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B Group Vitamins
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B GROUP VITAMINS - CURRENT USES AND PERSPECTIVES

Edited by **Jean Guy LeBlanc**
and **Graciela Savoy de Giori**

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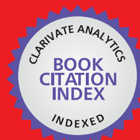

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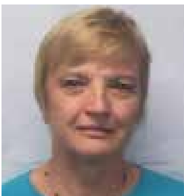

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Preface

This book contains eight chapters dealing with different aspects of B-group vitamins including their roles, uses, and perspectives. Authors from seven different countries (Argentina, India, Japan, Malaysia, Serbia, Turkey and the USA) have written original chapters relevant to these important micronutrients.

This multitopic book is divided into three sections: Folate, Thiamin, and Other B-Vitamins.

In the first section, there is discussion on the use of novel methods such as FTIR to detect folate (vitamin B9) in pregnant women, the use and role of folate in dentistry and the use of genotype notification, such as methylenetetrahydrofolate reductase polymorphisms C677T, to urge patients to increase their intake of foods rich in folates such as green-yellow vegetables with promising original data from the Sakado Folate Project.

The second section involves the description of thiamin (vitamin B1) metabolism in Archaea and the role that this vitamin plays in plants and the current perspectives on crop improvement.

The third section involves a description of riboflavin (vitamin B2) and innovations in improving blood safety and the role niacin (vitamin B3) plays in metabolic stress and insulin resistance in dairy cows.

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Section 1

Introduction

Chapter 1

Introductory Chapter: B-Group Vitamins

Jean Guy LeBlanc

Additional information is available at the end of the chapter

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1. Introduction

Vitamins are organic micronutrients, which are substances that must be present in small quantities and that are essential for the growth and development of the human body and are required in numerous metabolic reactions to maintain homeostasis. The 13 vitamins that are required by human metabolisms are divided as either being fat-soluble vitamins (such as vitamins A (retinols and carotenoids), D (cholecalciferol), E (tocopherols and tocotrienols), and K (quinones)) or water-soluble vitamins (which include vitamin C (ascorbic acid) and the B-group vitamins). In the latter group (B-group), these include: vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B7 (biotin), vitamin B9 (folic acid or folate), and vitamin B12 (cobalamins).

2. Vitamin deficiencies

Although only a very small amount of vitamins is required to avoid deficiencies, and that all 13 vitamins are found in a wide variety of foods, deficiencies are still very common in all parts of the world. There are numerous factors that can explain this problem; one of the most frequent causes is malnutrition, which not only includes the inadequate intake of foods for socioeconomic or psychological reasons, but also includes people that consume unbalanced diets. In this sense, a person can be obese and still not consume enough vitamins to avoid deficiencies, not because of an inadequate consumption of food, but because they might not be consuming a variety of foods that are essential to make sure that the recommended daily intakes are obtained. There are is no magic food that contains all the vitamins, the only way to avoid deficiencies is to consume a variety of foods, which is the base of all the nutritional

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guidelines. In addition to malnutrition, certain diseases and treatments have been shown to affect vitamin absorption or bioavailability. Furthermore, pregnant women and children have a greater need for vitamins because of their increased metabolism during cell replication.

3. Vitamin fortification programs

The consumption of a variety of foods might not be sufficient to meet the recommended intakes of vitamins; food preparation methods and storage conditions can also affect their contents. An example is the case of water-soluble vitamins that are lost during the boiling/cooking of foods. Because vitamin deficiencies are generalized in most populations, some governing bodies have adopted mandatory fortification of foods so that all consume sufficient amounts to avoid deficiencies. These types of programs consist of adding vitamins to foods of mass consumption, which can vary from region to region. In western diets, flours are used for fortification because they are the basis for most prepared foods, whereas in some Nordic countries, milk is fortified due to its high consumption rates, whereas in oriental countries, rice and its derivatives are used for micronutrient fortification.

Although there are numerous beneficial effects that have been demonstrated to result from these mandatory fortification programs, there are concerns that people who consume normal balanced diets might be exposed to elevated and potentially dangerous levels of vitamins. The chemical form of vitamins used in fortification programs has also been questioned by many. A good example is the case of folic acid fortification to decrease folate (vitamin B9) deficiencies, the excessive intake of folate cannot be adequately metabolized in the liver and thus causes an increased concentration of this substance in the blood stream and can cause several undesirable side effects such as masking vitamin B12 deficiencies and have even been associated with increased risks for colon, pancreatic, and breast cancers. These problems are becoming more frequent, not only because of mandatory food fortification programs, but also due to the increased consumption of food supplements such as multivitamin preparations, which can be very dangerous if the right doses are not consumed.

4. Alternatives to fortification programs

Food technologists and biotechnologists have been looking for methods to increase or preserve vitamins in foods by different methods. Reducing the exposure to light for the storage of milk using plastics or nontransparent bottles is an early method to reduce the loss of riboflavin (vitamin B2). Biotechnologists have been able to genetically modify crops so that these contain more elevated concentrations of vitamins, but because of current views by most that foods should not be genetically modified, these are not options that could easily be adopted in most countries. Another method of increasing B-group vitamin concentrations in foods is by fermentation. It has been shown that certain strains of fermentative microorganism have the ability to produce B-group vitamins; thus, the adequate selection of strains and fermentation conditions could lead to the development of novel bioenriched foods that contain elevated concentrations of natural forms of vitamins.

5. Conclusions

Even though the term "vitamin" was first coined by Casimir Funk in 1912 and that most research on vitamin requirements was made between the early ninetieth until the mid-twentieth centuries, there are many groups that are actively studying and demonstrating that the role of vitamins in human health, especially in diseases, is still widely misunderstood and need to continue so that our understanding of their roles can evolve from the initial period of discovery.

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The authors would like to thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0697) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2016-0301) for their financial support.

Conflict of interest

No conflict of interests exists with the publication of this chapter.

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Section 2

Thiamin

Chapter 2

Vitamin B1 (Thiamine) Metabolism and Regulation in Archaea

Julie A. Maupin-Furlow

Additional information is available at the end of the chapter

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Abstract

Thiamine is the water-soluble sulfur containing vitamin B1 that is used to form thiamine diphosphate (ThDP), an enzyme cofactor important in the metabolism of carbohydrates, amino acids and other organic molecules. ThDP is synthesized *de novo* by certain bacteria, archaea, yeast, fungi, plants, and protozoans. Other organisms, such as humans, rely upon thiamine transport and salvage for metabolism; thus, thiamine is considered an essential vitamin. The focus of this chapter is on the regulation and metabolism of thiamine in archaea. The review will discuss the role ThDP has as an enzyme cofactor and the catalytic and regulatory mechanisms that archaea use to synthesize, salvage and transport thiamine. Future perspectives will be articulated in terms of how archaea have advanced our understanding of thiamine metabolism, regulation and biotechnology applications.

