaling [95], and ERK1/ERK2 phosphorylation [96].

7. Conclusion

The incidence of GDM and macrosomia continues to grow worldwide and represents a major public health challenge. Except for genetic factors, physical inactivity and high caloric food are the major causing factors for these pathologies. Based on clinical studies, the *Dietary Guidelines for Americans 2005* report [97] and several international and professional organizations [98] have made recommendations for consumption of at least two meals, containing fish, per week or from 0.250 g to 1 g of EPA and DHA daily with a 5 : 1 ratio of n-6 fatty acid/n-3 fatty acid. There is no doubt concerning the beneficial effects of n-3 PUFAs in the improvements of hypertriglyceridemia and the reduction of cardiovascular risk [99]. Thus, the use of these fatty acids in combination with genuine drugs (lipid-lowering, anti-inflammatory, etc.) represents a new therapeutic strategy in fighting against diabetes and obesity.

Abbreviations

Th:	T helper
TNF-α:	The tumor necrosis factor-α
IL-6:	Interleukin-6
PPARα:	Peroxisome proliferator-activated receptor alpha
n-3 PUFAs:	n-3 polyunsaturated fatty acids
GDM:	Gestational diabetes mellitus
RDS:	Respiratory distress syndrome
VLDL:	Very-low-density lipoprotein
LDL:	Low-density lipoprotein
apoB100:	Apolipoprotein B 100
DHA:	Docosahexaenoic acid
EPA:	Eicosapentaenoic acid
PMNs:	Polymorphonuclear neutrophils
NK cells:	Natural killer cells
T1D:	Type I diabetic
Anti-IA2:	Islet Cell Autoantigen 512 Antibodies
IDDM:	Insulin-dependent diabetes mellitus
TCR alpha/beta:	Human T-cell receptor
STZ:	Streptozotocin
IFN-α:	Interferon alpha
Foxp3:	Forkhead box P3

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4
CCR4: C-C chemokine receptor type 4
PLC: Phospholipase C
TCR: T-cell receptor
MCP-1: Monocyte chemotactic protein-1
PAI-1: Plasminogen activator inhibitor
EGR-1: Early growth response protein-1
Runx-1: Runt-related transcription factor 1
ERK1/2: Extracellular signal-regulated kinases 1/2
PI3K: Phosphatidylinositol-3-kinase.

Competing Interests

All of the authors have nothing to declare as far as the conflict of interests is concerned.

Acknowledgments

The authors express their sincere thanks to Mrs. EVA Pop Askri for English language corrections.

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 2917065, 7 pages
http://dx.doi.org/10.1155/2016/2917065

Research Article

Active Holistic Surveillance: The Nutritional Aspect of Delayed Intervention in Prostate Cancer

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Received 24 December 2015; Accepted 7 April 2016

Academic Editor: Aziz Hichami

Purpose. Active surveillance is an emergent strategy for management of indolent prostate cancer. Our institution's watchful waiting protocol, Active Holistic Surveillance (AHS), implements close monitoring for disease progression along with various chemopreventive agents and attempts to reduce unnecessary biopsies. Our objective is to report on the treatment rates of men on our AHS protocol as well as determine reasons for progression. *Materials/Methods*. Low risk and low-intermediate risk patients were enrolled in AHS at Winthrop University Hospital between February 2002 and August 2015. Our IRB-approved study analyzed survival rate, discontinuation rates, and definitive treatments for patients in our AHS cohort. *Results*. 235 patients met inclusion criteria. Median age and follow-up for the cohort were 66 (44–88) years and 42 (3–166) months, respectively. The overall survival for the cohort was 99.6% and the disease specific survival was 100%. A total of 27 (11.5%) patients discontinued AHS. *Conclusion*. The incorporation of chemopreventive agents in our AHS protocol has allowed patients to prolong definitive treatment for many years. Longer follow-up and additional studies are necessary to further validate the effectiveness of AHS.

1. Introduction

Prostate cancer is the second most commonly diagnosed cancer amongst American men [1]. In combination with an aging population, the development of the PSA screening test resulting in early detection has driven the increase in prostate cancer incidence. Nevertheless, treatment of low risk prostate cancer remains unclear. As of July 2008, Cancer of the Prostate Strategic Urologic Research Endeavor (CaPSURE) reported that 95% of prostate cancer patients were receiving definitive treatment, even with most patients having low-grade disease [2]. Definitive treatment causes several side effects for patients, including incontinence, erectile dysfunction, and bowel complications [3]. However, many of these early detected cancers tend to be more indolent than life threatening, and often treatment of this nonlethal disease represents overtreatment. Clinicians thus found it necessary to develop an approach that embraced the concept of watchful waiting for low risk patients to prolong and possibly avoid definitive treatment [4].

This approach, known as active surveillance, is an emerging strategy for management of favorable-risk, localized prostate cancer. The active surveillance regimens that are commonly used involve closely monitoring low risk prostate cancer patients for any sign of disease progression with serial PSA tests, biopsies, and/or MRIs. The goal of any active surveillance program is to try and avoid or delay the side effects of treatment but also to maintain the option of delayed intervention.

At our institution, we have a large cohort of patients on an active surveillance protocol which uses a novel approach of diet and supplements. The protocol, called Active Holistic Surveillance (AHS), involves regular follow-up exams with PSA testing, review of symptoms, digital rectal examinations, and annual MRIs. The protocol does not have routine subsequent prostate biopsies unless there is a rapid rise in PSA or a change in MRI and/or DRE. This serves to decrease the side effects and discomfort of biopsy while attempting to still capture cancer progression before the window of cure is closed.

Epidemiological research has demonstrated that nutrition and lifestyle factors play a pivotal role in the initiation and progression of prostate cancer. However, chemopreventive agents such as nutrients, herbs, and dietary factors have been shown to reduce the aggressiveness of the disease. Implementing these chemopreventive agents into a treatment regimen may prevent progression and occurrence of prostate cancer. In addition to management of prostate cancer, these chemopreventive agents provide defense against various other malignancies and promote the overall health of the patient [5, 6].

This study is looking to determine the definitive treatment rates of the patients on our institution's AHS protocol and look at the reasons for progression. In addition, we also compare our rates of conversion to definitive therapy to other large series in the literature.

Diet's Role in Prostate Cancer Progression

Meat. The correlation between increased red meat consumption and increased risk for prostate cancer has been extensively studied. An internationally conducted epidemiological study revealed a positive correlation between prostate cancer death and fat consumption, specifically in fats obtained from meats and dairy [7, 16]. While the exact reason for the association with red meat and prostate cancer is unknown, studies suggest grilling or barbecuing meats at high temperatures can form heterocyclic amines and polycyclic aromatic hydrocarbons, both of which have carcinogenic properties in animal models [6].

Dairy Products. There is limited data on the relationship between dairy intake and prostate cancer. However, a high intake of saturated and trans fat often found in dairy products is positively correlated with incidence and mortality from prostate cancer [27]. One epidemiological study of 41 countries found milk to be the food most closely related to prostate cancer incidence [28]. While the exact mechanism by which fat induces tumorigenesis is not yet known, possible explanations include fat's effects on serum androgen levels which affect tumor growth. In addition, dairy products contain a significant amount of calcium. In high doses, calcium inhibits the formation of 1,25-dihydroxyvitamin D3, the active form of vitamin D which inhibits the proliferation of cancerous prostatic cells.

Soy. Epidemiological studies report a markedly lower incidence of prostate cancer in Asian countries, which may be attributable to the prevalence of soy based foods in a traditionally Asian diet [6]. Soy products contain numerous phytoestrogens. However, the predominant phytoestrogen comprising soy is genistein. In rat models, genistein has been proven to inhibit the growth and metastasis of prostatic cell carcinomas [29]. In recent years, the incidence rate of prostate cancer in Asian countries has risen, which may be due to the adaptation of western diets into the Asian lifestyle.

Vegetables. Studies comparing correlations between food groups and cancer mortality rates found a negative correlation between prostate cancer mortality and the consumption of vegetables [7, 8]. Vegetables are densely packed with antioxidants, which neutralize the effect of heterocyclic amines found in meats [6]. Cruciferous vegetables, which include broccoli, spinach, kale, and cauliflower, also contain glucosinolates. When digested, glucosinolates release numerous phytochemicals which induce the apoptosis of prostate cancer cells and prohibit these cells from proliferating. In addition, glucosinolates function to protect somatic DNA from carcinogenic damage.

Green Tea. Green tea contains epigallocatechin gallate (EGCG), an extensively studied catechin known for its anticarcinogenic and antioxidant properties. Like those found in vegetables, EGCG removes carcinogenic free radicals from the body. In addition, green tea exhibits anti-inflammatory properties, contributing to its value as a chemopreventive agent. Numerous cell culture model studies and animal studies have shown green tea's ability to attenuate development, progression, and metastasis of prostate cancer by inducing apoptosis and cell-growth inhibition [30]. Green tea's chemopreventive properties may be attributed to the low incidence of prostate cancer in Japanese and Chinese populations [31].

2. Methods

2.1. Cohort Enrollment. Between February of 2002 and August 2015, patients with low risk and low-intermediate risk prostate cancer have been enrolled in AHS at Winthrop University Hospital. This study has been approved by the Institutional Review Board.

Criteria for enrollment into AHS include the following: Gleason 6 (3 + 3), low volume Gleason 7 (3 + 4), nonpalpable disease on exam, stage T1 or T2, and no evidence of extracapsular extension on mpMRI. Exceptions were made for men with Gleason 7 (4 + 3) depending on significant comorbidities, shorter life expectancy, and other favorable disease characteristics (PSA, MRI). Exclusion criteria for AHS include men with serum PSA values >10, greater than 4 of 12 cores positive for cancer with tumor volume >50% in each core.

2.2. Holistic Regimen. Dietary recommendations include eliminating red meats, dairy products, fried foods, and refined carbohydrates from the patient's everyday diet. Our protocol emphasizes consuming poultry, fish, green tea, soy milk, red wine, and flaxseed in place of carcinogenic foods. In addition, patients are encouraged to add more fresh vegetables to their everyday diet, with an emphasis on cruciferous vegetables. AHS protocol replaces all cow milk with soy milk. At each visit, patients were reminded about the protocol and were asked if they were compliant with the diet and supplements as outlined in Table 1.

In addition, AHS patients were taking the following chemopreventive supplements to their diet (Table 1). Our institution has extensive first-hand experience with these particular supplements and thus recommends them to our patients as they have proven to be safe and effective. However, the supplements listed herein may be substituted with other

Table 1: Active Holistic Surveillance supplements.

Supplement	Directions	Rationale	Citations
BroccoProtect	3 capsules, daily	(i) Rich in glucosinolates and antioxidants (ii) Neutralizes the effect of heterocyclic amines found in meats (iii) Induces the apoptosis of prostate cancer cells and prohibits these cells from proliferating (iv) Protects somatic DNA from carcinogenic damage	[6–8]
Omega 3	2000 mg, daily	(i) Suppresses inflammation (ii) Inhibits growth factor induced proliferation in prostate cancer cells	[9, 10]
Zyflamend	3 capsules, daily	(i) Suppresses inflammation (ii) Reduces growth of prostate cell lines (iii) Induces apoptosis of prostate cancer cells	[11, 12]
Vitamin D3	5000 IU, daily	(i) Promotes differentiation of prostate cancer cells (ii) Induces apoptosis of prostate cancer cells (iii) Attenuates proliferation	[13–15]
Genikinoko (GCP)	1000 mg, twice daily	(i) Rich in genistein (ii) Causes cell cycle growth arrest (iii) Induces apoptosis of prostate cancer cells (iv) Antiangiogenesis properties in vitro and in vivo (v) Reduces serum PSA	[6, 16–19]
Active Hexose Correlated Compound (AHCC)	3 capsules, daily	(i) Boosts host immunity (ii) Protects against disorders induced by oxidative stress (iii) Reduces serum PSA	[20–24]
Lycocell	2 capsules, daily	(i) Lycopene complex (ii) Antioxidants protect DNA from damage (iii) Causes prostate cancer cell cycle arrest and apoptosis (iv) Induces differentiation (v) Reduces oxidative stress	[25, 26]

chemopreventive agents that have been proven to elicit the same host response.

2.3. Follow-Up. Our Active Holistic follow-up protocol includes a PSA every three months, digital rectal examination, and an annual mpMRI scan. If PSA doubling time (PSADT) was less than 12 months, repeat PSA testing was required to ensure accuracy. Criteria for progression on mpMRI includes extracapsular extension, development of a new focus of tumor, or an enlargement of an already existing tumor. Rise in PSA (PSADT < 12 months), unfavorable genomics result, and progression on mpMRI all warrant a biopsy confirmation or an opportunity for definitive treatment, depending on patient preference. If a confirmation biopsy is performed, Gleason upgrading to (3 + 4 or 4 + 3) and an increase of volume of the original tumor (involvement of more cores) are all indications for definitive treatment. Patient preference was an additional indication for obtaining definitive treatment.

2.4. Statistical Methods. Demographic data was obtained including: median age, follow-up, and initial PSA. Patients were stratified by Gleason score into Gleason 6 (3 + 3), Gleason 7 (3 + 4), and Gleason 7 (4 + 3). Average PSA over time for the cohort was calculated. The primary outcomes were overall and cancer specific survival and cessation of surveillance and initiation of definitive treatment. Kaplan-Meier estimates were generated using R-Project. The secondary outcomes included the reason for discontinuation and definitive treatments following AHS.

3. Results

235 patients met inclusion criteria. The distribution of age, follow-up, pre-Gleason score, and initial PSA are summarized in Table 2. Median age and follow-up for the cohort were 66 (44–88) years and 42 (3–166) months, respectively. On initial biopsy prior to AHS, 178 patients (76%) were found to have a Gleason score of 6 (3 + 3). 35 (14.9%) patients had a pre-AHS biopsy with Gleason score of 7 with 29 (12.3%) Gleason 3 + 4 and 6 (2.6%) having a Gleason score of 4 + 3, respectively. The median baseline PSA for patients enrolled in AHS was 4.1 ng/mL. Figure 1 shows the change in average PSA for the AHS cohort during surveillance.

The overall and prostate cancer specific survival for the cohort was 99.6% and 100% at a median follow-up of 42 months (range 3–166) (Figure 2).

At last analysis (December 2015), 27 patients (11.5%) received definitive treatment. Table 3 summarizes the rate of discontinuing AHS per year. The overall dropout rate was 11.5%. The probability of remaining on AHS was 94%, 82%, and 67%, at 2 years, 5 years, and 10 years, respectively (Figure 3(a)).

Table 4 evaluates the 27 patients who discontinued AHS. The median age of these men was higher than the overall

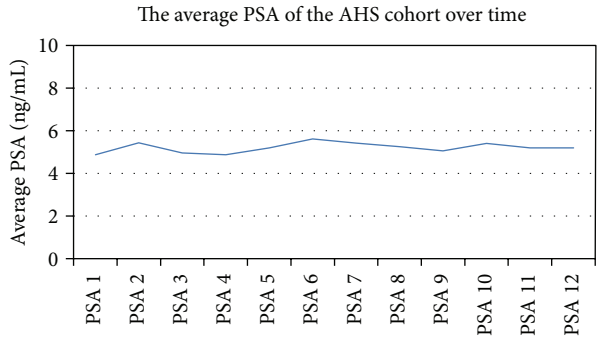

Figure 1: The average PSA of the total cohort while on AHS.

Table 2: Patient demographics ($N = 235$).

Age, median (range), years	66 (44–88)
Time on AHS, median (range), months	42 (3–166)
Gleason score	
6	178
7 (3 + 4)	29
7 (4 + 3)	6
N/R	22
Initial PSA, median (range), ng/mL	4.1 (0.5–15.6)

Table 3: The discontinuation rate for patients on AHS.

Year	Started AHS (n)	Total on AHS	Off AHS (n)	Rate off AHS
Before 2010	32	32	0	0.00%
2010	25	57	0	0.00%
2011	30	87	0	0.00%
2012	61	148	0	0.00%
2013	35	175	8	4.57%
2014	36	199	12	6.03%
2015	16	209	7	3.35%
Overall	235		27	11.50%

Table 4: Demographics of patients who discontinued AHS ($N = 27$).

Age, median (range), years	70 (57–80)
Time on AHS, median (range), months	30 (4–110)
Gleason score	
6	18
7 (3 + 4)	6
7 (4 + 3)	1
N/R	2
Initial PSA, median (range), ng/mL	5 (0.8–15.6)

cohort (70 years). 26% of these patients had a Gleason of 7 (both 3 + 4 and 4 + 3) compared to only 16% of the overall cohort. The initial median PSA, 5 ng/mL, was also higher than the overall cohort's. The reasons that patients discontinued AHS are found in Table 5. MRI progression with biopsy confirmation was the most common reason (40.47%). MRI

Table 5: Reason for intervention on AHS.

Reasons for discontinuing AHS	N (%)
Biopsy progression	4 (14.81)
MRI progression	8 (29.63)
MRI progression confirmed with biopsy	11 (40.74)
Patient preference	3 (11.11)
Deceased	1 (3.70)
Total	27 (100)

Table 6: Treatment options of the patients that discontinued ($N = 26$).

Cryotherapy	15
CyberKnife	8
Radical prostatectomy	1
ADT	1
N/A	1

progression without a biopsy and biopsy progression without an MRI were the next likely reasons patients discontinued AHS with 14.81% and 11.11%. Three patients (1.3%) left the protocol because of preferences which usually was a result of patient anxiety and/or fluctuations in PSA values. One patient died of an unrelated illness. A Kaplan-Meier curve illustrates the probability of patients to continue AHS stratified by these reasons shown on Figure 3(b).

Of the patients who opted for definitive intervention 58% had cryotherapy, 30% had CyberKnife, and 1 patient had a radical prostatectomy, shown in Table 6. In addition, 1 patient elected for androgen deprivation therapy.