Keywords: thiamine, vitamin B1, archaea, thiazole, thiazolium, pyrimidine, sulfur mobilization, riboswitch

1. Introduction

Thiamine or vitamin B1 consists of a thiazole/thiazolium ring [5-(2-hydroxyethyl)-4-methylthiazole, THZ] linked by a methylene bridge to an aminopyrimidine ring (2-methyl-4-amino-5-hydroxymethylpyrimidine, HMP) (**Figure 1A**). Thiamine diphosphate (ThDP) is the best-known form of thiamine, as it is a cofactor. Other natural thiamine phosphate derivatives include: thiamine monophosphate (ThMP), thiamine triphosphate (ThTP), adenosine thiamine triphosphate (AThTP) and adenosine thiamine diphosphate (AThDP) (**Figure 1A**) [1, 2]. These latter forms have yet to be analyzed in archaea and, thus, will not be a focus of this review.

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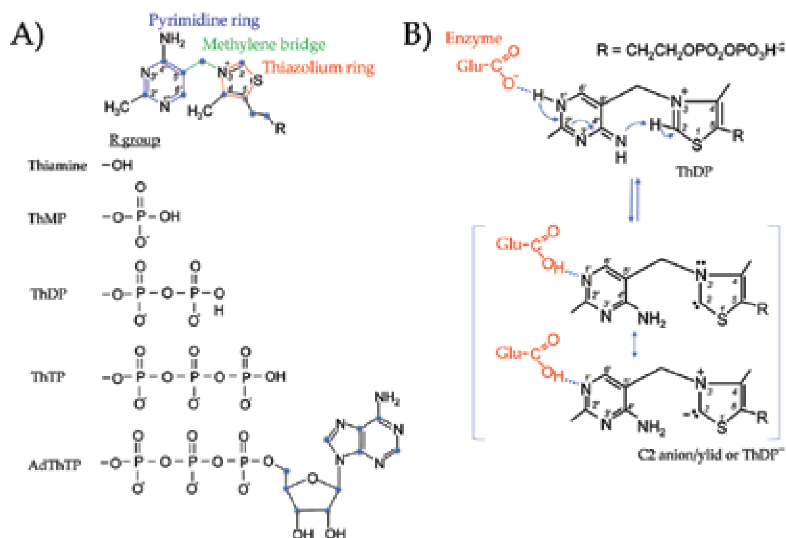

Figure 1. Thiamin (vitamin B1) and its natural forms. A) Thiamin and its natural derivatives thiamin monophosphate (ThMP), thiamin diphosphate (ThDP), thiamin triphosphate (ThTP), and adenosine thiamin triphosphate (AdThTP). The aminopyrimidine ring (blue), thiazolium ring (red) and methylene bridge (green) are highlighted with carbon indicated by C or blue balls. B) Thiamin diphosphate and its C2 anion/ylid form (ThDP-). Enzyme bound ThDP is in a V-conformation, which positions the 4′-amino group of the pyrimidine to abstract the C2-H proton of the thiazolium ring when activated by a conserved glutamate residue of the enzyme (in red). The two resonance structures of the anion/ylid are presented.

2. Thiamine diphosphate

ThDP is an enzyme cofactor found in all domains of life. In archaea and bacteria, ThDP is considered one of the eight universal cofactors along with NAD, NADP, FAD, FMN, S-adenosylmethionine (SAM), pyridoxal-5-phosphate (PLP, vitamin B6), CoA and the C1 carrier tetrahydrofolate or tetrahydromethanopterin [3]. The rare exceptions are the bacteria *Borrelia* and *Rickettsia*, which do not use ThDP as a coenzyme for metabolism [4].

ThDP-dependent enzymes catalyze the cleavage and formation of C-C, C-N, C-S and C-O bonds in a wide range of catabolic and anabolic reactions [5]. As a coenzyme, ThDP serves as an electrophilic covalent catalyst in the decarboxylation of 2-oxo acids (*e.g.*, pyruvate and 2-oxoglutarate) and in carboligation and lyase-type reactions [6–8]. The active species of ThDP is typically the C2 anion/ylid ($ThDP^-$) form, generated by dissociation of the C2-H proton from the thiazole ring (**Figure 1B**). $ThDP^-$ is the source of the catalytic power of ThDP-dependent enzymes, as it can add to unsaturated systems and serve as a sink for mobile electrons [9, 10]. ThDP typically requires Mg^{2+} or Ca^{2+} ions to bind the enzyme in a V conformation in which the 4′-amino group of the pyrimidine ring is positioned to abstract the C2-H proton from the thiazole ring (**Figure 1B**) [11–15]. This proton abstraction is often assisted by a conserved glutamate residue (Glu) of the enzyme that provides a carboxylate side chain for hydrogen bonding to the N1′ of the pyrimidine ring and for proton relay to form the $ThDP^-$ catalytic intermediate (**Figure 1B**). Thus, ThDP is fundamentally distinct among coenzymes in that both rings contribute to catalysis.

ThDP-dependent enzymes are used in pyruvate metabolism, the TCA cycle, the pentose phosphate pathway and branched chain amino acid biosynthesis (**Table 1**). Archaea commonly use ThDP-dependent 2-oxoacid: ferredoxin oxidoreductases (OFORs) to catalyze the oxidative decarboxylation of 2-oxoacids (*e.g.*, pyruvate, 2-oxoglutarate and 2-oxoisovalerate) into an energy rich CoA thioester [16–32] or the reverse reaction to fix CO_2 into cell carbon [33]. ThDP, Mg^{2+} and Fe-S cluster(s) are the intrinsic cofactors of OFORs with ferredoxin as the electron acceptor. OFORs (typically 270 kDa) are less complex than the 5-6 MDa 2-oxoacid dehydrogenases (ODHs) of mitochondria and aerobic bacteria; ODHs rely upon NAD^+ as the electron acceptor and are composed of E1p (ThDP-dependent 2-oxoacid decarboxylase), E2p (lipoate acetyltransferase) and E3p (dihydrolipoamide dehydrogenase) components [16]. While some archaea express mRNAs specific for all three ODH (E1p, E2p and E3p) homologs, ODH activity has yet to be detected in archaea [30]. Other ThDP-dependent enzymes of archaea include the non-oxidative 3-sulfopyruvate decarboxylase of coenzyme M biosynthesis [34, 35] and the acetohydroxyacid synthase of branch-chain amino acid (isoleucine, leucine and valine) biosynthesis [36, 37]. The transketolase activities of archaea [38] are presumed to be catalyzed by ThDP-dependent enzymes based on comparative genomics [39].

Archaea	Bacteria	Eukarya	EC	Enzyme (Abbreviation and Description)	
+	+	+	1.2.4.1	PDH	Pyruvate dehydrogenase (E1p component)
n.d.	+	+	1.2.4.2	OGDH	2-Oxoglutarate dehydrogenase (E1o component)
+ (rare)	+	+	1.2.4.4	BCOADH	Branched chain 2-oxoacid dehydrogenase (E1b component)
+	+	+	2.2.1.1	TK	Transketolase (glycolaldehyde transferase)
n.d.	+ (rare)	+	4.1.-.-	HACL	2-Hydroxyphytanoyl−/2-hydroxyacyl-CoA lyase
+	+	n.d.	1.2.3.3	POX	Pyruvate oxidase (phosphate-dependent)
+	+	n.d.	1.2.7.1	PFOR	Pyruvate: ferredoxin oxidoreductase
+	+	n.d.	1.2.7.3	KGOR	2-Oxoglutarate: ferredoxin oxidoreductase
+	+	n.d.	1.2.7.7	VOR	2-Oxoisovalerate: ferredoxin oxidoreductase
+	+	n.d.	1.2.7.8	IOR	Indolepyruvate: ferredoxin oxidoreductase
n.d.	+ (rare)	n.d.	1.2.7.10	—	Oxalate: ferredoxin oxidoreductase
n.d.	n.d.	+	2.2.1.3	DHAS	Dihydroxyacetone synthase (formaldehyde transketolase)
+	+	+	2.2.1.6	AHAS	Acetohydroxyacid synthase (acetylacetate synthase)
n.d.	+	+	2.2.1.7	DXPS	1-Deoxy-D-xylulose 5-phosphate synthase
+	+	+	2.2.1.9	MenD	2-Succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase
n.d.	+	n.d.	2.5.1.66	CeaS	N2-(2-carboxyethyl)arginine synthase
?	+	?	3.7.1.11	—	Cyclohexane-1,2-dione hydrolase
?	+	+	4.1.1.1	PDC	Pyruvate decarboxylase

Archaea	Bacteria	Eukarya	EC	Enzyme (Abbreviation and Description)	
+	+	n.d.	4.1.1.7	BFD	Benzoylformate decarboxylase
n.d.	+	n.d.	4.1.1.8	OXC	Oxalyl-CoA decarboxylase
?	?	+	4.1.1.43	—	Phenylpyruvate decarboxylase
n.d.	+	n.d.	4.1.1.47	GCL	Glyoxylate carboligase (tartronate semialdehyde synthase)
n.d.	+	n.d.	4.1.1.71	KGD	2-Oxoglutarate decarboxylase
+	+	n.d.	4.1.1.74	IpdC	Indolepyruvate decarboxylase
+	+	n.d.	4.1.1.79	ComDE	Sulfopyruvate decarboxylase
+ (rare)	+	+	4.1.1.82	PnPyDC	3-Phosphonopyruvate decarboxylase
n.d.	+	+	4.1.2.9	PHK	Phosphoketolase (D-xylulose-5-phosphate phosphoketolase)
?	+	?	4.1.2.38	BAL	Benzaldehyde lyase (benzoin aldolase)

Table 1. Thiamin diphosphate (ThDP)-dependent enzymes and their distribution among the three domains of life. Enzyme homolog detected (+), not detected (n.d.), or low homology (?) as indicated.

3. Thiamine biosynthesis *de novo*

Thiamine is synthesized *de novo* by generating thiazole and aminopyrimidine rings separately and then joining the rings to form ThMP, the precursor of ThDP. The *de novo* pathways rely upon energy input (ATP), carbon- and nitrogen-based intermediates and a source of sulfur (the latter incorporated into the thiazole ring).

3.1. Synthesis and phosphorylation of the aminopyrimidine ring of thiamine

ThiC (HMP-P synthase; EC 4.1.99.17) is the major enzyme used by bacteria [40, 41], plant chloroplasts [42] and archaea [43] to synthesize the aminopyrimidine ring of thiamine (**Figures 2**-**4**). ThiC converts 5′-phosphoribosyl-5-aminoimidazole (AIR) to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), thus, diverting carbon/nitrogen skeletons of purine metabolism to thiamine biosynthesis. ThiC is a radical SAM enzyme, that initiates this catalytic reaction by use of a $[4Fe\text{-}4S]^+$ cluster that reductively cleaves SAM to methionine and an 5′-deoxyadenosyl radical [40], a presumed oxidizing cosubstrate of the reaction [44].

THI5 forms the aminopyrimidine ring of thiamine from the substrates PLP and histidine in yeast [45, 46] (**Figure 3**). Only a subset of THI5 family (IPR027939) proteins have the conserved histidine residue needed for HMP-P synthesis [45] and appear restricted to yeast, fungi, plants (non-chloroplast) and select γ-proteobacteria. Bacterial ABC-type solute binding proteins for HMP precursor (ThiY) [47] and riboflavin (RibY) [48] transport are structurally related to THI5. Thus, the archaeal THI5 family proteins, which are devoid of the conserved histidine residue, are suggested to serve a similar role in transport.