4. Discussion

Today, the number of patients who are choosing to go on active surveillance rather than undergo definitive treatment is growing. In addition, there is increasing physicians' support of actively watching patients. As a result, numerous physicians and centers across the country have developed an active surveillance regimen. The AHS regimen differs greatly from other active surveillance protocols in the implementation of lifestyle changes, including the alteration of diet and the addition of supplements and physical activity. The dietary portion of the protocol adopts an anti-inflammatory, antioxidant chemopreventive approach where the patient reduces the factors that may contribute to chronic inflammation. Reduction of saturated and trans fats found in animal foods is implemented in the diet in order to reduce the risk of prostate cancer. Other bases of the holistic protocol are the addition of soy, flaxseed, AHCC, vitamin D3, and Omega 3 Fish Oil.

The ideal candidate for AHS at our institution matches that of many other active surveillance protocols that is characterized by a patient with low to intermediate prostate cancer. While early literature shown active surveillance to be promising for Gleason ≤6, recent studies have broadened the inclusion criteria [32]. In an active surveillance study performed at the University of Toronto, the cohort had successful overall outcomes with 25% of the patients classified

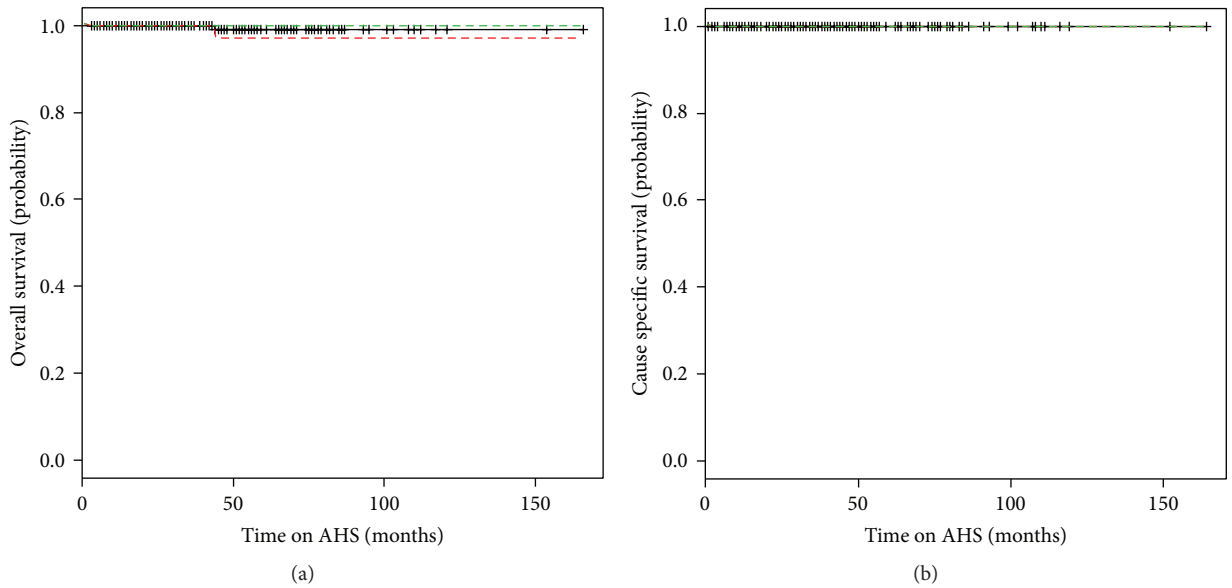

Figure 2: Kaplan-Meier survival curves. (a) The overall survival of the AHS cohort. (b) The disease specific survival of the AHS cohort (key: solid black line represents the probability; red and green lines represent the standard deviation of the curve).

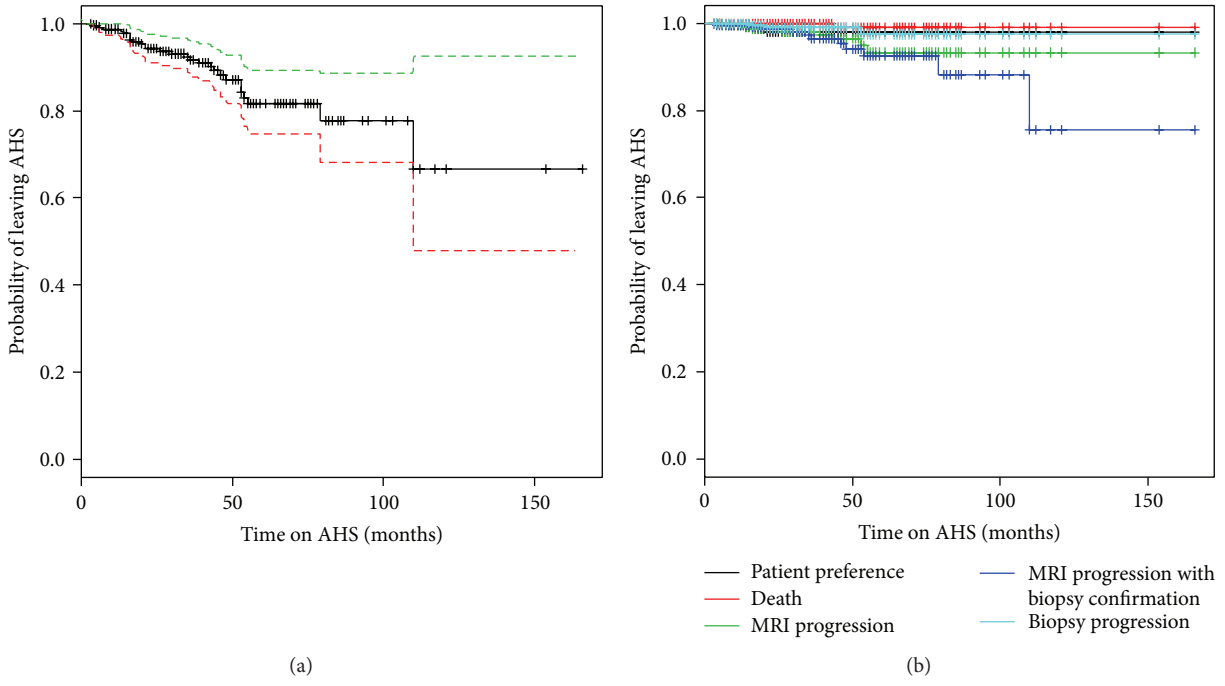

Figure 3: Kaplan-Meier survival curves. (a) The overall likelihood of patients remaining on AHS cohort (key: solid black line represents the probability; red and green lines represent the standard deviation of the curve). (b) The likelihood of patients remaining on AHS stratified by the reasons for intervention.

as intermediate risk and the remaining patients had low risk characteristics [4]. The majority of the AHS patients, 76%, would be classified as low risk according to the D'Amico risk groups, and the 24% would be considered intermediate risk. These numbers are comparable to the risk distribution at other institutions.

It has been reported that active surveillance is safe and nonlethal at duration of up to 15 years [4]. In a long term study of an active surveillance cohort of 993 patients with a median follow-up of 6.4 years (with a range of 0.2–19.8 years), the overall survival in the cohort was 80% at 10 years [32]. The overall survival of the AHS cohort is comparable if not better than the literature values at 99%. Similarly, the cause specific survival reported in previous literature has ranged from 0% to 1.8% [4, 32]. In the AHS cohort, no patients have died from prostate cancer. Thus, this study supports the results

of previous studies in that low to intermediate risk prostate cancer patients are the ideal candidates for active surveillance.

Overall, 11% of AHS patients went on to receive definitive treatment with a median follow-up of 3.5 years (with a range of 0.3–13.8 years). This is lower than both the Toronto and Hopkins groups, where 27% and 33.3% of their patients have been treated definitively with a median follow-up of 6.4 and 6.5 years [4, 32]. Despite the fact that these studies have a longer follow-up, our average dropout rate per year, 2.35%, is lower than the 8.8% rate previously reported [33]. Recent analysis from Klotz et al. published that 75.7%, 63.5%, and 55.0% of patients remained untreated and on surveillance at 5, 10, and 15 years, respectively [4]. Another study performed at the University of California San Francisco showed 2- and 5-year continuation on active surveillance to be 85% and 67%, respectively [34]. The AHS 2-year, 5-year, and 10-year continuation rates are 94%, 82%, and 67%, respectively. Thus, with 3.5-year follow-up, patients in the AHS cohort are more likely to remain on surveillance than previously reported.

There are limitations to this study. One is that there are many factors that go into AHS including patient selection, lifestyle changes, and means of follow-up. This makes it difficult to measure the impact that diet and lifestyle changes have on the low rates of progression seen in the AHS patients. A univariate and multivariate analysis should be performed in future studies. Furthermore, another major limitation of this study is the inability to measure patients' compliance to these recommended diet and lifestyle changes. Aside from patient confirmation there is no means to measure to what degree they have made these changes. Lastly, long follow-up analysis should be performed on the patients who discontinued AHS to establish rates of recurrence and metastasis.

5. Conclusion

In our cohort of patients that we selected for surveillance, the incorporation of evidence-based diet and supplements allowed men to stay on the protocol for many years, preventing the need for definitive therapy. Longer follow-up and perhaps a randomized trial comparing AS to AHS should be done to in the future.

Disclosure

The authors are solely responsible for the writing of this paper.

Competing Interests

The authors report no competing interests.

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 1373060, 13 pages
http://dx.doi.org/10.1155/2016/1373060

Clinical Study

Enteral Glutamine Administration in Critically Ill Nonseptic Patients Does Not Trigger Arginine Synthesis

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Received 24 October 2015; Revised 10 February 2016; Accepted 21 February 2016

Academic Editor: Najat Mokhtar

Glutamine supplementation in specific groups of critically ill patients results in favourable clinical outcome. Enhancement of citrulline and arginine synthesis by glutamine could serve as a potential mechanism. However, while receiving optimal enteral nutrition, uptake and enteral metabolism of glutamine in critically ill patients remain unknown. Therefore we investigated the effect of a therapeutically relevant dose of L-glutamine on synthesis of L-citrulline and subsequent L-arginine in this group. Ten versus ten critically ill patients receiving full enteral nutrition, or isocaloric isonitrogenous enteral nutrition including 0.5 g/kg L-alanyl-L-glutamine, were studied using stable isotopes. A cross-over design using intravenous and enteral tracers enabled splanchnic extraction (SE) calculations. Endogenous rate of appearance and SE of glutamine citrulline and arginine was not different (SE controls versus alanyl-glutamine: glutamine 48 and 48%, citrulline 33 versus 45%, and arginine 45 versus 42%). Turnover from glutamine to citrulline and arginine was not higher in glutamine-administered patients. In critically ill nonseptic patients receiving adequate nutrition and a relevant dose of glutamine there was no extra citrulline or arginine synthesis and glutamine SE was not increased. This suggests that for arginine synthesis enhancement there is no need for an additional dose of glutamine when this population is adequately fed. This trial is registered with NTR2285.

1. Introduction

Previously, numerous clinical studies demonstrated that supplementation with glutamine as free molecule or dipeptide results in a favourable clinical outcome as reflected by a reduction in infectious morbidity (trauma [1, 2] and medical [2–5] patients), mortality [3, 6], and a reduction in length of hospital stay in severely ill patients [4, 7–9]. However, the use of high dose glutamine in shock patients has been part of debate [10]. The underpinning mechanism of the clinical effects of exogenous glutamine administration has not been completely elucidated yet. The effects of glutamine could be partially explained by the substrate that glutamine is for the synthesis of citrulline and arginine. Citrulline may act as a radical scavenger and is also a potent arginine precursor [11]. Arginine is of great importance for wound healing and the immune system and it is the precursor of nitric oxide (NO) [12–16]. During trauma and sepsis, plasma concentrations of

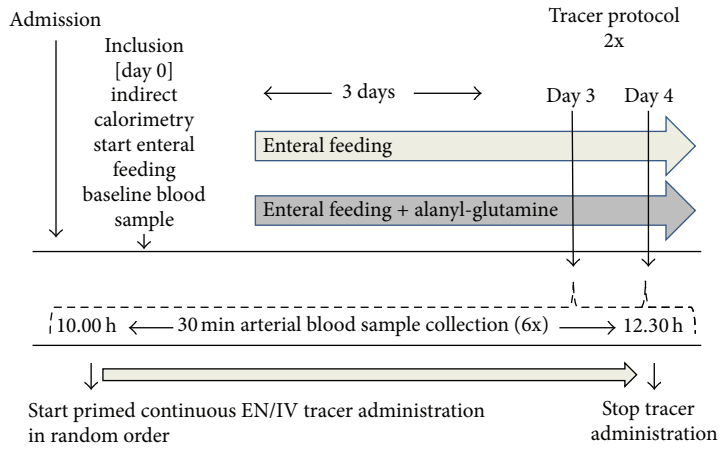

Figure 1: Study protocol.

arginine are decreased [17]. However, the action of arginine as a substrate for nitric oxide synthesis with potential subsequent hemodynamic instability and oxidative stress may be responsible for the reported adverse events of glutamine administration in severe critically ill patients [18–20]. Since endogenous glutamine can generate arginine by the citrulline pathway in the kidney, supplying glutamine may be a more physiologic and safe way to regulate arginine availability in the metabolically stressed ICU patient [21, 22]. However, in critically ill patients the metabolic fate of glutamine is still unclear. Possibly, generally altered metabolism could exist due to impaired enterocyte function because of injury, splanchnic ischemia, sepsis, and starvation.

Considering the observation that the gut preferentially takes up enterally provided glutamine, with subsequent higher intestinal release of citrulline, the precursor for arginine, we decided to provide L-alanyl-L-glutamine by the enteral route in this study, expecting to deliver glutamine most adequately to the interorgan pathway of glutamine into citrulline and arginine [23, 24]. Hence the objective of this clinical study was to investigate the effect of the enteral administration of a clinically relevant dose of L-glutamine, provided as L-alanyl-L-glutamine, on the synthesis of L-citrulline from L-glutamine and the subsequent synthesis of L-arginine from citrulline in critically ill nonseptic patients. Quantitatively, tracer methodology was used to determine exact turnover of these amino acids. A cross-over study design using intravenous and enteral tracers was chosen enabling splanchnic extraction calculations. We hypothesized that after enteral glutamine supply splanchnic glutamine uptake would increase as well as subsequent increases in citrulline and arginine synthesis.

2. Patients and Methods

2.1. Patients. Twenty critically ill patients considered stable were studied. All were expected to stay at the ICU for at least 5 days. Additional inclusion criteria were age, ≥18 years, BMI ≥ 18.5 and ≤35, ability to tolerate enteral nutrition, provided by postpyloric tube, meeting full protein/energy requirements based on indirect calorimetric measurements, and a protein intake of 1.2–1.7 g/kg/day.

Exclusion criteria were septic shock (defined according to the International Guidelines for Diagnosis of Sepsis [25]); need for high dose vasoactive medication such as norepinephrine higher than 0.2 μg/kg/min; PaO_2/FiO_2 ratio < 200; PEEP > 15 cm H_2O; liver failure (bilirubin levels > 100 μmol/L); hyperammonaemia (ammonia > 50 μmol/L); kidney failure (renal replacement therapy or increase in serum creatinine levels to >100 μmol/L), in the absence of primary underlying renal disease, associated with oliguria (defined as urine output < 150 mL in the previous 8 hours); urea cycle defects; chronic corticosteroids use (>7.5 mg/day >3 weeks); gastrointestinal malabsorption possibly interfering with intestinal absorptive function (celiac disease, Crohn's disease, presence of fistulas, major intestinal malabsorption disorder, or short bowel syndrome); pregnancy or lactation; admission after elective surgery; parenteral nutrition; use of medium chain triglycerides or glutamine/citrulline supplements.

Informed consent was obtained from all included patients or his/her legal representative. The Medical Ethical Committee of the VU University Medical Hospital approved the study protocol (MEtC VUmc 2009.083). The study complied with the Declaration of Helsinki (NTR2285).

2.2. Study Design. All twenty patients received enteral nutrition via postpyloric or nasogastric tube. Ten patients received an additional enteral dose of 0.5 g/kg/day L-alanyl-L-glutamine (ALA-GLN) (=0.325 g/kg glutamine/day) (Fresenius Kabi Nederland B.V. Den Bosch, Netherlands). Patients in the control group received isonitrogenous enteral nutrition without the additional glutamine (CON). Total nitrogen was compensated by using different enteral nutritional formulas containing different amounts of protein. Patients were investigated while being fed continuously. The study protocol is outlined in Figure 1.

Resting energy expenditure (REE) was measured with the Deltatrac Metabolic Monitor (Datex-Engstrom Division, Helsinki, Finland), calibrated every day. Energy requirement

Table 1: Enteral nutrition.

Enteral nutrition	Energy (kcal/L)	Protein (g/L)	Glutamine (g/L)	Citrulline (g/L)	Arginine (g/L)
Nutrison Standard	1000	40 g/L	4.6	0	1.6
Nutrison Protein Plus	1250	63 g/L	7.19	0	2.5
Promote	1000	63 g/L	2.3	0	2.3

Table 2: Tracer dosages.

Tracer	Prime (μmol/kg)		Infusate (μmol/kg/h)	
	CON mean (SEM)	ALA-GLN mean (SEM)	CON mean (SEM)	ALA-GLN mean (SEM)
GLN M + 1	232.0 (13.8)	236.7 (8.0)	18.4 (1.6)	16.0 (1.5)
CIT M + 5	627.2 (37.3)	639.8 (21.8)	1.2 (0.3)	1.0 (0.3)
ARG M + 2	141.1 (8.4)	144.0 (4.9)	1.3 (0.1)	1.2 (0.1)

was measured within 24 h before study or control feeding was started. During measurements, nutrition was not interrupted. Body height and weight were (self-)reported at admission. REE was measured for a minimum of one hour. Total energy expenditure (TEE) was calculated by adding 10% (activity factor) above REE [26]. Nutrition was based on TEE and total protein was aimed for at 1.5–1.7 g/kg/day but at least not under 1.2 g/kg/day [27]. To achieve these goals we used the following enteral formulas: Nutrison Protein Plus®, Nutrison Standard® (both from Nutricia, Zoetermeer, Netherlands), and Promote® (Abbott, Columbus, Ohio, US). Data on nutrition and nutritional requirements are listed in Tables 1 and S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/1373060).

Baseline characteristics and routine clinical blood variables were documented. APACHE II (Acute Physiology and Chronic Health Evaluation) scores were calculated as measures of severity of disease in ICU patients. A baseline blood sample was taken for amino acid concentration analysis.

All patients received stable isotopes both enterally and intravenously, on separate days (days 3 and 4, in random order). The enteral tracers were coadministered through a separate port on the tube; the intravenous tracers were administered in the antecubital vein. After 3 days of glutamine enriched or control feeding, if patients were considered stable, at approximately 10.00 am, an arterial baseline sample was collected to measure natural background enrichment followed by a primed continuous intravenous or enteral tracer infusion in random order. Blood samples were collected at 30-minute intervals for 2.5 hours. The same protocol ran the following day with the alternative route of tracer administration. In case stability of the patient was not guaranteed or clinical situation did not allow research, the tracer protocol was postponed by a maximum of one day.

Blood was collected in prechilled heparinized vacuum tubes (BD Vacutainer, Franklin Lakes, NJ) and immediately placed on ice. Blood was centrifuged (10 minutes, 3000 rpm, 4°C) and plasma was extracted and again centrifuged (10 minutes, 3000 rpm, 4°C) after which 500 μL of plasma was added to 20 mg dry sulfosalicylic acid (Across Inc., Geel, Belgium) to precipitate plasma proteins. After vortex mixing, deproteinized plasma samples were snap-frozen in frozen carbon dioxide and stored at −80°C until assayed.

2.3. Stable Isotopes. Stable isotope tracers of L-[2-^{15}N]-glutamine, L-[5-^{13}C-4,4,5,5^{2}H$_4$]citrulline, and L-[guanidino^{15}N$_2$]-arginine were used to investigate the effect of the enteral supplementation of glutamine on the metabolism of L-glutamine, L-citrulline, and L-arginine, as well as the conversions of L-glutamine into L-citrulline and L-citrulline into L-arginine. Tracers will be noted as glutamine [M + 1], citrulline [M + 5], and arginine [M + 2], respectively. Tracers were purchased from Cambridge Stable Isotope Laboratory (Woburn, MA, USA). The Department of Clinical Pharmacy at the Erasmus Medical Center in Rotterdam, Netherlands, prepared sterile and pyrogen-free stock solutions of the tracers. The glutamine tracer was prepared 1-2 days before tracer infusion, due to the limited stability of glutamine in solution (72 h). The stock solutions were diluted with physiological saline solution minutes before the start of the tracer administration.

The tracers were administered intravenously and enterally, to study splanchnic extraction of glutamine and to distinguish between the contribution of endogenous and exogenous L-glutamine to the metabolic interrelationship between L-glutamine, L-citrulline, and L-arginine.

Tracers and amounts are listed in Table 2. Tracer dosages were calculated by using previous results from Ligthart-Melis et al. and van de Poll et al. [24, 28]. Because these studies involved surgical patients in the postabsorptive state, a pilot was performed within the first two patients to confirm steady state within our continuously enterally fed critically ill patients [29]. This resulted in a small body weight related adjustment of the priming dose.

2.4. Laboratory Analyses. Amino acid concentrations in plasma and infusates were measured using high-performance liquid chromatography, as described elsewhere [30]. Isotopic enrichment was expressed as tracer to tracee (labeled versus unlabeled substrate) ratio (TTR, %), corrected for contribution of lower masses and for background TTR (determined in the baseline sample). Glutamine, citrulline, and arginine

TTRs were measured by liquid chromatography-mass spectrometry [30].

2.5. Calculations. Isotopic enrichment was adjusted for natural enrichment and for the contribution of overlapping isotopomer distributions of the tracee and tracers with lower masses to the measured TTR as described by Vogt et al. [31]. Metabolic conversions were calculated using established calculations [32]. Since all tracers were administered during enteral nutrition (no matter which route of administration), adjustments were made for tracee infusion, as explained beneath in the calculations and also used by Buijs et al. [33].

For each amino acid studied, arterial enrichment curves were fitted for each patient with the use of PRISM software (version 4.03; GraphPad Software Inc., San Diego, CA). Steady state was calculated by curve fitting plateau calculations. Primarily a first-order straight line was calculated (mean minus baseline). Hereafter an exponential decay function challenged the null hypothesis (first-order straight line), when steady state was in fact more likely to have optimized following a plateau after correction for possible occurring under- or overpriming (this would be a line that decays to a plateau with a constant rate K).

The plasma rate of appearance (WBRA: $\mu\text{mol} * \text{kg}^{-1} * \text{h}^{-1}$) of glutamine, citrulline, and arginine and the known infusion rate of these tracers are based on the following equation:

$$\text{WBRA} = \frac{I\,(\text{tracer})}{\text{TTR}}. \tag{1}$$

I(tracer) is the known infusion rate of the tracers and TTR is the tracer/tracee ratio. Knowing that enteral feeding and alanyl-glutamine infusion affect the RA, the WBRA calculation includes the exogenous infusion rate of tracee:

$$\text{WBRA} = \text{RA}\,(\text{endogenous}) + I\,(\text{tracee})\,. \tag{2}$$

I(tracee) reflects the exogenous amino acid (AA) supply (amino acids given by enteral nutrition):

$$I\,(\text{tracee}) = I\,(\text{AA}) * \left[\frac{\text{TTR}\,(\text{EN})}{\text{TTR}\,(\text{IV})}\right], \tag{3}$$

with TTR(EN) being the TTR with enterally administered tracers and TTR(IV) the TTR with intravenously administered tracers, corrected for splanchnic extraction of AA, reflected by splanchnic tracer extraction.

True RA (RA(endogenous)) is therefore calculated as follows [33]:

$$\begin{aligned}\text{RA}\,(\text{endogenous}) = &\left[\frac{I\,(\text{tracer})}{\text{TTR}\,(\text{IV})}\right] \\ &- \left[I\,(\text{AA}) * \left(\frac{\text{TTR}\,(\text{EN})}{\text{TTR}\,(\text{IV})}\right)\right].\end{aligned} \tag{4}$$

Calculation of the rate of WB plasma turnover (Q: $\mu\text{mol} * \text{kg}^{-1} * \text{h}^{-1}$) of glutamine into citrulline was performed by using the following equation from Castillo et al. adjusted for endogenous RA [13]:

$$\begin{aligned}&Q\,\text{gln} \longrightarrow \text{cit} \\ &\quad = \text{RA}\,(\text{endogenous})\,\text{CIT} \\ &\qquad * \left[\frac{\text{TTR CIT M} + 1}{\text{TTR GLN M} + 1}\right],\end{aligned} \tag{5}$$

where WBRACIT is the plasma WBRA of citrulline, calculated from the TTR of the infused CIT M + 5 tracer by using (1), and CIT M + 1 is the CIT M + 1 coming from GLN M + 1.

Likewise, calculation of the WB plasma turnover of citrulline into arginine (de novo synthesis) was performed by using the following equation:

$$\begin{aligned}&Q\,\text{cit} \longrightarrow \text{arg} \\ &\quad = \text{RA}\,(\text{endogenous})\,\text{ARG} \\ &\qquad * \left[\frac{\text{TTR ARG M} + 5}{\text{TTR CIT M} + 5}\right],\end{aligned} \tag{6}$$

where WBRaARG is the WBRa of arginine, calculated from the TTR of ARG M + 1 by using (1), and ARG M + 5 is the ARG M + 5 coming from CIT M + 5.

Splanchnic extraction (%) of glutamine, citrulline, and arginine was calculated as follows:

$$\left[1 - \left(\frac{\text{TTR}\,(\text{EN})}{\text{TTR}\,(\text{IV})}\right)\right] * 100. \tag{7}$$

2.6. Statistical Analyses. Data are expressed as mean ± standard error (SEM) in case of normally distributed data and as median ± interquartile range (IQR) when data were not normally distributed (tested by Shapiro-Wilk normality test). Independent sample t-test or Mann-Whitney test was used to compare control group with alanyl-glutamine group, according to distribution.

One sample t-test was used to test whether steady state of metabolic products differed from zero. Plasma values over time were compared using ANOVA and Bonferroni to determine specific time differences.

A p value of <0.05 (2-tailed) was considered as statistically significant. Statistical analysis was performed with SPSS 17.0 for Windows® (SPSS Inc., Chicago, IL, USA).

3. Results

Twenty patients were successfully included: ten received enteral alanyl-glutamine isocalorically and isonitrogenous enteral nutrition compared to 10 control patients. Results of one patient (CON) were not completely obtained due to detubation and subsequent removal of enteral tube at the last day. One patient got discharged and had his parenteral tracer administration accidently interrupted (ALA-GLN); therefore only enteral results could be obtained. Patient characteristics are summarized in Table 3. Baseline characteristics were not significantly different when comparing the two groups.

Table 3: Baseline characteristics.

Baseline parameter	CON *N* (%)/mean (SEM)/median (IQR)	ALA-GLN *N* (%)/mean (SEM)/median (IQR)
Demographics		
Sex: male/female (%)	6/4 (60/40)	6/4 (60/40)
Age (y)	65 (6.4)	57 (5.4)
Length (cm)	172.6 (3.7)	176.8 (3.0)
Weight (kg)	73.2 (5.8)	77.7 (4.1)
BMI (kg/m^2)	24.2 (1.0)	24.5 (3.3)
Clinical assessment		
Type of ICU admission		
Respiratory insufficiency	6 (60)	3 (30)
Cardiogenic shock	1 (10)	4 (40)
Neurotrauma	1 (10)	0
Multitrauma	1 (10)	2 (20)
Other	1 (10)	1 (10)
APACHE II score	27.0 (2.2)	25.3 (3.0)
Laboratory measurements at inclusion		
pH	7.42 (0.048)	7.44 (0.027)
pCO_2	41.4 (10.3)	43.9 (9.7)
Bicarbonate (mmol/L)	*32.9* (*22.0–33.0*)	30.5 (5.3)
Glucose (mmol/L)	6.6 (0.83)	7.5 (1.4)
Leukocytes (10^9 μmol/L)	11.0 (3.1)	11.8 (4.4)
Bilirubin (μmol/L)	*8.5* (*5.0–16.8*)	9 (4.0–11.3)
Creatinine (μmol/L)	78 (9.7)	81.8 (13.7)
Urine production (mL/24 h)	2301 (361)	2333 (295)

3.1. Nutrition. Energy expenditure was similar in both groups. All patients received 100% of the caloric target during the tracer infusions. During the whole study period 16 out of 20 received an actual nutrition of >97% of the aimed 5-day nutrition (tube feeding stopped or was lowered during the 5-day course), one patient received 89%, one 80%, one 70%, and one 66%. The last patient was eventually excluded due to unobtained steady state (described below). Mean energy intake during complete study period was 94.8% (SE 2.3) of target nutrition.

Nutrison Standard (lowest protein content) was used more often in patients in the alanyl-glutamine group, because the formula was chosen based on total amount of nitrogen (within the enteral formula) adjusted to the administered amount of alanyl-glutamine.

Although the alanyl-glutamine group received slightly more nitrogen, nitrogen per kg bodyweight was similar. Glutamine administration was as expected significantly higher in the alanyl-glutamine group. Citrulline administration was absent in both groups and total administered arginine was not different in both groups (Table 4).

3.2. Glutamine, Citrulline, and Arginine Metabolism. Alanyl-glutamine was not detectable in arterial plasma in both groups. Arterial plasma concentrations of glutamine, citrulline, and arginine were not significantly different at D0 for both groups. Plasma concentrations of glutamine and arginine did not differ during the study period or between groups. Plasma concentrations of citrulline increased significantly from baseline compared to 3-4 days in the control group (D0: 28 ± 4 μmol/mL, D3: 34 ± 3.3 μmol/mL, D4: 39 ± 5.5 μmol/mL, $p = 0.035$), without differences between groups.

In one patient, an isotopic steady state for glutamine, citrulline, and arginine tracers could not be reached with intravenous administration; therefore these results were excluded from analyses. This patient appeared to have higher bilirubin and creatinine levels (although not above exclusion level). Apart from this mentioned patient, steady state for the amino acid tracers could not be calculated for arginine M + 2 (EN: 1 case) and arginine M + 1 (EN: 2 cases). These results were therefore excluded from analyses as well. Steady state curves are presented in Figure S1.

TTR% for almost all infused tracers were higher when intravenously administered compared to enteral infusion, in both groups (Table 5). TTR% of infused tracers was not significantly different between control and alanyl-glutamine group. TTR% of metabolic products differed in case of citrulline M + 1 for both the intravenous and enteral experiments (Table 5).

The TTR% of the metabolic products of [15N]glutamine metabolism—[15N]citrulline and [15N]arginine—were significantly different from zero in both groups with either way of administration. However, TTR% of the metabolic product of L-[5-^{13}C-4,4,5,5^{2}H$_4$]citrulline metabolism, [5-^{13}C-4,4,5,5^{2}H$_4$]arginine, was below detection level in 6 and 7 control patients (iv and enteral tracer administration, resp.)

Table 4: Nutrition characteristics.

Nutrition parameter	CON mean (SEM)	ALA-GLN mean (SEM)	Sig. between groups (p)
Calorimetry			
REE (kcal/24 h)	1847 (165)	1998 (85)	0.429
TEE (kcal/24 h)	1998 (165)	2176 (80)	0.350
VCO_2 (mL/min)	223 (17.0)	234 (10.7)	0.587
VO_2 (mL/min)	269 (24.8)	292 (12.4)	0.577
RQ	0.84 (0.03)	0.81 (0.016)	0.237
Nutrition			
Nutrison Protein Plus/Nutrison Standard/Promote	3/5/2 (30/50/20)	0/1/9 (0/10/90)	
Received % of nutritional target (study period)			
Median (IQR)	98.5 (84.3; 100)	100 (98.8–100)	0.136
Energy (kcal/d)	1999 (165)	2190 (88)	0.325
Received energy (kcal/d)	1844 (183)	2129 (67)	0.170
Nitrogen			
(g/d)	101.5 (7.2)	122.7 (5.8)	0.034
(g/kg/d)	1.41 (0.08)	1.59 (0.11)	0.066
Received nitrogen			
(g/d)	93.1 (7.9)	119.4 (5.1)	0.014
(g/kg/d)	1.30 (0.11)	1.54 (0.02)	0.055
Received glutamine			
(g/d)	10.2 (1.0)	35.2 (0.17)	(*<0.000*)
(mmol/kg/d)	0.98 (0.09)	3.11 (0.03)	(*<0.000*)
Citrulline (g/d)	—	—	—
Received arginine			
(g/d)	3.6 (0.32)	3.2 (0.13)	0.278
(mmol/kg/d)	0.29 (0.08)	0.24 (0.01)	0.066
Baseline plasma glutamine (μmol/mL)	521 (66)	497 (37)	0.743
D3	539 (21)	518 (34)	0.592
D4	525 (32)	510 (38)	0.755
Baseline plasma citrulline (μmol/mL)	28 (4)	32 (3)	0.365
D3	33 (3)	37(2)	0.349
D4	39 (5)	38 (3)	0.865
Baseline plasma arginine (μmol/mL)	53 (6)	72 (8)	0.076
D3	64 (6)	62 (6)	0.833
D4	68 (7)	64 (5)	0.645

and 4 and 3 patients in the alanyl-glutamine group (iv and enteral tracer administration, resp.).

Endogenous rates of appearance were not significantly different for all administered tracers in the alanyl-glutamine group as compared to the control group (Table 5).

Splanchnic extraction of glutamine and citrulline was not significantly different for both groups (Figure 2).

Whole body plasma turnover gln → cit (Q: $\mu\text{mol} * \text{kg}^{-1} * \text{h}^{-1}$) was not significantly higher in glutamine-administered patients. In contrast, in control patients, 47.8% (±7.8) of the citrulline was derived from glutamine, versus 24.8% (±4.4; $p = 0.018$) in the alanyl-glutamine group. The percentage of citrulline that served as substrate for arginine was 0% (range 0–10.8) versus 6.5% in the alanyl-glutamine group (range 1.3–12.5 ns). The percentage of glutamine that was converted into arginine was 1.3% (range 1.0–1.4) versus 0.7% (range 0.3–1.3, ns) (Table 5 and Figure 3).

4. Discussion

The primary aim of the present study was to quantify the effect of a therapeutically relevant dose of enteral L-glutamine on the synthesis of L-citrulline and subsequent L-arginine in critically ill patients receiving enteral nutrition. In contrast with our working hypothesis, we did not demonstrate a significantly higher turnover of glutamine into the substrates citrulline and arginine in this group.

Table 5: Tracer dynamics.