ThiD domain proteins are used as bifunctional HMP kinase (EC 2.7.1.49)/HMP-P kinase (EC 2.7.4.7) enzymes in thiamine biosynthesis and salvage (**Figures 2**-**4**). Bacterial ThiD [49, 50] and yeast THI20 and THI21 (N-terminal ThiD domain proteins) [51] phosphorylate HMP-P to HMP-PP in the *de novo* pathway and successively phosphorylate HMP to HMP-PP in the

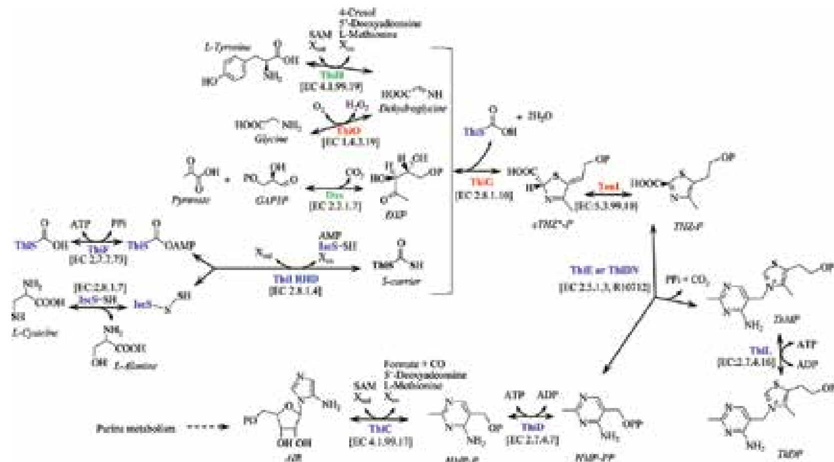

Figure 2. Thiamin (vitamin B1) biosynthesis in bacteria. Enzymes are discussed in text and colored by phylogenetic distribution (red, restricted to one domain of life; blue, found in all domains of life; green, apparent homologs in all domains of life but no direct evidence). Abbreviations: AIR, 5-aminoimidazole ribotide; SAM, S-adenosyl-methionine; GAP3P, D-glyceraldehyde 3-phosphate; HMP-P, 4-aminohydroxymethyl-2-methylpyrimidine phosphate; HMP-PP, 4aminohydroxymethyl-2-methylpyrimidine diphosphate; ThMP, thiamin monophosphate; ThDP, thiamin diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; cTHZ-P, 2-[(2R,5Z)-2-carboxy-4-methylthiazol-5(2H)-ylidene]ethyl phosphate; THZ-P, 4-methyl-5-(β-hydroxyethyl)thiazolium phosphate; X, electron carrier.

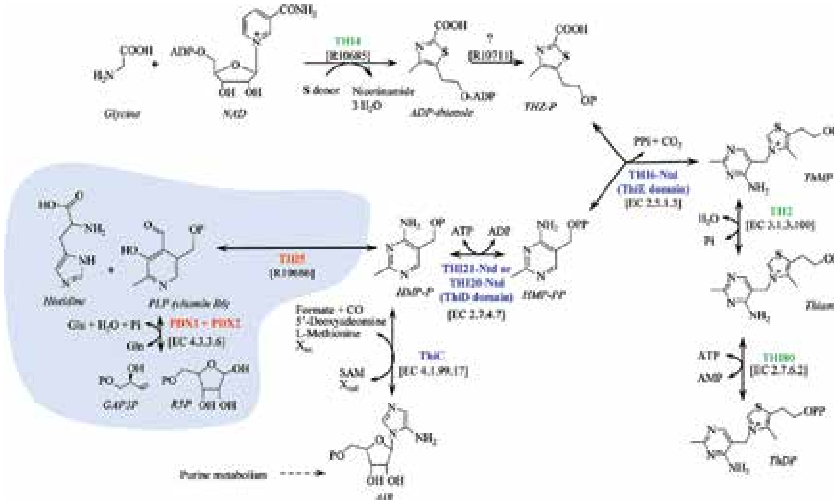

Figure 3. Thiamin (vitamin B1) biosynthesis in eukaryotes. Blue shading indicates restricted to yeast. Abbreviations: ADP-thiazole, ADP-5-ethyl-4methylthiazole-2-carboxylate; PLP, pyridoxal phosphate; R5P, D-ribose 5-phosphate.?, not determined to date. For additional abbreviations and coloring scheme see **Figure 2**.

salvage pathway. Proteins with an unusual ThiD2 domain (standalone or fused to ThiE) are identified in bacteria to catalyze only HMP-P kinase activity, potentially to avoid misincorporation of damaged and/or toxic analogs of HMP into ThDP-dependent enzymes [52]. ThiD homologs (IPR004399) are widespread in all domains of life, including organisms that only salvage HMP and do not synthesize thiamine *de novo*. Archaeal ThiD proteins are standalone or fused to a ThiN-type ThMP synthase domain (see later discussion) [43, 53, 54].

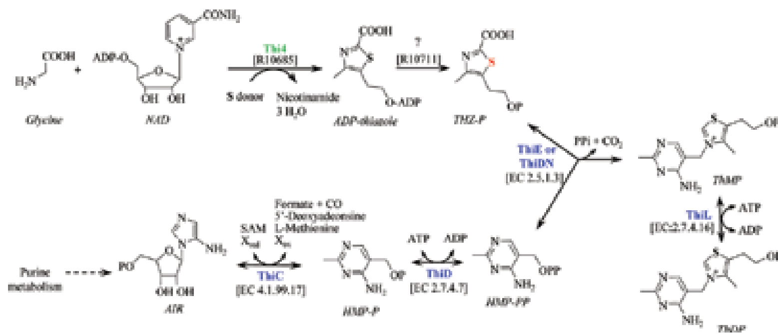

Figure 4. Thiamin (vitamin B1) biosynthesis in archaea. For abbreviations and coloring scheme see **Figures 2**, **3**.

3.2. Synthesis of the thiazole ring of thiamine

De novo biosynthesis of the thiazole ring can be classified into two fundamentally distinct pathways based on the type of thiazole synthase (ThiG vs. Thi4) used. While similar in nomenclature, the ThiG- and Thi4-type thiazole synthases differ in terms of structure and function. The ThiG-dependent pathway relies upon at least six steps to form THZ-P and appears limited to bacteria based on the phylogenetic distribution of ThiG (EC 2.8.1.10) (**Figure 2**). By contrast, the Thi4-type branch for thiazole biosynthesis is simpler in having only two steps (**Figures 3-4**) and appears more widespread, as Thi4-homologs (KEGG K03146) are represented in all domains of life and are demonstrated to function in thiazole ring biosynthesis in yeast [55] and archaea [56, 57].

3.2.1. Synthesis of the thiazole ring of thiamine by the ThiG-pathway

To form the thiazole ring, ThiG uses three substrates: () dehydroglycine, (ii) 1-deoxy-D-xylulose-5-phosphate (DXP) and (iii) thiocarboxylated ThiS [58–61] (**Figure 2**).