Tracer parameters and calculations	CON mean (SEM)/median (IQR)	ALA-GLN mean (SEM)/median (IQR)	Difference CON versus ALA-GLN (p)
Glutamine			
RA GLN M + 1			
IV	364.8 (57.5)	390.4 (40.0)	0.720
EN	787.5 (91.6)	606.3 (88.3)	0.173
Endogenous RA GLN	335.3 (62.3)	322.04 (39.4)	0.856
TTR% GLN M + 1			
IV	4.88 (0.38)	4.88 (0.33)	0.997
EN	2.54 (0.35)	2.44 (0.25)	0.820
Splanchnic extraction GLN	48.2 (4.6)	48.4 (3.6)	0.965
Citrulline			
RA CIT M + 5			
IV	26.5 (4.7)	28.5 (5.5)	0.780
EN	51.7 (23.7; 69.2)	29.5 (20.8; 64.3)	0.462
Endogenous RA CIT	26.5 (4.7)	28.5 (5.5)	0.780
TTR% CIT M + 1			
IV	1.99 (0.33)	1.13 (0.16)	***0.031***
EN	4.44 (0.47)	2.64 (0.46)	***0.015***
TTR% CIT M + 2			
IV	0.29 (0.14)	0.21 (0.08)	0.622
EN	1.08 (0.21)	0.50 (0.19)	0.057
TTR% CIT M + 5			
IV	4.31 (0.85)	4.06 (0.34)	0.791
EN	2.49 (2.25; 2.94)	1.98 (1.82; 2.29)	0.207
Splanchnic extraction CIT	33.0 (7.7)	44.8 (3.1)	0.185
Arginine			
RA ARG M + 2			
IV	76.6 (12.2)	75.0 (6.0)	0.907
EN	135.4 (106.8; 240.3)	121.3 (17.0)	0.374
Endogenous RA ARG	26.9 (25.0; 58.0)	31.8 (6.1)	0.336
TTR% ARG M + 1			
IV	0.44 (0.13)	0.35 (0.09)	0.583
EN	0.97 (0.25)	0.83 (0.15)	0.624
TTR% ARG M + 2			
IV	6.33 (0.76)	6.00 (0.50)	0.717
EN	3.73 (0.78)	3.26 (0.46)	0.593
TTR% ARG M + 5			
IV	0.33 (0.06)	0.35 (0.07)	0.898
EN	0.25 (0.05)	0.30 (0.05)	0.500
Splanchnic extraction ARG	45.0 (6.2)	41.8 (6.4)	0.725
Conversion rates			
Q Gln → Cit	13.4 (4.2)	6.3 (1.3)	0.121
Q Cit → Arg	0 (0–2.2)	1.3 (0.5–3.1)	0.135
Q Gln → Arg	3.2 (1.0)	2.4 (0.9)	0.539
Q Gln → Cit% of Gln	4.45 (0.91)	2.06 (0.42)	0.026
Q Gln → Cit% of Cit	47.8 (7.8)	24.8 (4.4)	0.018
Q Cit → Arg% of Cit	0 (0–10.8)	6.5 (1.3–12.5)	0.370
Q Cit → Arg% of Arg	0 (0–7.4)	5.3 (2.2–10.4)	0.131
Q Gln → Arg% of Gln	1.3 (1.0–1.4)	0.7 (0.3–1.3)	0.370

RA in μmol/kg/h, endogenous RA in μmol/kg/h, splanchnic extraction in %, TTR in %, and Q in μmol/kg/h.

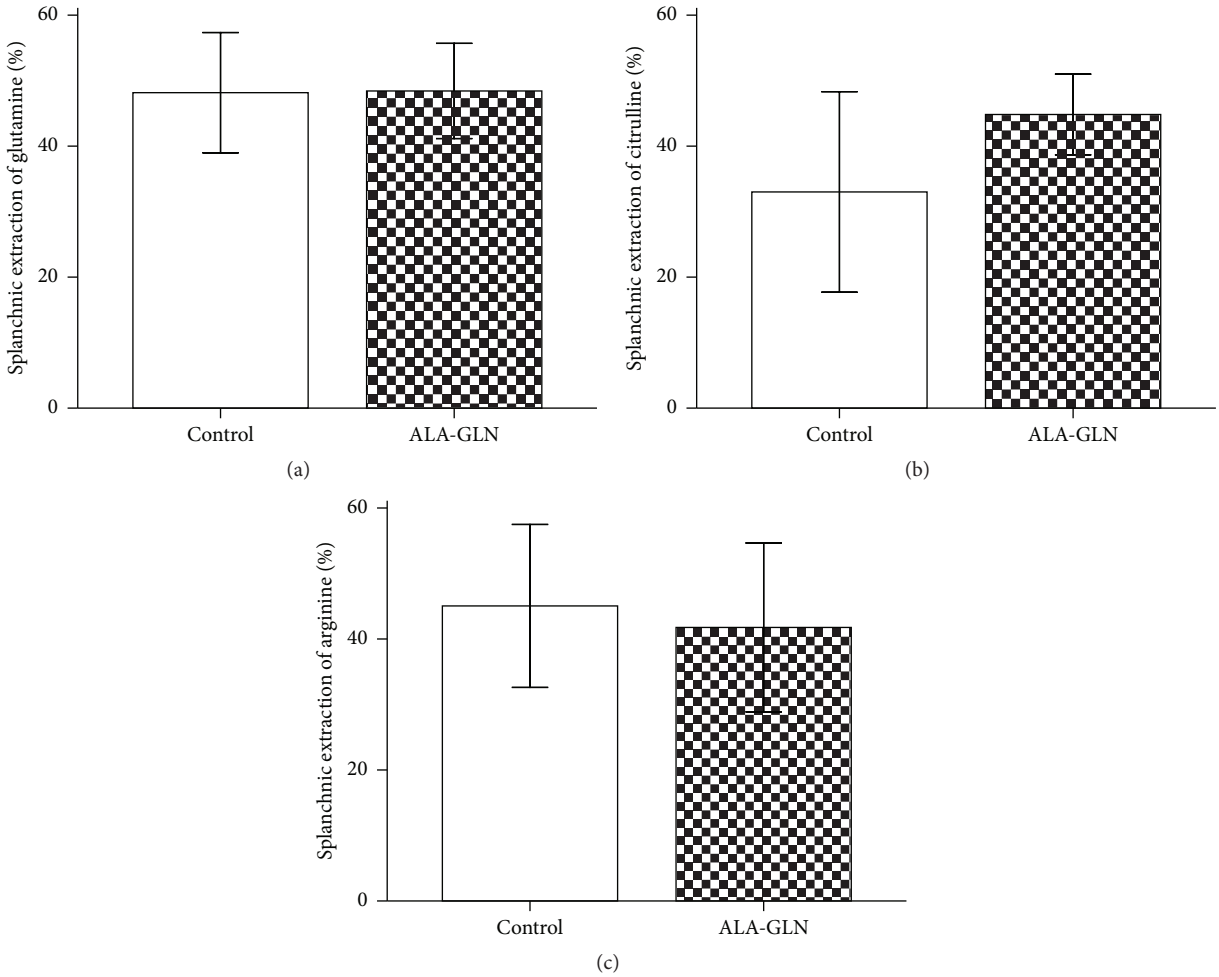

Figure 2: Splanchnic extraction: splanchnic extraction of glutamine (a), citrulline (b), and arginine (c) expressed in mean ± SE.

Glutamine is one of the most abundant amino acids in the human body. In healthy adults, the small intestine is the major organ of glutamine utilization. Enterocytes extract both arterial glutamine and in a greater extent luminal glutamine.

Intestinal glutamine degradation starts with deamination into glutamate and ammonia. Ammonia is released into the portal vein, after which it can be taken up by the liver serving ureagenesis and glutamine synthesis. Glutamate is released into the portal vein, either as glutamate, as alanine, or as α-ketoglutarate after transamination with pyruvate, or it is converted to citrulline (approximately 12%, which is 60%–80% of the total citrulline) in which the amino-group and the carbon skeleton of the original glutamine molecule are preserved. The major part of this citrulline is released into the portal vein and subsequently taken up by the proximal tubular cells of the kidney for arginine de novo synthesis [22].

Citrulline itself is scarce in a regular human diet. It was first identified in the 1930s and its name is based on the juice of watermelon (*Citrullus vulgaris*) [34]. In contrast to other amino acids, it is not used in protein synthesis, so it was long time considered to function solely as a metabolic intermediate, specifically in the urea cycle. However, with extensive research performed over the past decades, citrulline appears to play a considerable role in the regulation of nitrogen homeostasis and in the cardiovascular system as regulator of immunity.

After hepatic escape and renal extraction, an ammonia group from aspartate is incorporated to form arginine (catalysed by argininosuccinate synthase [ASS] and argininosuccinate lyase [ASL]) in the proximal convoluted tubules of the kidney. Citrulline is the only precursor for de novo arginine synthesis, of which the majority is executed in the kidneys. This pathway is called the "intestinal-renal axis." The synthesized arginine from citrulline accounts for 60% of the de novo whole body arginine synthesis; however this only represents 5%–15% of the total circulating arginine [35]. This indicates that most of the plasma arginine is derived from proteolysis and food intake. This arginine pool is sufficient to provide the body's full arginine requirements in physiological conditions.

The relationship between glutamine and arginine has been subject of research in our group since the early nineties [1, 36]. Since then, extensive research has been evolved on

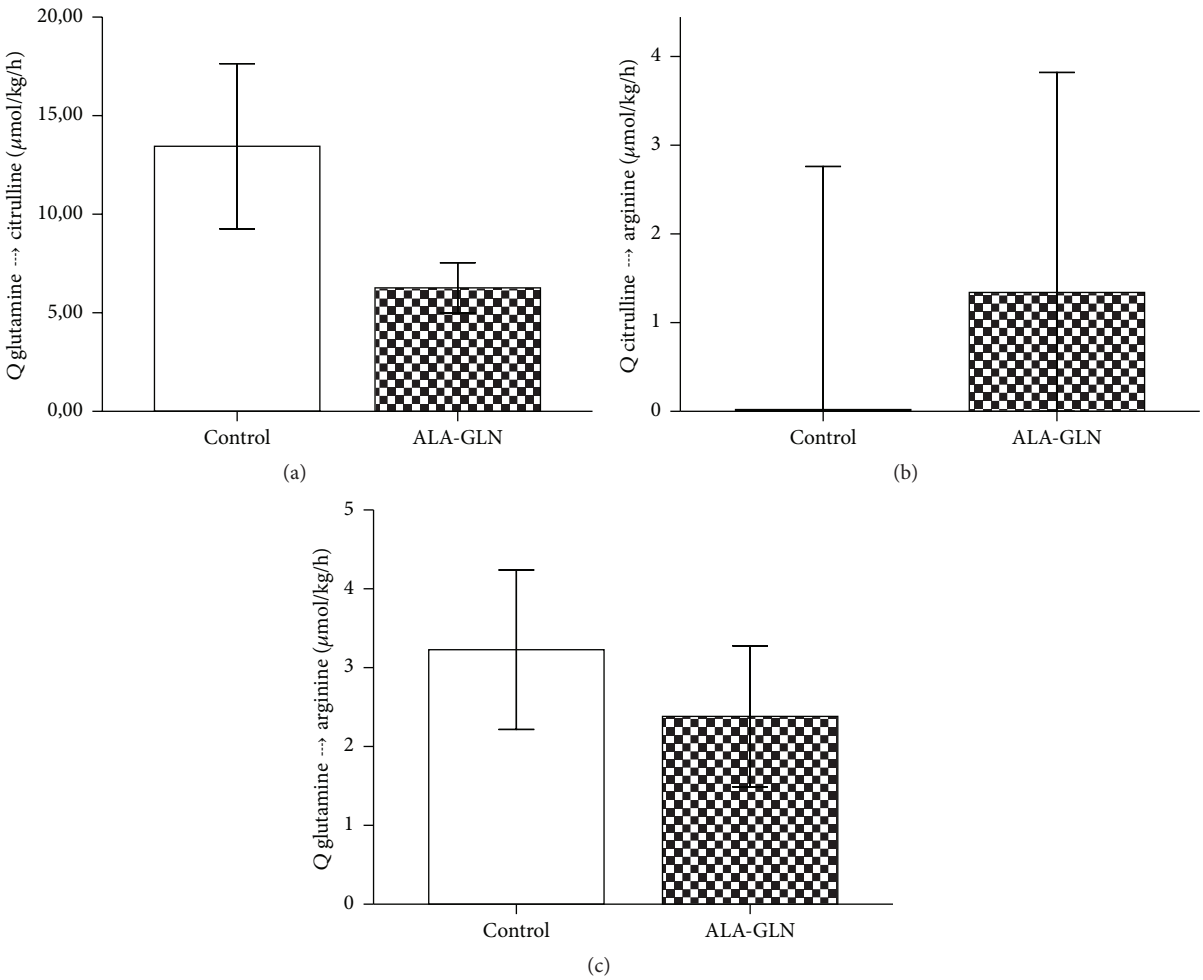

Figure 3: Conversion of glutamine into citrulline and arginine. Conversion rates in μmol/kg/h of glutamine into citrulline (a), citrulline into arginine (b), and glutamine into arginine (c). (a) and (c) are expressed in mean ± SE and (b) is expressed in median ± 95% CI.

using tracer methodology on this topic by us and others. Although the existence of the relationship between glutamine, arginine, and citrulline is clear, we learned that (A) mice metabolism is unequal to human metabolism [35, 37, 38], (B) enteral glutamine administration does not have the same effect as intravenous glutamine supply [23], and (C) critically ill patients behave differently as opposed to healthy volunteers [39–41]. At least three matters have remained unclear: Does postabsorptive glutamine handling differ from the postprandial state? Do critically ill patients metabolize an additional enteral dose of glutamine differently than (so far investigated) trace dosages? And do differences exist between septic and relatively stable ICU patients? We attempted to provide the answers to the first two questions. Kao et al. and Luiking et al. investigated amino acid metabolism in septic ICU patients using stable isotope methodology. Kao et al. show an altered glutamine metabolism in fasted septic patients compared to healthy volunteers [39]. With enteral administration, they show a more pronounced glutamine to citrulline conversion, as was observed earlier in non-critically ill patients [23]. Both investigators observed diminished de novo arginine synthesis. These findings strongly suggest that arginine availability is indeed at risk in septic patients [40, 42].

Since Heylands recent publication on glutamine and antioxidant supplementation in critically ill patients, concerns were raised about glutamine supplementation within their study population [10]. It has now been argued that safety is not guaranteed when high dosages (0.35 g/kg/d parenterally and 30 g/d enterally) of glutamine are administered to patients with multiple organ failure. Given the fact that liver and/or kidney failure impairs protein clearance, glutamine is probably best given to either surgical or medical critically ill patients but should not be given in case of liver or kidney failure [43, 44].

Our results could be explained by a number of considerations as follows. Primarily, the patients were well fed and not glutamine, citrulline, or arginine deficient. Therefore the use of additional glutamine may not have been as effective as within truly depleted patients. Attributing to this, most severely ill (and possibly most depleted) patients could not be included, due to the five-day study period in which dropout must be avoided.

Secondly, due to adapted nutritional formulas, control patients received an average of 10.4 grams of glutamine per day. Given the equal amounts of glutamine splanchnic extraction rates and the equal endogenous rates of appearances, the gut does not seem to metabolize glutamine differently when it comes to different amounts of enteral delivery.

The absence of glutamine promoting arginine synthesis was unexpected. In fact, control patients had a relatively larger glutamine into citrulline conversion rate (13.4 versus 6.3 μmol/kg/h) with significantly higher CIT M + 1 TTR% in the control group. Since glutamine and citrulline compete for the same transporter (neutral amino acid system N transporter: SN1), similar or more citrulline splanchnic extraction can be explained with little glutamine supply [45, 46]. Some studies have previously demonstrated the capability of the liver to take up citrulline [28]. However, this uptake was associated with a release of the liver as well, so unidirectional uptake was never demonstrated.

Unfortunately, due to study design, we were unable to provide any insight neither on hepatic versus intestinal nor on renal citrulline metabolism.

Remarkably, in our experiments, citrulline to arginine turnover and glutamine to arginine turnover were lower compared to Ligthart-Melis, with a conversion rate of 0–6.5% and 0.7–1.3%, respectively, differing with a factor of 5–10% compared to earlier experiments. Again, splanchnic extraction and enteral administration partly account for this.

Remarkably, in the control group, the median conversion of citrulline to arginine was calculated zero while having higher glutamine to arginine conversion rates. This is because the TTR% of the metabolic product of L-[5-^{13}C-4,4,5,5^{2}H$_4$]citrulline metabolism, [5-^{13}C-4,4,5,5^{2}H$_4$]arginine, was below detection level in most of the control patients. The glutamine to arginine production probably finds its origin in the gut by the enzymes argininosuccinate synthase and argininosuccinate lysase. Circumstantial induction seems evident since this has been subject of discussion earlier [24, 47].

Furthermore, as we know renal citrulline metabolism is autoregulated [21]: in the presence of adequate arginine concentrations, arginine de novo synthesis is diminished, whereas at low concentrations renal arginine de novo synthesis is promoted. The faith of the "unused" citrulline is unclear, but it can be used for many systems.

Most importantly, our experiments prove that excessive arginine production after glutamine supplementation does not occur; hence the safety of 0.5 g/kg/day enteral alanyl-glutamine administration is proven in relatively stable critically ill patients without sepsis, kidney, or liver failure.

4.1. Methodological Perspective. Glutamine stable isotope studies have generated intense debate recently, since Marini et al. and Tomlinson et al. published multiple tracer results in mice and fed volunteers, respectively [38, 48]. Marini et al. found discrepancies between nitrogen and carbon labeled glutamine in mice implying that glutamine provides nonspecific carbon for the citrulline ureido group. Tomlinson et al. found overestimation of the glutamine contribution to arginine synthesis with the labeled nitrogen and found a contribution of 56% when using labeled carbon (compared to the 64% found earlier in fasted surgical patients). In the light of the first study and earlier published results it can be concluded that there are interspecies differences. With Tomlinson et al.'s study, the overestimation of recycling tracers due to splanchnic extraction remains quantitatively unclear, because no correction was made with whole body rates of appearances calculated solely with intravenous tracers. Our results show 48% glutamine splanchnic extraction, with lower enterally administered glutamine M + 1 TTR%, resembling results of Bourreille et al. [49] and lower glutamine to citrulline conversion rates (24.8–47.8%). Importantly the glutamine systemic delivery (endogenous infusion rate) after splanchnic extraction and corrected for steady state nutrient (tracee) delivery was not different in both groups. In contrast RA (not corrected) with enteral administration almost doubled intravenous administration (Table 5). Therefore, overestimation due to splanchnic extraction is proven by our experiments and future tracer studies should not use the dilution equations on solely enteral tracer experiments, as also addressed by Ligthart-Melis and Deutz [50]. This also implies that when correctly using the dilution equations it is still not definite which glutamine tracer should best be used for future studies. We suggest additional research on this topic using transition LC-MS/MS enabling differentiation between different fragments of the labeled amino acids; however this method includes similar quantitative pitfalls. A multistep approach with multilabeled amino acids could probably be the golden standard, but then inevitable setting associated bias (as discussed below) also disqualifies this approach.

4.2. Strength and Limitations of the Study. The cross-over study design enabling correcting for splanchnic extraction and enteral feeding is a strength although it can also be seen as a weakness. Although patients were considered stable, within the ICU stability and clinical condition of patients can vary every minute. Therefore an approach with two separate study days does not cover small metabolic changes that may have occurred in the meantime. Additionally, different metabolic phases with different energy needs are observed within this patient population [27]. The initial metabolic phase after administration was covered by the three-day administration of TEE-based nutrition with or without glutamine. Furthermore, by randomizing the administration order we attempted to outbalance potential metabolic differences. An alternative approach in which simultaneous administration through both routes is studied has the disadvantage of different tracer usage (often giving rise to different metabolic outcome) or (when given sequentially on the same day) different timing within circadian rhythm.

The study design disqualified the use of a control group, due to a five-day continuous enteral feeding regime while being immobilized to mimic minimal basal energy expenditure.

The heterogeneity of the studied population means that interpretation should be with caution. It also means that this is a reflection of the exact population that is able to receive full enteral nutrition: no instability, no bowel surgery, no sepsis, and no expected quick discharge. Therefore these results are

useful as a pilot for larger investigation on enterally enriched nutrition.

Unfortunately two patients could not fulfil their second tracer study day. This is a risk that goes hand in hand with the clinical setting. Stable isotope studies are usually performed with 5-6 patients (per group), due to complexity and expenses of the method.

In conclusion, these results prove that, in critically ill non-septic patients receiving optimal enteral nutrition including a clinically relevant dose of glutamine, the relationship between glutamine, citrulline, and arginine is still present. However in the glutamine receiving group there was no extra citrulline or arginine synthesis and splanchnic glutamine extraction was not increased. Arginine synthesis was not promoted by glutamine administration indicating that in this population glutamine supplementation is safe. This also suggests that for arginine synthesis enhancement there is no need for an additional dose of glutamine when these patients are adequately fed. Furthermore, we proved that overestimation of calculated metabolic products can be reduced by correcting for splanchnic extraction and enteral nutrition.

Abbreviations

NO:	Nitric oxide
REE:	Resting energy expenditure
TEE:	Total energy expenditure
APACHE II:	Acute Physiology and Chronic Health Evaluation II
TTR:	Tracer to tracee ratio
(WB)RA:	(Whole body) rate of appearance
I:	Infusion rate
AA:	Amino acid
Q:	Plasma turnover
CON:	Control group
ALA-GLN:	Group receiving alanyl-glutamine.

Competing Interests

Paul A. M. van Leeuwen has served as a speaker, a consultant, and an advisory board member for Fresenius Kabi. The study was partly financed by Fresenius Kabi. Other than this grant, Mechteld A. R. Vermeulen, Saskia J. H. Brinkmann, Albertus Beishuizen, Pierre M. Bet, Alexander P. J. Houdijk, and Johannes B. van Goudoever declare that there are no competing interests regarding this paper.

Authors' Contributions

Mechteld A. R. Vermeulen designed and performed the study, performed calculations and statistical analyses, and wrote the paper. Saskia J. H. Brinkmann helped performing the study and critically reviewed the paper. Nikki Buijs critically reviewed the study. Albertus Beishuizen helped designing the study and performed the study; Pierre M. Bet was responsible for ad hoc tracer preparation and pharmaceutical handling of the tracer solutions. Alexander P. J. Houdijk helped designing the study; Johannes B. van Goudoever helped designing the study, performed calculations, and critically reviewed the paper. Paul A. M. van Leeuwen was responsible for all parts of the study. All authors read and approved the final paper.

Acknowledgments

The authors would like to thank Sigrid de Jong for her help with the laboratorial handling, Hans van Eijk for determining the TTRs, Erna Albers and Ingrid van den Hul for their assistance at the Intensive Care Unit, Klara Bruyn for ad hoc glutamine tracer preparation, and Gerdien Ligthart-Melis for her advice. This study was supported by a Yoshimura Grant, Norman Yoshimura Grant, 2009 and 2010, and Project "The Contribution of L-Glutamine to L-Citrulline and L-Arginine Synthesis When Alanyl-Glutamine Is Supplied in an Enteral Dose of 0.5 g/kg, in Critically Ill Patients."

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 8468594, 7 pages
http://dx.doi.org/10.1155/2016/8468594

Clinical Study

Fortified Iodine Milk Improves Iodine Status and Cognitive Abilities in Schoolchildren Aged 7–9 Years Living in a Rural Mountainous Area of Morocco

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Received 25 December 2015; Accepted 21 February 2016

Academic Editor: Aziz Hichami

Iodine is required for the production of the thyroid hormones essential for the growth and development of the brain. All forms of iodine deficiency (ID) affect the mental development of the child. Our study aims to assess the impact of ID on the intellectual development of Moroccan schoolchildren and to evaluate the effect of consumption of fortified milk on reducing ID. In a double-blind controlled trial conducted on schoolchildren, children were divided into two groups to receive fortified milk (30% of cover of RDI iodine) or nonfortified milk for 9 months. Urinary iodine was analyzed using the Sandell-Kolthoff reaction, a dynamic cognitive test using Raven's Standard Progressive Matrices to assess learning potential was performed at baseline and end line, and anthropometric assessment was done only at baseline. The study included schoolchildren who were severely iodine deficient. The prevalence of malnutrition was high in both groups; in this study, we found improvements in iodine status and in cognitive abilities among Moroccan schoolchildren. Our study showed that the consumption of fortified milk led to a clear improvement in iodine status and also appeared to have a favorable effect on the cognitive ability of Moroccan schoolchildren in a rural mountainous region.

1. Introduction

2 billions of world's population have insufficient iodine intake, and the majority of the world's children have iodine deficiency (ID) with higher rates in developing countries [1, 2]. And all degrees of ID (mild: iodine intake of 50–99 μg/day, moderate: 20–49 μg/day, and severe: <20 μg/day) have many adverse effects on growth and mental development [3]. These effects are collectively termed iodine deficiency disorders (IDD). The damage increases with the degree of the deficiency, and the most serious adverse effect of ID is endemic cretinism [4]. These effects are due to inadequate production of thyroid hormone due to an insufficient iodine intake [1]. In Morocco, the severity of the IDD is considered as moderate; indeed a regional prevalence survey done in mountainous areas of Azilal in 1992 revealed that 65% of children examined were goitrous [5, 6]. Iodine deficiency results in a global loss of 10–15 Intellectual Quotient (IQ) points at a population level and constitutes the world's greatest single cause of preventable brain damage and mental retardation [3]. The relationship between iodine deficiency and early cognitive development has captured recent attention because iodine is related to specific physiological processes [7]. Studies on the effect of iodine deficiency on children's cognition and

behavior are selectively reviewed, looking for evidence of a causal relationship [4, 8]. Most correlational studies have found associations between ID and poor cognitive and motor development and behavioral problems. Longitudinal studies consistently indicate that children having iodine deficiency in infancy continue to have poorer cognition, school achievement, and more behavior problems in middle childhood [4, 8]. However, the possible confounding effects of poor socioeconomic backgrounds prevent causal inferences from being made. In nearly all countries, the best strategy to control iodine deficiency is iodization of salt, which is one of the most cost-effective ways to contribute to economic and social development [1]. Nowadays, and after 20 years of salt fortification with iodine in Morocco, the results are far from being satisfactory [9]. International efforts to control iodine deficiency disorders are slowing down and reaching the third of the worldwide population that remains deficient is still a major challenge. Fortification of basic food such as dairy products was developed as an alternative approaches for combating IDD [10, 11]. Thus, in collaboration with the Foundation for Child Nutrition, a leading manufacturer in the distribution of dairy products in Morocco, we have undertaken the current study to evaluate iodine status and its effect on cognitive ability (learning potential) after consumption of iodine fortified milk among schoolchildren aged 7 to 9 years, living in rural mountain region of Morocco.

2. Materials and Methods

2.1. Study Design. This study is a longitudinal interventional, double-blind (participants and assessors), and controlled one conducted among Moroccan schoolchildren, aged 7–9 years between February and October 2012, in a rural mountainous region. A total of 200 schoolchildren were recruited from three primary satellite schools and were divided into two groups to receive daily 200 mL either the fortified or the nonfortified milk. A distance of 52 km separated the two sites to avoid errors of distribution and/or exchange of milk batches between schoolchildren. To be included in the study, children had to be aged between 7 and 9 years and should not take supplements during the study period. Children with severe malnutrition needing nutritional rehabilitation or having chronic or severe illness requiring hospitalization or treatment were excluded from the study (and transferred to a local health center for follow-up). The study was conducted with respect to ethical and legal aspects, and written informed consent was obtained from each parent of recruited children.

2.2. Sample Size. The calculation of sample size was based on the standard deviation (2.5 μg/L) of the rate of urinary excretion of iodine [12]. To observe a difference of 2 μg/L with 5% level of significance and 90% power between the intervention group and placebo and after accounting for 15% dropouts, sample size of 40 children per group was required [13].

2.3. Milk Composition. The milk used was developed and produced specifically for this study by the Foundation for Child Nutrition to meet the purpose of the survey. It was whole, flavored with vanilla and sterilized by Ultrahigh Temperature (UHT). Both fortified milk and nonfortified milk were identical in taste and smell; the containers had the same appearance and packaging and they were distributed in schools during the 9 months of this study (including weekends and vacation days) [9]. The quality and quantity of nutrients of each batch of milk were doubly checked by AQUANAL (Laboratoire Aquitaine Analyses) in France and LOARC (Laboratoire Officiel d'Analyses et de Recherches Chimiques de Casablanca) in Morocco before their use in the study.

2.4. Data Collection. At recruitment, data regarding socioeconomic status (SES) were administered to each child and anthropometric parameters were measured. Cognitive ability was evaluated dynamically and the random urine samples were collected in the morning to assess urinary iodine using the Sandell-Kolthoff reaction at baseline and after 9 months of intervention.

2.5. Socioeconomic Status Assessment. Data regarding SES were collected at the beginning of the study from parents in all subjects groups, using questionnaires including level of parental education, household size, and monthly alimentary expenses.

2.6. Anthropometric Measurements. Anthropometric measurements were taken following standard procedures [9, 14] at baseline. Underweight and stunting were defined as weight-for-age Z-scores (WAZ) and height-for-age Z-scores (HAZ) < -2, respectively, according to the World Health Organization (WHO) [15].

2.7. Psychometric Test. In our study, the cognitive ability was evaluated dynamically by using a dynamic procedure instrument using the nonverbal part of Standard Progressive Matrices of Raven (SPMR) as a starting point. The SPMR, used in our study, are suitable for children from the age of six [16]. Dynamic testing is intended to assess children's learning potential or ability to benefit from instruction (Raven test-intervention-retest paradigm). The dynamic tests look at people's ability to learn while they are being tested [17]. This Kind of testing procedure involves an initial test without instruction and then a pretest which provides data on the children's current level of functioning; an instruction component is then given with the aim of familiarizing children with test demands, equalizing their experiences, and teaching the necessary problem solving skills; and finally the student's new level of functioning is tested during a posttest session. The intervention phase should give children, who did not have an adequate opportunity to develop their academic potential, a greater chance to achieve a fair test result.

The opening test consisting of set A of SPMR (items A1 to A12) was used to introduce children to the test material; the first two items of test 1 were used as items of examples. Test 2 (pretest and posttest) was built based on the items in sets B and C of SPMR (items B1 to B5, B7, B9, and B11; items C1 to C4, C6, C8, C10, and C12); the first two items of test 2 were also used as items of examples. Instruction component (test

3) was performed using the items B6, B8, B10, C5, C7, C9, and C11; the evaluators gave standardized explanations on how to solve the problems to all children (using a paper-pen support, drawing in blackboard, and giving explanations to children one by one).

The test protocol was translated into Moroccan dialect. Two health workers were trained to conduct the test which was pretested on a homogenous sample of $n = 48$ children (not included in the study) to ensure appropriateness of the test materials, to check item difficulties, and to standardize the passing protocol, especially the instruction phase. Instructions were explained to the children in Moroccan dialect and local Berber dialect (tachelhit). The passing of the test was made collectively in small groups of half-classes in a class room which was quiet and free of distractions; the maximum duration of the dynamic assessment was 1 hour and 30 min.

2.8. Urine Sampling. Random urine samples were collected in the morning, between 10 a.m. and 11 a.m. to assess urinary iodine [18]. These samples were aseptically collected in 40 mL capped polypropylene tubes, aliquoted in 4 mL Cryovial tubes, and stored at −20°C until analysis. Urinary iodine was determined spectrophotometrically using the Sandell-Kolthoff reaction [19]. According to the level of iodine in urine, the iodine deficiency is classified into three classes; normal iodine status: >100 μg/L; mild iodine deficiency: 50–99 μg/L; moderate iodine deficiency: 20–49 μg/L; and severe iodine deficiency [20].

2.9. Statistical Analysis. Data analysis was done by the software IBM SPSS Statistics version 20 (Statistical Package for the Social Sciences). Anthropometric Z-scores were calculated using WHO standards. The distribution normality of the quantitative variables was tested by Kolmogorov-Smirnov test. The variables normally distributed were presented as mean ± standard deviation. The nominal variables were presented as proportion and 95%. Chi-square test was used to test independence between nominal variables. In the case of cells with a theoretical frequency $n < 5$, we take the p value of Fisher. Two-sided p values < 0.05 were considered significant.

For cognitive tests, Quade's rank-transformed analysis of covariance was used [16], linear regression of the ranks of the 9th month posttest score was run on the ranks of the covariates (baseline posttest score, baseline urinary iodine), then the unstandardized residuals were saved, and finally a one-way analysis of variance was run using as a dependent variable, the unstandardized residuals of the ranks of the 9th month posttest score and the grouping factor (fortified milk/nonfortified milk) as the factor. The linearity was assessed and homogeneity of variance was met with Levene's test.

3. Results

The rural and mountainous region where the study was conducted is characterized by a community with low to medium income, more than half of parents being analphabet

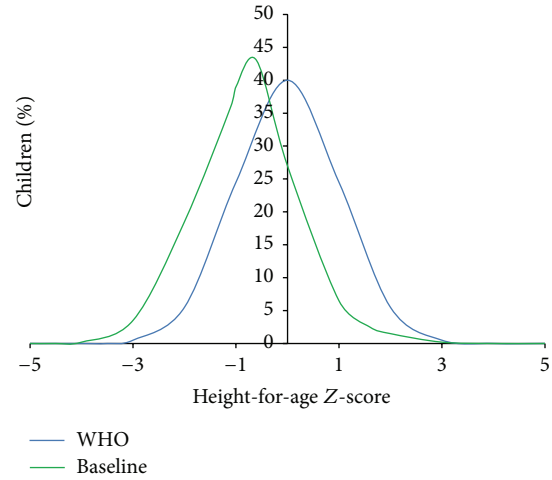

Figure 1: Distribution of the index height-for-age in schoolchildren studied at baseline compared to the reference population (WHO).

[9], and a relatively high prevalence of iodine deficiency [21]. Furthermore, over a third of children in the region suffer from stunting knowing that the national prevalence is 14.9% [22]. The mean age of the studied population is 8.0 ± 6.7 years with sex ratio 1.10.

Figure 1 shows the distribution of the anthropometric index height-for-age in schoolchildren 7 to 9 years compared with the reference population. The analysis of the figure shows that 8.5% of children are stunted (HAZ score < −2 SD). The distribution of the ratio height-for-age among children examined is moved to the left and is below the median compared to the distribution of the reference population (Figure 1).

Figure 2 shows the distribution of the anthropometric index weight-for-age in children examined aged 7 to 9 years compared with the reference population. The analysis of the figure shows that 3.4% of children are underweight (WAZ score < −2 SD). We also noted that our population is shifted to the left, thus being below the median of the reference population (Figure 2).

Figure 3 illustrates the prevalence of severe, moderate, and mild iodine deficiency among fortified and nonfortified groups at the beginning of the survey. We observed that before intervention the studied groups have approximately the same level of different classes of iodine deficiency and no statistical difference was registered between groups (χ^2-test, $p > 0.05$).

Figure 4 indicates that after 9 months of consumption of fortified or nonfortified milk among Moroccan schoolchildren the status of severe, moderate, and mild iodine deficiency was improved.

The results of dynamic cognitive test scores after receiving either iodine fortified milk or noniodine fortified milk are shown in Table 1. The results from Quade's test after controlling for confounding variables showed a significant p value between the fortified group and the nonfortified group at the end of the study, with the fortified group being better ($p = 0.020$).

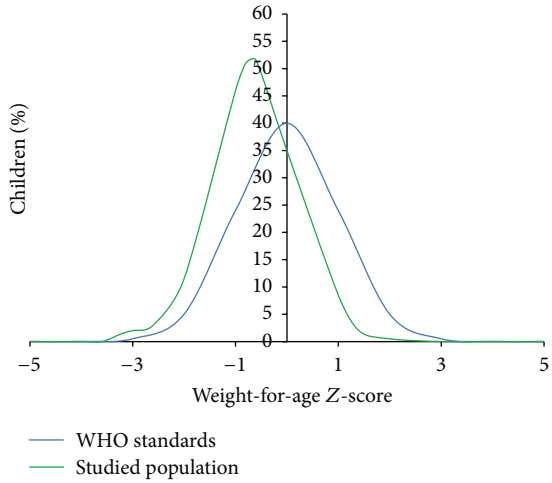

Figure 2: Distribution of the index weight-for-age in schoolchildren studied at the beginning of the study compared to the reference population (WHO).

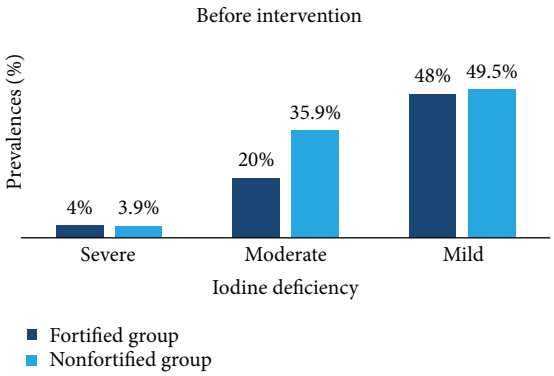

Figure 3: Prevalence of iodine deficiency among both groups before intervention.

Table 1: Analysis of covariance table for posttest scores at the end line.