(i) Dehydroglycine is synthesized by either oxygen-dependent (ThiO; EC 1.4.3.19) or SAM radical enzymes (ThiH; EC 4.1.99.19), both of which are broadly distributed in bacteria but generally absent in archaea and eukaryotes. The ThiO glycine oxidase catalyzes the oxidative deamination of glycine to form the dehydroglycine required for thiazole ring synthesis [62–65]. By contrast, the ThiH tyrosine lyase forms a 5′-deoxyadenosyl radical that initiates cleavage of the C alpha-C beta bond of tyrosine to generate the dehydroglycine (needed for thiamine biosynthesis) and p-cresol (the byproduct) [66–68].

(ii) The 1-deoxy-D-xylulose-5-phosphate synthase (Dxs; EC 2.2.1.7) is a ThDP-dependent enzyme that condenses the (hydroxyethyl)-group derived from pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate (GAP3P) to generate DXP and CO_2 [69, 70]. Dxs homologs (IPR005477) are widespread in bacteria, green algae, higher plants and protists but rare in archaea. Dxs generates the DXP precursor of thiamine, pyridoxol and non-mevalonate isoprenoid biosynthesis pathways [69, 70]. DXP is used for thiamine biosynthesis in bacteria but not in eukaryotes or archaea (**Figure 2**).

(iii) The ThiG-dependent pathway uses a protein-based relay system to mobilize sulfur to the thiazole ring. Sulfur is transferred from L-cysteine to an active site cysteine residue of a sulfurtransferase (*e.g.*, IscS-SH) [71] to form an enzyme persulfide intermediate (*e.g.*, IscS-S-SH) [72].

In a separate reaction, the E1-like ThiF adenylates the C-terminus of the ubiquitin-fold protein, ThiS, in a mechanism resembling the activation step of ubiquitination [73]. This modification step readies the C-terminus of ThiS for thiocarboxylation. The sulfur is relayed from IcsS-S-SH to ThiS through the ThiI rhodanese (RHD) domain [71, 74–76]. The resulting thiocarboxylated ThiS serves as the sulfur donor for the ThiG mediated synthesis of the thiazole ring [58–61].

3.2.2. Synthesis of the thiazole ring of thiamine by the Thi4-pathway

The Thi4-pathway used to form the thiazole ring (**Figures 3**, **4**) is distinct from that of ThiG (**Figure 2**). Key to the pathway is Thi4-mediated formation of ADP-thiazole, which is then hydrolyzed to THZ-P by a presumed NUDIX hydrolase [55]. Thi4 family (IPR002922) proteins are distributed in all domains of life and generally absent from ThiG-containing bacteria. Although initially annotated as ribose-1,5-bisphosphate isomerases (R15Pi) based on indirect assay [77], archaeal Thi4 homologs are found to be distinct from archaeal R15Pi of the e2b2 family [78, 79] and demonstrated to catalyze thiazole synthase activity [56] that is transcriptionally repressed when thiamine and THZ levels are sufficient [43] and is required for thiazole ring formation [57]. *In vitro*, yeast Thi4 operates by a suicide mechanism by mobilizing the sulfur of its active site cysteine (C205) to form ADP-thiazole from NAD and glycine [55]. By contrast, the methanogen Thi4, uses an active site histidine residue and iron to catalyze the synthesis of ADP-thiazole from NAD, glycine and sulfide [56]. Thi4 enzymes of archaea, yeast [80] and plant [81] are related based on X-ray crystal structure; in addition, yeast Thi4 modified to use an active site histidine residue can operate by a catalytic mechanism with iron similarly to the methanogen Thi4 [56, 80].

3.2.3. Condensation of the aminopyrimidine and thiazole rings to form ThMP

Once formed, the thiamine ring precursors (*i.e.*, THZ-P and HMP-PP) are condensed to ThMP by a ThMP synthase of the ThiE- or ThiN-type (EC 2.5.1.3).

ThiE-type ThMP synthases are widespread in all domains of life (IPR036206) and are found to catalyze the substitution of the diphosphate of HMP-PP with THZ-P to yield ThMP, CO_2 and diphosphate (PPi) in bacteria [82, 83], plants [84] and yeast [85]. ThiE homologs are often bifunctional, fused to an additional catalytic domain such as HMP-P kinase (EC 2.7.4.7) [52, 84, 85]. ThiE serves as a ThMP synthase in certain archaea based on its requirement for growth of haloarchaea in the absence of thiamine, HMP and/or THZ [43].

ThMP synthases of the ThiN-type are also identified in archaea and bacteria, but absent in eukaryotes. ThiN domain (IPR019293) proteins are of three major types: I) fused to an N-terminal DNA binding domain (ThiR type), II) fused to an N- or C-terminal catalytic domain (*e.g.*, ThiD) and III) standalone ThiN domains. The ThiDN proteins are ThMP synthases based on *in vitro* assay and complementation of *ΔthiE* mutants for growth in the absence of thiamine [43, 53, 54]. Fusion of the ThiN domain to the HMP/HMP-P kinase domain (ThiD) is suggested to minimize the release of HMP-PP prior to its condensation with THZ-P and, thus, channel substrate to the ThMP product [43]. ThiN domains that lack a conserved α-helix near the active site histidine are not ThMP synthases and instead can serve as apparent ligand binding sites for transcriptional regulation as in ThiR (see later discussion) [43].

3.2.4. Formation of ThDP from ThMP or thiamine

Thiamine diphosphate (ThDP), the biologically active form of thiamine, is produced from ThMP by two routes. ThMP is commonly phosphorylated to ThDP by the ATP-dependent ThiL ThMP kinase (EC 2.7.4.16 of IPR006283) in bacteria [86] and archaea [87]. Alternatively, ThMP is hydrolyzed to thiamine, and thiamine, is converted to ThDP by a Mg^{2+}-dependent thiamine pyrophosphokinase TPK (THI80) that catalyzes thiamine + ATP ⇋ ThDP + AMP (EC 2.7.6.2) in eukaryotes [88–91]. Consistent with this latter route, TPK is required for the *de novo* biosynthesis of thiamine in yeast [89, 90] and the ThMP phosphatase TH2 can hydrolyze ThMP to thiamine in plants [92]. TPK is also used to salvage thiamine to ThDP in eukaryotes [91, 93] and certain bacteria (TPK homolog YloS) [93]; by contrast, γ-proteobacteria use a thiamine kinase (ThiK, EC 2.7.1.89) to phosphorylate thiamine to ThMP [93] prior to ThiL-mediated phosphorylation of ThMP to ThDP. While TPK (IPR036759) homologs are conserved in some archaea, ThiK is not. Puzzling then is that certain archaea (*e.g.*, haloarchaea and *Pyrobaculum*) have ThiBQP thiamine transport and ThiL ThMP kinase homologs but do not have ThiK or TPK homologs or activities (*e.g.*, *Pyrobaculum californica*) [87]. Furthermore, archaea lacking TPK and ThiK homologs can transport thiamine and generate ThDP as demonstrated by growth of a ThMP synthase mutant, *Haloferax volcanii ΔthiE*, when supplemented with thiamine but not THZ or HMP [43, 57]. These findings suggest that certain archaea use an alternative pathway to salvage thiamine to ThDP.