	Sum of squares	df	Mean square	F	p value*
Between groups	47164.297	1	47164.297	5.564	0.020
Within groups	1356334.459	160	8477.090		
Total	1403498.756	161			

* p value was calculated using Quade's test.

The comparison of cognitive test scores between the two study groups showed an improvement of learning abilities in favor of the fortified group over 9 months of intervention period (Figure 5).

4. Discussion

The study was designed to investigate whether regular consumption of multiple micronutrients fortified milk over a period of 9 months could improve the iodine status and the effects of iodine deficiency on school children's cognitive abilities. To our knowledge, this is one of the first studies

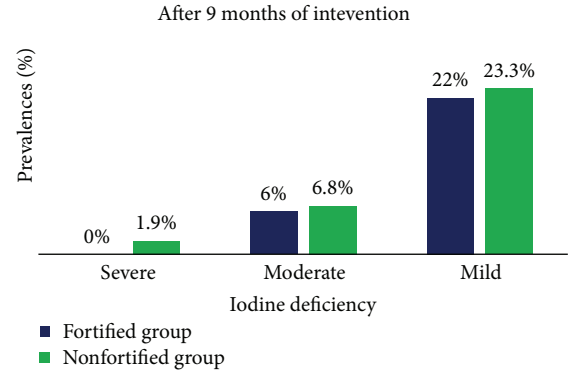

Figure 4: Prevalence of classes of iodine deficiency among fortified and nonfortified milk groups after 9 months of consumption of milk.

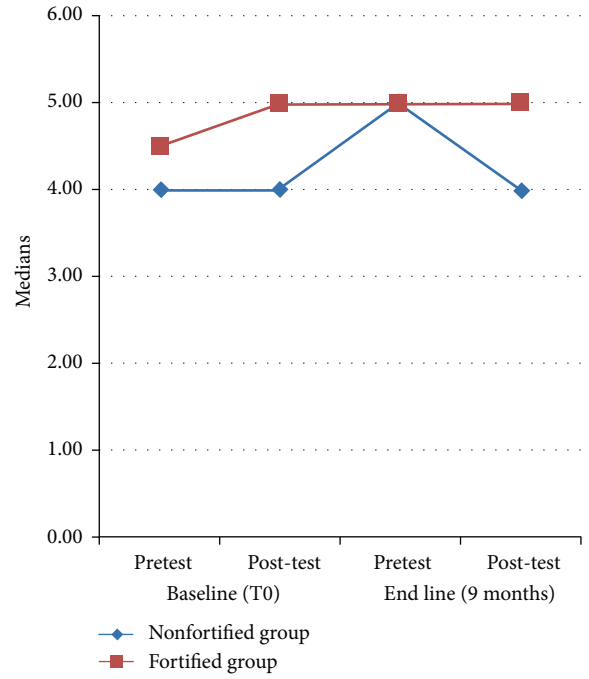

Figure 5: Comparison of cognitive test scores between the two studied groups.

that used milk as a vehicle for delivery of micronutrients and evaluated the impact among school children in a double-blind Randomized Controlled Trial (RCT) in Morocco. After 9 months of intervention, our results showed that the consumption of milk fortified with potassium iodide and other micronutrients is efficacious in reducing the prevalence of iodine deficiency and improving iodine status indicators in a sample of 7–9-year-old children. The increase in the control group at the end of the study can be explained by the fact that the unfortified milk also contained some iodine. A systematic review [23] evaluated the effect of the multimicronutrient (MMN) fortification of foods compared to unfortified foods on the micronutrient status of school children and measured a statistically significant improvement in iodine status in the intervention group compared to the control group, taking baseline values into account. In South

Africa, fortified biscuits resulted in a significant improvement in the iodine status of primary school children from a poor rural community after 12 months of intervention [24]. A study conducted in Filipino schoolchildren showed that consumption of a multiple-micronutrient-fortified beverage for 16 weeks had significant effects on iodine status.

On the other hand, the link between iodine deficiency and cognitive development is direct but can be prevented through public health methods, making iodine deficiency the most preventable cause of mental retardation in the world [25]. In Morocco, there is no interventional study that has evaluated the effect of a school based micronutrient fortification on biochemical status as well as functional health outcomes including cognitive performances/abilities. Very few studies in Morocco have assessed the cognitive performances of school children in static way [26, 27]. The results of our study showed better effect for the iodine fortified milk group as compared with the nonfortified iodine milk group for dynamic testing after 9 months of intervention. The beneficial effect may have been due to improvements in myelination of central nervous system, particularly in the frontal cortex which is responsible for higher-order cognition and fluid intelligence, mediated by an increased supply of thyroid hormone [28] or by effects on neurotransmitters and/or glucose metabolism through better thyroid function [29].

In a meta-analysis of 18 observational studies, which compared children based on whether they lived in an iodine-deficient area or not, children who lived in iodine-deficient areas had deficits in cognitive functioning [30]. In a well-controlled observational study in Bangladesh, investigators found that children with mild hypothyroidism had deficits in spelling and reading compared to healthy controls [31]. Although evidence from these studies is compelling, families who live in iodine-deficient areas are often more impoverished than families in areas where iodine status is adequate. Similarly, in two placebo controlled, double-blind interventional trials in Albania and New Zealand [32, 33], increasing iodine intakes over several months improved cognition in school-aged children who presumably grew up under conditions of iodine deficiency. In Albania, moderately iodine-deficient 10–12-year-old children were randomized to receive either 400 mg of iodine as oral iodized oil or 400 mg of placebo for 6 months. Treatment with iodine improved iodine status and significantly improved information processing, fine motor skills, and visual problem solving [32]. In New Zealand, 10–13 y children were given a daily tablet containing 150 μg iodine or placebo for 28 wk. Iodine improved scores on picture concepts and matrix reasoning [33]. In Filipino schoolchildren, salt iodization, accompanied by adequate intakes of energy, protein, and foods rich in thiamin and riboflavin, contributed to improved mental performance assessed by psychomotor and cognitive function tests (Bender-Gestalt and Raven's Colored Progressive Matrices) [34]. Furthermore, many observational studies have compared children in iodine-sufficient and iodine-deficient areas and nearly all have found poorer psychomotor or cognitive development in children living in iodine-deficient areas [35]. Iodine-deficient areas are generally more remote, poorer, and lacking in facilities compared with iodine-sufficient areas and these differences themselves could account for the children's poor development. Some authors have raised the problems of assessment of subclinical effects of ID and recommended the need for dynamic psychometric methods that reflect the contribution of sociocultural variables and skills acquired by learning to measure learning ability for assessing ID effect on the learning potential of children in endemic areas [36, 37]. However, information on iodine deficiency effect on children's learning potential in endemic areas remains rare [36].

5. Conclusion

We showed that milk can be used successfully as a vehicle for nutrient fortification in school feeding programs. Consumption of fortified milk resulted in a significant improvement in iodine status and also appeared to have a favorable effect on the cognitive ability of Moroccan schoolchildren in a rural mountainous region. The high prevalence of ID in the school aged population from poor rural background should be of concern and underlines the compelling need for implementing corrective and preventive measures to fight against this deficiency in school aged children from disadvantaged areas in Morocco.

Our study has already determined the feasibility and the efficacy of a geographical and targeted fortified school milk program in reducing the prevalence of ID among schoolchildren. Dynamic testing seems to be more appropriate for assessing children with poor educational backgrounds and could help to better detect the micronutrient fortification effect on cognitive ability.

Additional Points

The major limitations of the study are the small size of the study population due to recruitment of children from only 3 schools.

Abbreviations

ID:	Iodine deficiency
IDD:	Iodine deficiency disorders
IQ:	Intellectual Quotient
UHT:	Ultrahigh Temperature
AQUANAL:	Laboratoire Aquitaine Analyses
LOARC:	Laboratoire Officiel d'Analyses et de Recherches Chimiques de Casablanca
SES:	Socioeconomic status
SPMR:	Standard Progressive Matrices of Raven
SPSS:	Statistical Package for the Social Sciences
FG:	Fortified group
NFG:	Nonfortified group
RCT:	Randomized Controlled Trial
MMN:	Multimicronutrients.

Competing Interests

The authors declare that they have no competing interests. None of the authors was affiliated in any way with an entity involved with the manufacturing or marketing of milk.

Acknowledgments

The authors are grateful to acknowledge the contributions of local authorities, regional medical representatives, school head masters, teaching staff, and parent's union representatives in schools and schoolchildren who participated in this study. They would like also to gratefully acknowledge the contribution of Foundation for Child Nutrition for providing UHT milk used in the survey.

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Hindawi Publishing Corporation
Journal of Nutrition and Metabolism
Volume 2016, Article ID 3530971, 10 pages
http://dx.doi.org/10.1155/2016/3530971

Research Article

Hepatoprotective Activity of Herbal Composition SAL, a Standardize Blend Comprised of *Schisandra chinensis*, *Artemisia capillaris*, and *Aloe barbadensis*

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Received 6 October 2015; Revised 12 February 2016; Accepted 21 February 2016

Academic Editor: Azeddine Ibrahimi

Some botanicals have been reported to possess antioxidative activities acting as scavengers of free radicals rendering their usage in herbal medicine. Here we describe the potential use of "SAL," a standardized blend comprised of three extracts from *Schisandra chinensis*, *Artemisia capillaris*, and *Aloe barbadensis*, in mitigating chemically induced acute liver toxicities. Acetaminophen and carbon tetrachloride induced acute liver toxicity models in mice were utilized. Hepatic functional tests from serum collected at T24 and hepatic glutathione and superoxide dismutases from liver homogenates were evaluated. Histopathology analysis and merit of blending 3 standardized extracts were also confirmed. Statistically significant and dose-correlated inhibitions in serum ALT ranging from 52.5% ($p = 0.004$) to 34.6% ($p = 0.05$) in the APAP and 46.3% ($p < 0.001$) to 29.9% ($p = 0.02$) in the CCl_4 models were observed for SAL administered at doses of 400–250 mg/kg. Moreover, SAL resulted in up to 60.6% and 80.2% reductions in serums AST and bile acid, respectively. The composition replenished depleted hepatic glutathione in association with an increase of hepatic superoxide dismutase. Unexpected synergistic protection from liver damage was also observed. Therefore, the composition SAL could be potentially utilized as an effective hepatic-detoxification agent for the protection from liver damage.

1. Introduction

Liver diseases as a result of habitual repeated alcohol consumption, exposure to some xenobiotics, and/or drug interactions are some of the major causes of morbidity and mortality where variable symptoms manifested ranging from asymptomatic elevation of the liver enzyme to sudden hepatic failure. Liver is susceptible to alcohol-induced injury as both alcohol and its primary metabolite acetaldehyde produce reactive oxygen species (ROS) and hydroxyl radicals (OH), altering hepatic antioxidant defense system [1]. While damage of oxidative stress affects the whole body as a system, the impact becomes more detrimental when it involves vital organs such as the liver where primary detoxification takes place to remove and metabolize harmful toxins such as alcohol. The most frequently observed pathological conditions of the liver such as fatty liver, hepatitis, fibrosis, and cirrhosis are common findings in alcohol-linked liver disorders as a result of recurrent exposure of alcohol. These outcomes in conjunction with cellular lipids, proteins, and DNA oxidation have been demonstrated in multiple experimental animal models [2]. Among these models, the acetaminophen and carbon tetrachloride induced hepatotoxicity models have been most frequently used in assessing hepatoprotective activity of nutraceuticals.

Various xenobiotics are known to cause hepatotoxicity, among which acetaminophen (n-acetyl-p-aminophenol or APAP) and carbon tetrachloride (CCl_4) are generally

utilized to develop an animal model that mimics the human type of liver toxicity with similar mechanisms of actions. Acetaminophen (n-acetyl-p-aminophenol, APAP, also known as Tylenol, Paracetamol) is a very safe and effective analgesic and antipyretic drug at therapeutic dosage. However, APAP overdose can cause severe liver toxicity characterized by depletion of GSH, protein adduct formation [3, 4], generation of highly active free radicals, mitochondrial damage, and nuclear DNA fragmentation [5] that leads to cell death and hence necrosis. While some species like rat are relatively resistant to APAP toxicity, the mouse is the preferred model as several studies have demonstrated dose dependent response to either oral or intraperitoneal APAP challenge [6, 7]. Similarly, CCl_4, a halogenated alkane with restricted usage as industrial chemical/solvent, is a well-known hepatotoxin that is widely used to induce acute toxic liver injury in a large range of laboratory animals. CCl_4 toxicity is initiated by cytochrome P450s (CYP) primarily of 2E1 [8], to yield reactive metabolic products trichloromethyl free radicals (CCl_3^-), which can initiate lipid peroxidation and ultimately results in the overproduction of reactive oxygen species (ROS) and hepatocyte injuries [9, 10]. These radicals can also react with oxygen to form the trichloromethylperoxy radical CCl_3OO^-, a highly reactive species that could initiate the chain reaction of lipid peroxidation leading to cell death. Therefore, it could be inferred that, regardless of the chemical agents used to induce the hepatotoxicity, both the acetaminophen and carbon tetrachloride models share the critical step in oxidative stress induced by reactive oxygen species generated by excess intermediate metabolites leading to protein oxidation, lipid peroxidation, and DNA damage.

Historically, some botanicals containing phenolic compounds have been reported to be associated with antioxidative actions in biological systems, acting as scavengers of free radicals rendering their usage in herbal medicine. We hypothesized that combining such plant materials with historical efficacy and safety data would give a beneficial boost in their indication for overall liver health. In the screening process, we selected and tested 38 plant extracts (plants list not shown) collected through legacy mining leading to the discovery of a composition designated as SAL which comprised *Schisandra chinensis*, *Artemisia capillaris*, and *Aloe vera*.

Schisandra chinensis, also known as Wuweizi and Wurenchum, is traditionally used for conditions of lung and kidney insufficiency. It is also indicated in cases of chronic cough and dyspnea, diarrhea, night sweats, wasting disorders, irritability, palpitations, and insomnia, as well as a general tonic for treating fatigue associated with illness [11]. In modern pharmacotherapy, mounting experimental and clinical evidences suggest the hepatoprotective nature of *Schisandra* extracts preventing carbon tetrachloride induced hepatotoxicity and glutathione depletion and stimulating the activity of glutathione reductase [12–14]. The major active principles of *Schisandra* are lignans called schisandrins, which have energizing properties by increasing the activity of some enzymes which participate in the oxidative phosphorylation process and also increased superoxide dismutase and catalase activities in rat liver cytosol and were able to inhibit gossypol-induced superoxide anion generation in rat liver microsomes [15]. The hepatoprotective effect of *Schisandra* fruit extracts has been reported in Chinese literature for the treatment of patients with hepatitis, in a clinically controlled trial resulting in 68% (72/107) and 44% (36/72) improvement in serum ALT levels within 4 weeks and 8 weeks [16].

Artemisia capillaris, with the common name "yinchen" or "yinchenhao" in Chinese depending on the different collection season, also known as "yinjin" in Korean, is one of the commonly used TCM included in various ancient Chinese dispensatories. The earliest record of *Artemisia capillaris* was recorded in *Shen Nong Ben Cao Jing* (*The Classic of Herbal Medicine*)—a Chinese book of agriculture and medicinal plants—for treating jaundice, removing the dampness, and use as a diuretic. Both aqueous extracts and ethanol extracts have been reported with hepatoprotective efficacy in both *in vitro* assays and *in vivo* animal studies [17, 18]. Catechins, coumarins, flavonoids, organic acids, water soluble polysaccharides, and polypeptides have been reported as active components responsible for the liver protective activities of *Artemisia capillaris* [18].

Aloe vera N-931 is a composition containing a unique combination of 1–4% aloesin and 96–99% 200 : 1 *Aloe vera* inner leaf gel powder with not less than 8% polysaccharides blended via a conventional method (Aloecorp, USA). Chromones isolated from various *Aloe* species have been reported to have diverse biological activity. A C-glycosyl chromone isolated from *Aloe barbadensis* demonstrates anti-inflammatory activity [19] and antioxidant activity similar to that of alpha-tocopherol based on a rat brain homogenates model [20]. Aloesin is a C-glycosylated 5-methylchromone with a potent antioxidation activity [21].

Here we implemented the most frequently used animal model with practical clinical implications such as acetaminophen and confirmed findings with the classic carbon tetrachloride induced hepatotoxicity model to assess the effect of the composition SAL in protecting liver from such damage. In addition, the merit of combining *Schisandra chinensis*, *Artemisia capillaris*, and *Aloe barbadensis* was also evaluated.

2. Materials and Methods

2.1. The Composition. SAL is a novel synergistic composition containing *Artemisia capillaris* extract, *Schisandra chinensis* extract, and *Aloe vera* composition, N-931. *Artemisia capillaris* extract was produced as 70% ethanol extracts of the aerial parts with no less than 3% chlorogenic acid. *Schisandra chinensis* extract was standardized as 70% ethanol extract of the fruits containing at least 2% total schisandrins. N-931 is a unique combination of 1–4% aloesin and 96–99% 200 : 1 *Aloe vera* inner leaf gel powder with not less than 8% polysaccharides provided by Aloecorp, Mexico. *Schisandra* extract, *Artemisia* extract, and *Aloe* N-931 were blended at a ratio of 4 : 8 : 3 to produce the standardized SAL composition containing no less than 0.2% schisandrins from *Schisandra chinensis* and 1.0% chlorogenic acid from *Artemisia capillaris* and N-931 with the above specifications.

2.2. Animals and Housing. Purpose bred female CD-1 mice, weighing 18–24 g, were purchased from USDA approved laboratory animal vendor (Charles River Laboratories, Inc., Wilmington, MA) and acclimated upon arrival for a week. Individual cages were identified with a cage card indicating project number, test article, dose level, group, and animal number. The Harlan T7087 soft cob bedding was used and changed at least twice/week. Animals were provided with fresh water and rodent chow diet #T2018 (Harlan Teklad, 370W, Kent, WA) *ad libitum* and were housed in a temperature controlled room (22.2°C) on a 12-hour light-dark cycle. All animal experiments were conducted according to institutional guidelines congruent with the guide for the care and use of laboratory animals and approved by IACUC with approval #SAL-441-14/15.

2.3. Model Inductions. A balanced therapeutic schedule was generated and optimized as follows to address prophylaxis and intervention: for APAP-induced hepatotoxicity model, APAP (Lot #MKBQ8028V, from Sigma) at a dose of 400 mg/kg dissolved in warm saline (Lot #132908 from G-Biosciences, Lot #720729 from Quality Biological) (heated to 60°C and cooled down to ambient temperature) was orally administered to overnight fasted CD-1 mice to induce toxicity [6, 7]. For the CCl_4 induced hepatotoxicity model, CCl_4 (Lot #SHBD5351V, from Sigma) at a dose of 25 μL/kg dissolved in corn oil was administered intraperitoneally to overnight fasted CD-1 mice to induce toxicity [14, 22, 23]. For both models, materials were administered at −48 hr, −24 hr, and −2 hr before APAP or CCl_4 administration and +6 hr after induction. Materials were administered at (a) 400 mg/kg, 325 mg/kg, and 250 mg/kg of composition SAL for dose-response study; (b) 106.7 mg/kg, 213.3 mg/kg, 80 mg/kg, and 400 mg/kg of *Schisandra chinensis*, *Artemisia capillaris*, N-931, and composition SAL, respectively, for synergy determinations; (c) 300 mg/kg of each *Schisandra chinensis*, *Artemisia capillaris*, N-931, and composition SAL for functional tests and comparative study; and (d) 400 mg/kg and 50 mg/kg of composition SAL and UDCA, respectively, for activity confirmation tests. In total, the mice received 3 doses before the chemical induction and a dose after the chemical induction. 10% Tween-20 (Lot #0134C141 from Amresco) was used as a carrier vehicle for all the materials. Control mice with or without APAP or CCl_4 received carrier vehicle only.

2.4. Hepatic Function Test. Serum was isolated from blood drawn at T24 using serum separator tube after 30-minute room temperature clotting and spun at 3000 rpm for 10 minutes for ALT (alanine aminotransferase), AST (aspartate aminotransferase), total bilirubin, conjugated and unconjugated bilirubin, bile acid, total protein, albumin, globulin, and alkaline phosphatase monitoring in an automated colorimetric assay using Beckman Coulter AU2700 at Phoenix Laboratories (Everett, WA).

2.5. Glutathione (GSH) and Superoxide Dismutases (SODs) Measurements. Liver tissues were collected immediately after necropsy and were kept in dry ice until transferred to −80°C. Materials were then shipped to a contract laboratory (Brunswick Laboratories, 200 Turnpike Road, MA 01772, USA) in dry ice for final specimen processing and biomarker analysis. (A) Sample preparation: Frozen tissue was ground to a course powder using a pulverizer. 1 mL of PBS containing 19.6 μM EDTA was added to ~0.2 g of ground tissue and homogenized for 1 min in ice bath using a homogenizer from Omni International. The mixture was then centrifuged for 15 min at 10,000 rpm at 4°C. A portion of the supernatant was used for SOD and protein analysis. The rest of supernatant was further processed as follows for GSH analysis. (B) GSH analysis: portion of the supernatant was mixed with the same volume of 100 mg/mL MPA solution to deproteinized in order to avoid interference from proteins. The mixture was allowed to stand at room temperature for 5 min after vortexing and then centrifuged for 15 min at 10,000 rpm at 4°C. The deproteinated supernatant was evaluated for GSH content. Glutathione (GSH) is a key intracellular tripeptide thiol that helps protecting cells from free radical damage by providing reducing equivalents for the reduction of lipid hydroperoxides. During this process, oxidized glutathione (GSSG) forms as a reaction product. GSH level has been used as indicative biomarkers of *in vivo* oxidant and oxidative stress level in cells and tissues. In this analysis, the sulfhydryl group of GSH reacts with DTNB (5,5′-dithio-bis-2-(nitrobenzoic acid)) to produce a a yellow colored 5-thio-2-nitrobenzoic acid (TNB) product. The amount of GSH in the deproteinated supernatant is determined via measurement of the absorbance of TNB at 410 nm. A Glutathione Assay Kit from CAYMAN Chemical Co., Inc. (Ann Arbor, Michigan), was used for analysis. (C) SOD and protein analysis: superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. SOD is considered one of the most important antioxidant enzymes *in vivo*. The SOD assay is a colorimetric assay, which utilizes a tetrazolium salt to measure the dismutation of superoxide radicals that were induced by xanthine oxidase and xanthine, and the activity of SOD in a given sample is quantified by the standard curve generated using the SOD standards. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radicals. A Superoxide Dismutase Assay Kit from CAYMAN Chemical Co., Inc., was used for analysis. The protein concentrations of the tissue homogenates were determined by assessing protein concentrations of the suppressants via a Pierce™ BCA Protein Assay Kit. (D) Materials and equipment: Homogenizer (cat number TH-01) from Omni International (Kennesaw, GA); Hard Tissue Omni Tip™ Plastic Homogenizing Probes (7 mm × 110 mm) from Omni International (Kennesaw, GA); Refrigerated Centrifuge (model number 5402) from Eppendorf (Hauppauge, NY); and Microplate Reader (model number Synergy HT) from US Biotek (Shoreline, WA) were used.

2.6. Histopathology. Liver samples were fixed in 10% buffered formaldehyde and embedded in paraffin wax for histological examination. All microsectioned (5 μm) slides were stained with haematoxylin/eosin and analyzed under the microscope system (Olympus BX 51 microscope and DP72 digital camera,

Table 1: Dose-correlated liver protection of the SAL composition in APAP/CCl_4 induced hepatotoxicity model.

Composition	N	Dose (mg/kg)	Dose (mg/kg) L498/R684/N-931	APAP (400 mg/kg)		CCL_4 (25 μL/kg)	
				Serum ALT (mean ± SD)	p values	Serum ALT (mean ± SD)	p values
Control (−)	10	—	0	37.4 ± 8.7	—	21.4 ± 4.7	—
APAP/CCL_4	10	—	0	8558.6 ± 2297.6	—	10616.4 ± 3386.3	—
SAL	10	250	66.7/133.3/50	5600.4 ± 3399.8	0.05	7445.3 ± 2472.2	0.02
	10	325	86.7/173.3/65	4406.0 ± 3040.5	0.007	6417.8 ± 2421.0	0.003
	10	400	106.7/213.3/80	4065.1 ± 2046.9	0.004	5697.3 ± 2697.4	<0.001

Mice (n = 10) were orally given composition SAL at doses of 250, 300, and 400 mg/kg at −48 hr, −24 hr, −**and** 2 hr before APAP or CCl_4 administration and +6 hr after induction of model suspended in 10% Tween-20. Hepatotoxicity models were induced using 400 mg/kg and 25 μL/kg APAP and CCl_4, respectively. APAP/CCL_4: the vehicle group of mice was induced by APAP/CCL_4 and did not receive the SAL. Serum ALT was determined at T24. Data are expressed as mean ± SD. L498 = *Schisandra*, R684 = *Artemisia*, N-931 = *Aloe* polysaccharide with aloesin.

Table 2: Unexpected synergistic effect of *Schisandra chinensis*, *Artemisia capillaris,* and N-931 in liver protection.

Composition	Material	Dose (mg/kg)	N	Percent inhibition of vehicle	
				APAP (400 mg/kg)	CCL_4 (25 μL/kg)
SAL	*Schisandra* (L498) (X)	106.7	10	18.4	17.5
	Artemisia (R684) (Y)	213.3	10	20.8	22.8
	Aloe (N-931) (Z)	80.0	10	20.8	15.0
	Expected**	400	—	48.8	45.9
	Observed¥	400	10	52.8	46.3

Data of serum ALT are presented as percentage change of vehicle. Mice (n = 10) were given composition SAL (400 mg/kg), *Schisandra* (106.7 mg/kg), *Artemisia* (213.3 mg/kg), N-931 (80 mg/kg), and vehicle at −48 hr, −24 hr, and −2 hr and +6 hr after induction of model suspended in 10% Tween-20. ** Calculated value according to Colby's equation = $A + B - C$; that is, $A = (X + Y + Z)$, $B = (XYZ)/10000$, and $C = ((XY) + (XZ) + (YZ))/100$. ¥Data observed when a composition was orally administered at 400 mg/kg, when observed ≥ expected = unexpected synergy.

Olympus Optical Co., Ltd., Tokyo) with a magnification ×200.

2.7. Statistical Analysis. Data were analyzed using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA). The results are represented as mean ± standard deviation. Statistical significance among groups was calculated by means of single factor analysis of variance (ANOVA) and by t-test. p values less than or equal to 0.05 ($p \leq 0.05$) were considered as significant. When normality test failed, for nonparametric analysis, data were subjected to Mann-Whitney sum ranks for t-test and Kruskal-Wallis one-way ANOVA on ranks for ANOVA.

3. Results

3.1. Dose-Response Effect of SAL. The optimum dosage of the composition SAL that incurs significant liver protection was evaluated both in APAP and in CCl_4 induced models. Mice were given orally the composition SAL at doses of 400 mg/kg, 325 mg/kg, and 250 mg/kg suspended in 10% Tween-20. As seen in Table 1, in the APAP group, dose-correlated inhibitions in serum ALT were observed for the composition. 52.5% ($p = 0.004$), 48.5% ($p = 0.007$), and 34.6% ($p = 0.05$) inhibitions were observed for mice treated with doses of 400 mg/kg, 325 mg/kg, and 250 mg/kg SAL, respectively. Similarly, in the CCl_4 group, dose-correlated inhibitions in serum ALT were observed for the composition. 46.3% ($p < 0.001$), 39.5% ($p = 0.003$), and 29.9% ($p = 0.02$) inhibitions were observed for mice treated with doses of 400 mg/kg, 325 mg/kg, and 250 mg/kg SAL, respectively. There was a 100% survival rate for all the groups in both models. Compared to the normal control animals that received 10% Tween-20, administration of APAP and CCl_4 caused a 229- and 496-fold increase in serum ALT, respectively. The composition SAL has provided statistically significant protection from liver damage at a dosage level as low as 250 mg/kg as determined by serum ALT level when compared to vehicle treated diseased mice.

3.2. Unexpected Synergy. The efficacy of individual plants was tested including *Schisandra*, *Artemisia*, and N-931 at a dosage equivalent to each plant ratio in the composition of SAL as they appear in 4S : 8A : 3L at the highest dose tested (400 mg/kg). An average of 20% inhibition with 70–80% survival rates was observed for these plants at the given dose. Colby's equation [24] was utilized to evaluate the benefit of combining *Schisandra chinensis*, *Artemisia capillaris*, and N-931 in both APAP and CCL_4 model. As shown in Table 2, the observed values were greater than the expected hypothetical values in both the models indicating the existence of synergy in formulating three ingredients at a specific ratio in SAL. The merit of blending *Schisandra*, *Artemisia*, and N-931 was confirmed by their synergistic protection from liver damage caused by APAP and CCl_4 induction.

3.3. Liver Protection Activity of Composition SAL Compared to Its Individual Components. Both APAP and CCl_4 induced liver toxicity models were utilized to compare the liver

Table 3: Effect of SAL (300 mg/kg) on the major biomarkers of liver in APAP model.

Group	Material dose (mg/kg)	N	Survival rate	Analyte				
				AST (U/L)	Bile acid (μmol/L)	T. bilirubin (mg/dL)	Albumin (g/dL)	T. protein (g/dL)
Control	0	10	100	77.7 ± 28.3	1.0 + 0.0	0.1 + 0.0	2.67 + 0.09	4.70 + 0.24
APAP (400 mg/kg)	0	10	60	4707.7 ± 2899.1	76.2 + 24.8	0.5 + 0.2	2.33 + 0.20	4.43 + 0.22
SAL	300	10	90	1855.7 ± 1859.6*	15.1 + 5.7*	0.3 + 0.1*	2.71 + 0.12*	4.84 + 0.12*

Mice ($n = 10$) were orally given composition SAL at doses of 300 mg/kg at −48 hr, −24 hr, and −2 hr before APAP administrations and +6 hr after induction of model suspended in 10% Tween-20. Hepatotoxicity models were induced using 400 mg/kg APAP administered orally. Serum was collected at T24. Data are expressed as mean ± SD. * $p \leq 0.05$.

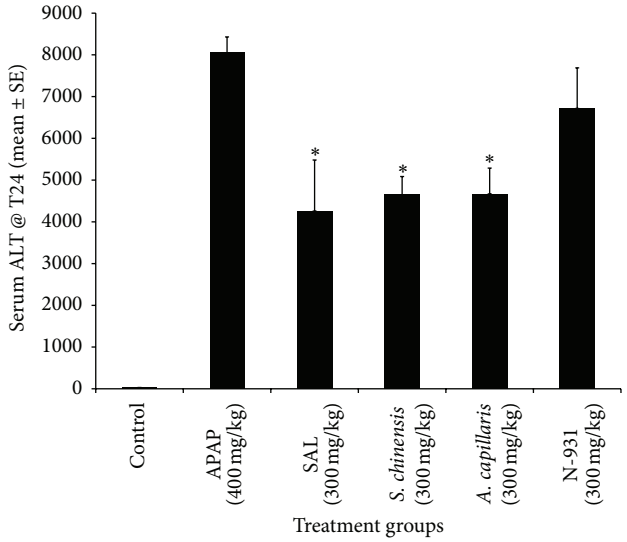

Figure 1: Liver protection activity of the composition of SAL against its individual components at a Dose of 300 mg/kg in APAP-induced hepatotoxicity model. * $p \leq 0.05$.

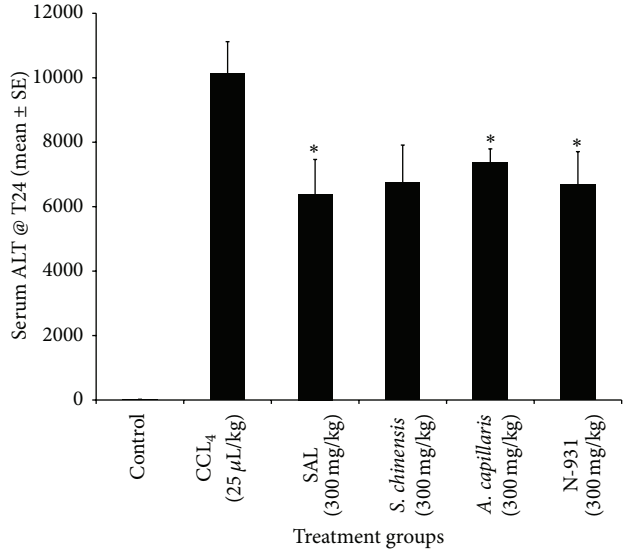

Figure 2: Liver protection activity of the composition of SAL against its individual components at a dose of 300 mg/kg in CCl_4 induced hepatotoxicity model. * $p \leq 0.05$.

protection activity of the SAL composition against its individual components at a dose of 300 mg/kg using reduced serum ALT level as a measure of efficacy. As seen in Figures 1 and 2, the composition (SAL) showed enhanced liver damage protection compared to vehicle in the APAP model. Statistically significant 47.1, 42.2, 42.0, and 16.6% reductions in serum ALT were observed for mice treated with SAL, *Schisandra*, *Artemisia*, and N-931 compared to vehicle group, respectively. In this study, the lowest survival rate (50%) was observed for mice treated with *Artemisia* while survival rates of 90, 70, and 70 were observed for the composition, *Schisandra*, and N-931, respectively. Further substantiating the evidence observed in APAP model, the composition SAL showed greater liver protection than each individual component at a dose of 300 mg/kg in the CCl_4 model using serum ALT as a measure of efficacy. Reductions of 37.0, 33.6, 37.4, and 34.2% were observed for SAL, *Schisandra*, *Artemisia*, and N-931, respectively. There was a 100% survival rate for all the groups in this model.

3.4. Moderation of Hepatic Functional Panel. Liver panels such as AST, ALT, total bilirubin, conjugated and unconjugated bilirubin, bile acid, total protein, albumin, globulin, and alkaline phosphatase have been used as a standard screen method for liver health. Both APAP and CCl_4 induced liver toxicity models were utilized to compare the liver protection activity of the composition of SAL (400 mg/kg) against pharmaceutical drug ursodeoxycholic acid (50 mg/kg) using liver panel data as a measure of efficacy. In this report only the CCl_4 data have been depicted for the UDCA comparison. As depicted in Tables 3 and 4, statistically significant moderations in these major biomarkers were observed when induced mice were treated with the composition of SAL at a dose of 400 mg/kg. In the APAP model, 60.6% and 80.2% reductions in serums AST and bile acid, respectively, were observed for mice treated with the composition when compared to vehicle treated induced mice. Statistically significant increases in serums albumin and total protein were observed in the same model. The 90% survival rate observed in the SAL treated group, compared to the 60% in the APAP model, is a reflection of liver protection that occurred as a result of the composition. In the CCl_4 model, 34.0, 44.5, 26.6 and 63.6% reductions in serums ALT, AST, bile acid, and direct bilirubin, respectively, were observed for mice treated with the composition (Table 4). For the comparison purpose,

Table 4: Effect of SAL (400 mg/kg) on the major biomarkers of liver in CCl_4 model.