4. Thiamine transport

Thiamine is a micronutrient that is actively transported into cells against a concentration gradient. Transport of thiamine and its precursors alleviates the need for *de novo* biosynthesis of thiamine. Thiamine transporters are predicted in archaea based on homology to bacterial transport systems or identification of putative transporter genes that are either in genomic synteny with thiamine biosynthesis genes or downstream of ThDP-binding riboswitch (THI- box) motifs [57, 94–96].

Bacterial transporters of thiamine and thiamine precursors, conserved in archaea, can be classified into: (i) ABC-type transporters (*e.g.*, ThiBPQ and ThiYXZ) [47, 97, 98], (ii) a new ABC-type class termed energy coupling factor (ECF) importers [95, 99], (iii) NiaP transporters [100] of the major facilitator superfamily (MSF, IPR036259) that use an ion gradient [101] and (iv) PnuT transporters that mediate the facilitated diffusion of thiamine [102, 103]. ABC and ECF are primary active transporters that hydrolyze ATP in thiamine uptake by use of conserved ATPases (**Figure 5**). ECF and ABC transporters are distinguished by the type of protein used to bind solute: ECF uses a transmembrane substrate-capture protein (S component, ThiT) while ABC uses an extracytoplasmic solute binding protein (*e.g.*, ThiB or ThiY) [95, 99]. ECF systems are typically modular in that ThiT and other S-components (*e.g.*, the biotin specific BioY) interchangeably bind to the transmembrane (T) component of the system [95, 99, 104]. By comparison, ABC systems are not modular and have solute binding proteins (ThiB/Y) that bind to the extracytoplasmic domain of the transporter [47, 48, 105, 106].

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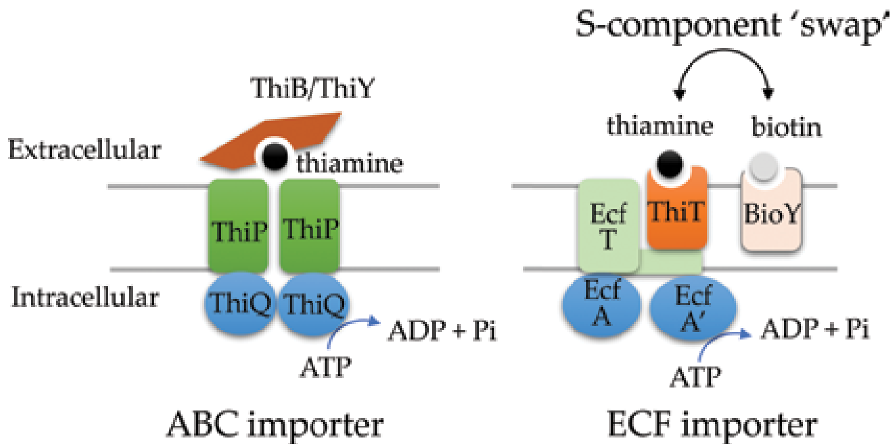

Figure 5. Comparison of thiamin transport by ABC and ECF importers. The nucleotide-binding domains that hydrolyze ATP and drive transporter are shown in blue. The ABC-type transmembrane domain protein (ThiP) and ECF-type Tcomponent (EcfT) are in shades of green. The soluble binding protein (ThiB, ThiY) of the ABC importer is in dark orange. The ECF importer S-components of thiamin (ThiT) and biotin (BioY), which can be swapped, are in shades of orange.

5. Thiamine salvage

Thiamine and its derivatives are salvaged from the outside and inside of a cell to replenish and repair the ThDP cofactor for metabolism. Thiamine salvage pathways are widespread in all domains of life and overcome the need for *de novo* biosynthesis of thiamine, minimize energy cost, and reduce the misincorporation of thiamine breakdown products into ThDP-dependent enzyme active sites [107].

Archaea are found to salvage thiamine and its derivatives (HMP and THZ) from the environment [43, 57] and repress the *de novo* biosynthesis of thiamine when thiamine levels are sufficient [43, 108]. Archaeal salvage pathways are predicted to include enzymes of *de novo* biosynthesis (*i.e.*, ThiD, ThiE or ThiDN, and ThiL) with enzymes specific for salvage such as ThiM (THZ kinase, EC 2.7.1.50), TenA (aminopyrimidine aminohydrolase, EC 3.5.99.2) and/or YlmB (formylaminopyrimidine deformylase, EC 3.5.1.-) the latter speculative as it clusters to a family of proteins (IPR010182) that includes succinyl-diaminopimelate desuccinylase and YodQ of N-acetyl-beta-lysine synthesis [57] (**Figure 6**). ThiM is a THZ kinase in bacteria [49, 109–111], protists [112], and plants [113] and is predicted in archaea (*e.g.*, UniProtKB D4GV40) based on conserved active site residues [114]. TenA homologs are subclassified into TenA_C and TenA_E [115], based on conserved active site cysteine and glutamate residues, respectively. Both types of TenA proteins are conserved in archaea. TenA_C is demonstrated to be an aminohydrolase that works in concert with the YlmB deformylase to regenerate HMP from thiamine degradation products and to function as a thiaminase II that hydrolyzes thiamine to THZ and HMP in bacteria [94, 116]. Note that thiaminase I (EC 2.5.1.2) which is secreted by certain bacteria to degrade thiamine [117, 118] is distinct from TenA. In plants, TenA_E is bifunctional in catalyzing deformylase and aminohydrolase activities to regenerate

Figure 6. Thiamin (vitamin B1) salvage in archaea. Abbreviations: Formylaminio-HMP, N-formyl-4-amino5-aminomethyl-2-methylpyrimidine; amino-HMP, 4amino-5-aminomethyl-2-methylpyrimidine; HMP, 4amino-5-hydroxymethyl-2-methylpyrimidine; THZ, 4methyl-5-(2-hydroxyethyl)thiazole. For additional abbreviations and coloring scheme see **Figures 2-4**.