Analyte	Control ($n = 10$)	CCL_4 (25 μL/kg) ($n = 9$)	CCl_4 (25 μg/kg) + SAL (400 mg/kg) ($n = 9$)	CCl_4 (25 μg/kg) + UDCA (50 mg/kg) ($n = 10$)
ALT (U/L)	20.0 ± 6.5	9796.5 ± 2223.4	6466.6 ± 2696.5*	7352.1 ± 3157.4
AST (U/L)	69.9 ± 16.1	5031.8 ± 1510.2	2794.0 ± 1427.2*	2957.3 ± 1451.6*
T. bilirubin (mg/dL)	0.17 ± 0.05	0.40 ± 0.11	0.31 ± 0.09	0.36 ± 0.10
Direct bilirubin (mg/dL)	0.00 ± 0.00	0.11 ± 0.03	0.04 ± 0.05*	0.06 ± 0.05*
Indirect bilirubin (mg/dL)	0.17 ± 0.05	0.29 ± 0.09	0.27 ± 0.07	0.30 ± 0.08
ALP (U/L)	76.6 ± 15.7	139.7 ± 65.5	115.0 ± 19.5	111.5 ± 33.7
Bile acid (μmol/L)	1.2 ± 0.4	30.1 ± 8.6	22.1 ± 7.4*	28.9 ± 12.2
T. protein (g/dL)	4.50 ± 0.19	4.62 ± 0.20	4.61 ± 0.18	4.63 ± 0.18
Albumin (g/dL)	2.42 ± 0.13	2.64 ± 0.07	2.60 ± 0.09	2.60 ± 0.12
Globulin (g/dL)	2.08 ± 0.14	1.98 ± 0.15	2.01 ± 0.18	2.03 ± 0.14

Mice ($n = 10$) were orally given composition SAL at doses of 400 mg/kg and UDCA at doses of 50 mg/kg at −48 hr, −24 hr, and −2 hr before intraperitoneal CCl_4 injection and +6 hr after induction of model suspended in 10% Tween-20. Hepatotoxicity models were induced using 25 μL/kg of CCl_4. Serum was collected at T24. Data are expressed as mean ± SD. * $P \leq 0.05$.

Table 5: Effect of composition SAL on oxidative stress biomarkers in liver homogenates collected from CCl_4induced hepatotoxicity model.

Group	Dose (mg/kg)	N	GSH (nmole/mg of protein)	SOD (U/mg of protein)
Control	0	10	38.26 ± 9.52	19.04 ± 4.20
CCl_4 (25 μL/kg)	0	9[a]	57.87 ± 10.85	15.21 ± 6.09
SAL	400	9[b]	72.91 ± 14.93*	22.89 ± 7.95*
UDCA	50	10	69.07 ± 10.09*	19.48 ± 4.64

* $P \leq 0.05$; [a]Misdosed and hence data for one mouse was excluded; [b]Not enough blood to match liver homogenate data and hence one mouse was excluded. UDCA = ursodeoxycholic acid. Mice received three doses of the composition before model induction and a single dose after model induction.

the pharmaceutical drug UDCA (ursodeoxycholic acid) was tested and showed 25.0, 41.2, 4.0, and 45.5% reductions in serums ALT, AST, bile acid, and direct bilirubin, respectively, when compared to the vehicle control. Among these, only the AST and direct bilirubin values were statistically significant. These reductions were statistically significant. There was a 100% survival rate in the CCl_4 models for both the intervention and vehicle treated groups.

3.5. Effect on Oxidative Stress Biomarkers in Liver Homogenates. Additional confirmatory assays were carried out to assess the effect of the composition of SAL in protecting liver using CCl_4 induced hepatotoxicity model. Mice were given the composition SAL at 400 mg/kg. As shown in Table 5, the composition SAL replenished the depleted hepatic glutathione in association with an increase in hepatic superoxide dismutase. While an intraperitoneal injection of CCl_4 at a dose of 25 μL/kg to mice caused a 20.1% depletion in SOD, 51.3% increases in GSH were found in the liver tissues of these mice. The composition SAL restored the depleted SOD by 50.5% compared to vehicle treated CCl_4 challenged mice. Similarly, a 25.9% increase in tissue GSH was also observed for these mice compared to vehicle treated CCl_4 injected mice. The pharmaceutical drug, UDCA, increased the GSH and SOD level by 19.4% and 28.1% compared to the CCl_4 administered animals. When these biomarker changes observed as a result of UDCA were subjected to a head-to-head comparison against the composition SAL, there were 5.56% and 17.5% increases in the GSH and SOD level, respectively, for the mice treated with the composition SAL, indicating the significance of the composition. These findings, in conjunction with previously disclosed liver panel data, strongly suggest that the SAL composition possesses liver protection activity from oxidative stress elicited by CCl_4 induced liver damage.

3.6. Histopathology Findings. As seen in Figure 3, the liver tissues of the untreated control animals showed normal architecture of hepatic cells with clear cytoplasm, normal Kupffer cells, and normal large nuclei. In the vehicle treated APAP and CCl_4 induced mice the liver tissue showed distorted architecture with extensive area of necrosis, cytoplasmic condensation, and marked nuclei shrinkage. Some degenerative ballooning and vacuolation were also observed in these groups. On the other hand, discernible normal cellular architecture and lesser degrees of structural changes were evident in mice treated with SAL in both models (Figure 3).

4. Discussions

Hepatoprotective plant extracts have been traditionally used for treatments of liver diseases over centuries. Furthermore, in modern pharmacotherapy, mounting experimental and clinical evidences suggest the hepatoprotective nature of Schisandrae. For example, oral pretreatment of rats with

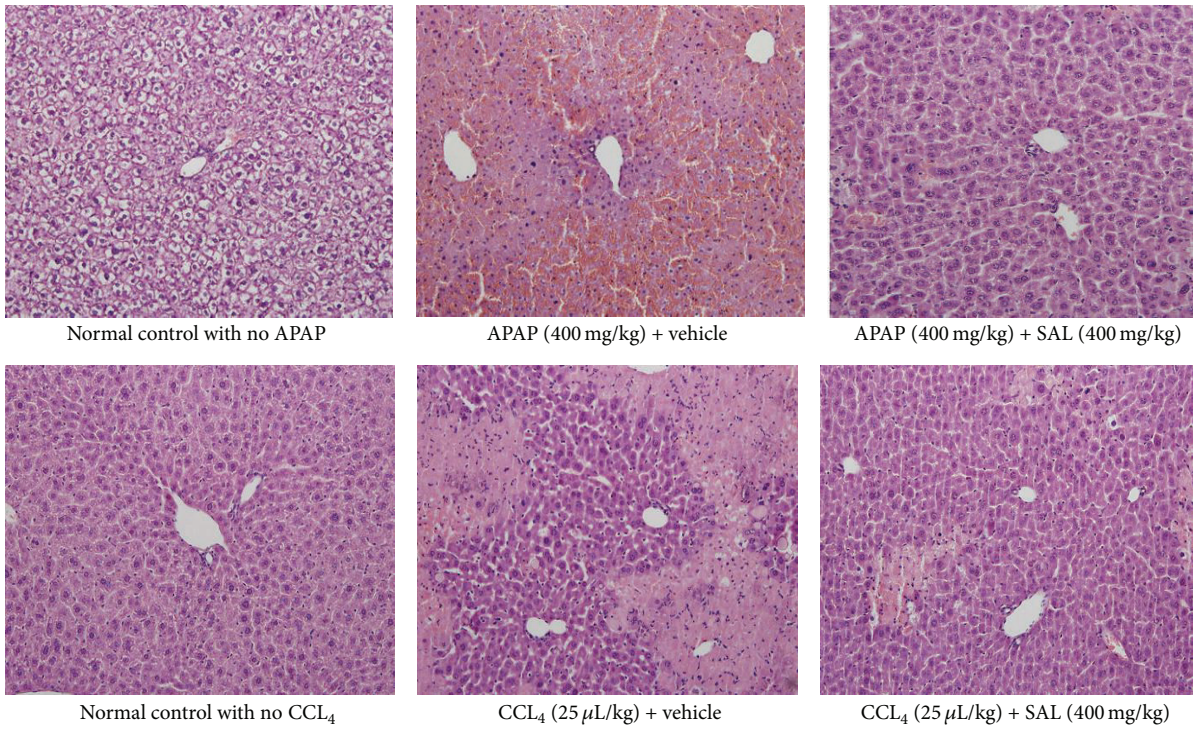

Figure 3: Histopathology of liver tissues from APAP and CCl_4 induced hepatotoxicity models.

a lignan-enriched extract from the fruit of *Schisandra* at a dose of 1.6 g/kg for 3 days prevented carbon tetrachloride induced hepatotoxicity and glutathione depletion and stimulated the activity of glutathione reductase [12]. One of the active constituents of *Schisandra*, gomisin A, administered at an oral dose of 50 mg/kg an hour before acetaminophen injection resulted in statistically significant reduction in serums ALT and AST at 18 and 24 hours after induction minimized degeneration and necrosis also observed in this study [13]. In another publication, administration of gomisin A at a dose of 12.5–50 mg/kg in CCl_4, d-galactosamine, and orotic acid induced hepatotoxicity also showed improved bile flow and liver function in rats [14]. Similarly pretreatment or concurrent administration of wuweizisu C to rats has showed reduced serum transaminase activities and improved histological changes such as fatty degeneration, cell necrosis, and inflammatory cell infiltration, in liver injuries induced by a single or repeated administration of carbon tetrachloride (CCl_4), d-galactosamine, and dl-ethionine [25]. Schisandrol A or schisandrin B administered orally at a dose of 200 mg/kg for 3 days reduced liver malondialdehyde formation when mice were sacrificed 12 hours after liver toxicity induced by administration of 50% ethanol. These compounds also increased superoxide dismutase and catalase activities in rat liver cytosol and were able to inhibit gossypol-induced superoxide anion generation in rat liver microsomes [15]. The mechanism by which schisandrin B exerts its hepatoprotectant effect appears to be through the enhancement of the hepatic mitochondrial glutathione antioxidant status in mice with CCl_4 induced hepatotoxicity [22]. Pretreating mice with schisandrin B at a daily dose of 1 mmol/kg for 3 days protected against menadione-induced hepatic oxidative damage in mice, as evidenced by decreases in plasma ALT (78%) and hepatic malondialdehyde level (70%), when compared with the menadione intoxicated control [26]. In CCl_4 induced liver injury, the hepatoprotective effect of the major components isolated from Schisandrae is believed to be due to their inhibitory effect on lipid peroxidation and the binding of CC14-metabolites to lipids of liver microsomes [23].

More than 5000 case studies of hepatoprotective effects of fruit extracts from *Schisandra* have been reported in Chinese literature with patients with hepatitis; in a clinically controlled trial involving 189 patients with chronic viral hepatitis B and elevated ALT levels, an ethanol extract of the fruits, containing 20 mg of lignans (equivalent to 1.5 g of the fruits), resulted in 68% (72/107) and 44% (36/72) improvement in serum ALT levels within 4 weeks and 8 weeks, in patients receiving the extract and control group, respectively [16].

Besides the description of hepatoprotective effect from *Schisandra* extract, the physiological adaptogenic properties of extracts from *Schisandra* have also been reported. This mechanism will make the host prone to adapt to a state of nonspecific resistance which leads to biochemical changes at the time of exposure to harmful external or internal factors resulting in a more rapid and effective response to the stimuli [27]. By doing so, the extracts increase the host resistance to a wide range of physical, chemical, and emotional stresses while promoting improved overall moderation of physiological processes. Enhancement of liver protections observed in the compositions SAL could be, in part, contributed by the adaptogenic characteristics of *Schisandra*.

In addition to treating various hepatic disorders in traditional oriental medicine, significant experimental reports have been documented to show the hepatoprotective activities of *Artemisia capillaries* as the other active ingredient of the present composition. For example, when aqueous extract of *A. capillaries* was administered orally at a dose range of 50–100 mg/kg for 10 days to mice at which liver injuries were induced by oral administration of 30% alcohol (10 mL/kg, twice/day) plus pyrazole (PRZ, 30 mg/kg), statistically significant reductions in ALT, AST, and malondialdehyde (MDA) levels in serum and liver tissues were observed for mice treated with the extract. In addition, it (a) moderated microvesicular steatosis and necrosis in hepatic histopathology; (b) replenished the antioxidant components including glutathione content and total antioxidant capacity and activities of glutathione peroxidase (GSH-Px) and catalase and SOD; (c) normalized levels of tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) in hepatic tissues; and (d) attenuated the alterations of aldehyde dehydrogenase (ALDH) level in serum and hepatic gene expressions of ALDH and alcohol dehydrogenase (ADH) were the principal markers positively impacted suggesting both enhancement of antioxidant activities and modulation of proinflammatory cytokines as the possible mechanisms that could be involved during the hepatoprotective activity of *A. capillaries* [17]. In 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) induced oxidative stress, rats pretreated with aqueous extract of *A. capillaries* for 7 days at a dose of 7.5 g/kg significantly attenuate serums ALT and AST and improved glutathione levels and enhanced the production of catalase and significantly attenuated the accumulation of thiobarbituric acid-reactive substances in both plasma and liver tissues compared with those of rats given AAPH alone [18]. In an optimal dosage finding study carried out using subacute hepatotoxicity model induced by 10-week injection of carbon tetrachloride to rats, aqueous extract of *A. capillaris* (at 200 mg/kg) resulted in moderations in transaminase activities, MDA, and hydroxyproline concentrations [28]. Knowing the fact that ethanol (EtOH) is almost exclusively metabolized by the liver, a human hepatoma cell lines (Hep G2 cell) were used to study effect of an aqueous extract of *A. capillaris* on alcohol-induced hepatotoxicity *in vitro*. In this study, *A. capillaris* at a concentration range of 0.5–5 μg/mL inhibited the secretion of EtOH-induced interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), IL-1α, and TNF-α-induced cytotoxicity and inhibited the EtOH-induced apoptosis of Hep G2 cells [29]. In a bile duct ligation- (BDL-) induced cholestatic fibrosis model, aqueous extracts of *A. capillaris* administered daily at a dose of 25 or 50 mg/kg for two weeks significantly reduced serum malondialdehyde and liver hydroxyproline levels and restored depleted glutathione content and glutathione peroxidase activity and attenuate cholestatic liver injury and collagen deposition and suppressed expression of fibrogenic factors suggesting the antifibrotic properties through both upregulation of antioxidant activities and downregulation of extracellular matrix protein production [30].

The third ingredient used in formulation of the SAL composition was *Aloe vera* N-931, containing *Aloe* chromone aloesin and *Aloe* polysaccharide. Chromones isolated from various *Aloe* species have been reported to have diverse biological activity. Aloesin is a C-glycosylated 5-methylchromone with a potent antioxidation activity [21, 31]. In a recent study where the phytochemical profile of *Aloe barbadensis* was investigated using colorimetric assays, triple quadrupole and time-of-flight mass spectrometry, focusing on phenolic secondary metabolites in the different leaf portions, the outer green rind that contains aloesin was identified as the most active in radical scavenging activity, which is better than the inner parenchyma in stable radical DPPH test and ORAC assay. Further tests using isolated pure secondary metabolites confirmed that the 5-methylchromones aloesin were among the most active chromones [32].

Moreover, polysaccharides, the major constituents of *Aloe vera* gel, have been utilized for varieties of human disease and suggested for liver protection, in part, because of their antioxidant activities. For instance, strong antioxidant activities have been reported for purified polysaccharides from *Aloe barbadensis* gel when tested in DPPH, hydroxyl, and alkyl radical scavenging assays [33]. Similarly, in *Aloe* plant age and function related study, polysaccharides from three-year-old *Aloe* extract were found showing the strongest radical scavenging activity (72.19%) which was significantly higher than that of synthetic antioxidants butylated hydroxytoluene (70.52%) and α-tocopherol (65.20%) at the same concentrations of 100 mg/L via DPPH assay [34]. Polysaccharides isolated from *A. vera* have also been found to possess high antioxidant efficiency as demonstrated with a decrease in the oxidative stress marker MDA and an increase in the hepatic nonenzymatic antioxidant GSH and enzymatic antioxidant SOD *in vivo* in chronic alcohol-induced hepatotoxicity in mice [35].

Based on analyses of above results and data, it is reasonable to postulate that a composition comprised of these three plant materials possesses significant antioxidant activity and hence protects the liver from oxidative stress caused damage. To the best of our knowledge, these three plant extracts have never been reported to combine together before at specific ratios to formulate the SAL composition on the basis of literature search. In the present study, each component of the composition showed strong individual performances in modulating toxicities induced by the disclosed chemicals reinforcing the idea of combining these plant extracts for a better outcome in both models. This hypothesis needs to be confirmed in a way that the composition of SAL should demonstrate a boosted protection from liver damage elicited by both APAP and CCl_4. When the combinations of these three plant materials were tested, clearly interesting yet, an unexpected synergy was observed from the SAL composition which exceeded the predicted effect based on simply summing the effect observed for each individual ingredient at the given ratio. In fact, none of the individual ingredients showed liver protection activity at the magnitude equivalent to the one noted for the composition in both models separately. Furthermore, data from liver function test including AST, ALT, bile acid, total protein, total bilirubin, conjugated bilirubin, albumin, and total protein demonstrated that the composition has indeed liver protection activity when compared to the vehicle

treated control animals with liver injury. This is supported by the comparison of the effect of SAL composition against a pharmaceutical drug Ursodeoxycholic acid (UDCA). Moreover, as reflected from data of the liver homogenate, the composition SAL also replenished the depleted hepatic glutathione in association with an increased activity in hepatic superoxide dismutase. Glutathione is a key intracellular tripeptide thiol that helps protecting cells from free radical damage by providing reducing equivalents for the reduction of lipid hydroperoxides. Similarly, SODs are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. As a result, SOD is considered one of the most important antioxidant enzymes *in vivo*. These phase II enzymes substantiate each other to provide the strong antioxidant activity of the composition. These findings were substantiated by the histopathological observations signifying a liver protection capability of SAL composition in both APAP and CCl_4 models.

5. Conclusions

Collectively, based on analyses of data from the hepatic function test, antioxidation biomarkers, and histopathological findings, we strongly believe that combining these traditionally well-known folk medicinal plants *Schisandra chinensis*, *Artemisia capillaris*, and *Aloe vera* N-931 into the ratio of 4S : 8A : 3L provided a significantly enhanced liver protection activity to the composition. Therefore, the composition of SAL could potentially be considered as a mitigating agent for alcohol and/or chemical induced hepatotoxicity.

Competing Interests

All authors are current Unigen/Univera employees and therefore with financial interests.

Acknowledgments

The authors would like to express their best gratitude to Dr. Wenwen Ma, Mrs. Lidia, Brownell, and Unigen team for their incalculable support for the completion of this research. The authors would like to extend their utmost gratitude to Mr. Bill Lee, the owner of Econet/Unigen, Inc., who supported the project described in this paper.

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