HMP from thiamine breakdown products, thus, overcoming the need for YlmB [115]. TenA_C and TenA_E are conserved in archaea and likely to function in thiamine salvage.

6. Thiamine regulation

Thiamine biosynthesis, salvage and/or transport pathways are regulated by THI-box riboswitches in bacteria [119–121], eukaryotes [122–125], and a few archaea (based on Rfam RF00059) [43, 96]. The THI-box riboswitch is a regulatory element of an mRNA/pre-mRNA aptamer that binds a thiamine metabolite and an expression platform that transduces the ligand binding to control gene expression [126]. In bacteria, when ThDP levels are sufficient, ThDP binds the 5′ untranslated region (UTR) of the THI-box and triggers the formation of a stem-loop structure that masks the Shine-Dalgarno (SD) sequence of the mRNA and inhibits translation initiation [119–121]. The major targets of this regulation are the mRNAs of the thiamine metabolic operons (*e.g.*, *thiCEFSGH* and *thiMD* in *E. coli*) [119–121] and the ABC-type thiamine transporter (*thiBPQ*), with the latter based on motif analysis (Rfam RF00059). Eukaryotes (plants, fungi, and algae) also use a THI-box riboswitch to regulate expression of thiamine metabolism but do so by modulating the alternative splicing of pre-mRNAs [42, 122–125, 127–130]. In these eukaryotic systems, ThDP or HMP-PP binds the THI-box riboswitch of an intron located in the 5′- or 3′-UTR and causes mispairing of the splice donor (GU) and acceptor (AG) of the pre-mRNA (*e.g.*, *THIC* and *THI4*). This incorrect pairing promotes alternative mRNA slicing and, thus, reduces thiamine biosynthesis.

Thiamine metabolism is also regulated by transcription factors, as exemplified by organisms that synthesize thiamine *de novo* but do not have a THI-box riboswitch motif including yeast and many archaea. In yeast, three proteins (Thi2p, Thi3p, and Pdc2p) coordinate the induction of thiamine biosynthetic (*THI*) gene expression in response to thiamine starvation [131–136].

Thi3p serves as the thiamine sensor for the two transcription factors (Thi2p and Pdc2p) that bind specific DNA sequences upstream of the *THI* genes. When thiamine is low, Thi3p forms a ternary complex with Thi2p and Pdc2p that activates transcription of the *THI* genes. Once the levels of thiamine are sufficient, Thi3p binds ThDP, triggering dissociation of Thi3p from the ternary complex and reduced expression of the *THI* genes. In archaea from the phyla *Euryarchaeota* [43] and *Crenarchaeota* [108], a novel transcription factor, ThiR, is found to repress thiamine metabolic gene (*thi4* and *thiC*) expression when the levels of thiamine are sufficient. ThiR is composed of an N-terminal DNA binding domain and C-terminal ThiN domain. The ThiN domain of ThiR is not catalytic, as it is missing an α-helix extension and conserved Met near the active-site His that are needed for the thiazole synthase activity of ThiDN proteins [43]. Instead the ThiN domain of ThiR serves as an apparent sensor of thiamine metabolites that triggers ThiR-mediated repression of *thi4* and *thiC* transcription during thiamine sufficient conditions. This type of transcriptional regulation appears common in archaea based on the widespread phylogenetic distribution of ThiR homologs vs. THI-box motifs.

7. Future perspectives and conclusions

Thiamine is an important vitamin for improving human health [137], is a strategic nutritional supplement [138, 139], is targeted for production in probiotics [140], is useful in drug discovery including developing antimetabolites to treat cancer or fungal infections [141–144], has potential for use as antitoxic agent in the food industry [145], may improve crop resistance [146], is a starting point for design of novel riboswitches [147], functions in central metabolism and unusual biocatalytic reactions [6–8, 148–151], may modulate global nutrient cycles [152], and holds promise for other applications.

Discovery of the metabolic route for the *de novo* biosynthesis of thiamine in archaea opens a new window for the use of extremophiles in thiamine-related biotechnology applications. Archaea are designated as GRAS (generally recognized as safe) by the FDA, are amenable to genetic manipulation [153], and can readily express ThDP-dependent enzymes from foreign systems (*e.g.*, bacterial pyruvate decarboxylase) [154]. Thus, archaea provide a useful resource to discover and optimize ThDP-dependent biocatalysts for the generation of renewable fuels and chemicals.

Archaea also provide an evolutionary perspective on the origins of thiamine biosynthesis pathways. The aminopyrimidine biosynthesis branch, composed of the radical SAM enzyme ThiC and the HMP/HMP-P kinase ThiD, appears ancient based on its functional conservation in all three domains of life. By contrast, thiazole biosynthesis can be divided into two major pathways: ThiG- and Thi4-dependent. Of these two divisions, the Thi4-type is suggested to be fairly ancient as Thi4 depends on Fe for catalytic activity, can use sulfide as a source of sulfur for thiazole ring formation, is functionally conserved in archaea and eukaryotes, and is predicted to function in certain bacteria (including anaerobes) based on genome sequencing.

Identification of genes needed to transport, synthesize, and salvage thiamine (from the three domains of life) improves understanding of how vitamin B1 may be trafficked in the environment. Finding that Thi4 is important for thiazole ring formation in eukaryotes and archaea provides new perspective on defining the organisms that synthesize thiamine *de novo*. Microbes that produce thiamine and thiamine precursors are suggested to be of benefit to

other microbial taxa that cannot produce thiamine yet require this vitamin as a cofactor for their metabolic activity [152]. Thus, interspecies vitamin transfer may influence the metabolism of microbial consortia and global/carbon energy cycles.

Finally, thiamine is damaged by extreme conditions such as oxidation. Plant and yeast have a hydrolase (Tnr3, YJR142W) that converts the oxy- and oxo-damaged forms of ThDP into monophosphates to avoid misincorporation of the damaged thiamine molecules into the ThDP-dependent enzymes [155]. Many archaea thrive in conditions of extreme thermal and oxidative stress suggesting these microbes use unique mechanisms to avoid and/or repair damaged ThDP for use as a cofactor.

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Conflict of interest

The author has no conflict of interest to declare.

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The Role of Thiamine in Plants and Current Perspectives in Crop Improvement

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Abstract

Current research is focusing on selecting potential genes that can alleviate stress and produce disease-tolerant crop variety. The novel paradigm is to investigate the potential of thiamine as a crop protection molecule in plants. Thiamine or vitamin B_1 is important for primary metabolism for all living organisms. The active form, thiamine pyrophosphate (TPP), is a cofactor for the enzymes involved in the synthesis of amino acids, tricarboxylic acid cycle and pentose phosphate pathway. Recently, thiamine is shown to have a role in the processes underlying protection of plants against biotic and abiotic stresses. The aim of this chapter is to review the role of thiamine in plant growth and disease protection and also to highlight that TPP and its intermediates are involved in management of stress. The perspectives on its potential for manipulating the biosynthesis pathway in crop improvement will also be discussed.

Keywords: thiamine, vitamin B1, plant protection, stress, crops

1. Introduction

Thiamine also known as vitamin B_1 was the first vitamin type B identified [1]. Free thiamine, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) are the three most predominant forms of B_1 that exist in the cells [2]. Vitamin B_1 is a colourless, water-soluble vitamin made solely by plants and microorganisms and act as essential micronutrient in the human diet [3].

Thiamine occurrence in plants is widely distributed across organs, namely, leaves, flowers, fruits, seeds, roots, tubers and bulb [4]. Studies in *Arabidopsis* plant showed that the most abundant vitamer is TPP followed by TMP and thiamine, respectively [5]. The concentration

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of thiamine vitamer can be increased by supplementation of hydroxyethyl-thiazole (HET) and hydroxy-methylpyrimidine (HMP) [3]. The highest concentration of vitamin B in plants can be secured up to μ g/g relatively [6]. Diverse B_1 sources include yeast, cereal grains, beans, nut and meat [7].

2. Role of thiamine

In plants, thiamine is known to have its role as a cofactor for important metabolic activities [8]. Thiamine is known to be an essential regulator that plays an important role in plant's primary regulatory system [9]. Living organisms require the active form of thiamine which is known as thiamine pyrophosphate (TPP) in order to play the role as an important cofactor. TPP is a crucial component required in many metabolic activities such as acetyl-CoA biosynthesis, amino acid biosynthesis, Krebs cycle and Calvin cycle [10].

3. Role of thiamine in plant protection

In plants, thiamine plays a role as a response molecule towards abiotic and biotic stresses, and data from the literature suggest that boosting thiamine content could increase resistance to stresses [11]. Biotic stress is usually involve in the damage of plants caused by living organisms, while abiotic stress is due to environmental factors which cause a series of morphological, physiological, biochemical and molecular changes to plants that will affect the plants' growth, development and productivity [12].

Previous study that the effect of the infection of *Ganoderma boninense*, a pathogenic fungus, to the expression of *ThiC* gene in oil palm suggests that thiamine may play an important role in dealing with biotic stress [13]. Comprehensive studies on the effect of abiotic stresses on the regulation of thiamine in oil palm were also done where various types of stresses, namely, oxidative, salinity and osmotic stresses, have been induced in oil palm where an increase in gene expression and also total thiamine content was observed post-stress inductions [14–17]. A study by Kamarudin et al. explored the application of an endophytic fungus, *Hendersonia toruloidea*, in elevating the expression of thiamine biosynthesis genes in oil palm post-fungal application and also in the accumulation of thiamine and its intermediates in the plant [18, 19]. It was clear that a fungal endophyte could also boost thiamine content in oil palm. Current work is providing data on thiamine accumulation in oil palm seedlings upon application of beneficial endophytic bacteria, namely, *Pseudomonas aeruginosa* and *Burkholderia cepacia*.

Besides that, a study on the impact of *ThiC* promoter as well as its riboswitch on thiamine regulation in *Arabidopsis* sp. showed that the transcript of *ThiC* gene is highest at the end of the light period and lowest at the end of dark period [20]. Other than that, the responses of thiamine biosynthesis genes under several types of abiotic stresses such as salt and osmotic stress in *Arabidopsis* were examined, and it was found that these conditions have caused the

upregulation of the expression of the genes, thus eventually causing significant changes in thiamine level [5].

A study by Croft et al. revealed the declination of *ThiC* gene expression upon exogenous application of thiamine, which suggests a feedback regulation system in thiamine biosynthesis of green alga, *Chlamydomonas reinhardtii* [21]. On the other hand, Mcrose et al. proved that the relative gene expression of prasinophyte algae, *Emiliania huxleyi*, was significantly increased when thiamine supply was exhausted [22]. It has been demonstrated in *Cassava* sp. plant that the application of exogenous thiamine led in the formation of splicing variants of *ThiC* gene suggesting the presence of TPP riboswitch [23].

Thiamine possesses an antioxidant capacity as it has O_2^-/OH^- scavenger properties [24]. Vitamin B_1 is responsible for the recycling of vitamin C through the synthesis of nicotinamide adenine dinucleotide phosphate (NADPH) [4]. The antioxidant properties of thiamine were seen in a study on *Arabidopsis* sp. where paraquat-treated plant caused reduction in protein carbonyls and dichlorofluorescein diacetate (indicator of oxidative stress) when thiamine was applied [25]. Thiamine pyrophosphate indirectly acts as antioxidant by supplying NADH and NADPH to tackle oxidative stress [25]. However, out of all the studies conducted, scientists still find difficulties to unravel the cellular mechanism of B_1 as an antioxidant either through indirect effect of cofactor or as direct effect as antioxidant [4].

Recently, it has been reported that thiamine formed an indirect role in enhancing anti-oxidative capacity in plants, which is important in defence responses [26]. In addition, systemic acquired resistance (SAR) in *Oryza sativa*, *Arabidopsis thaliana*, *Nicotiana* sp. and *Cucumis sativus* was shown to be induced when thiamine was applied to these plants [27].

4. Thiamine biosynthesis is regulated by TPP riboswitch

In the past years, we recognise DNA as the main key on every single reaction that occurs in the cellular environment. The paradigm has been shifted to RNA nowadays. Since RNA sequences can carry out diverse tasks and are amenable to engineering both *in vitro* and *in vivo*, they are particularly attractive for controlling cell behaviour [28].

5. Thiamine pyrophosphate: the dominant class of riboswitch

Riboswitch is a natural RNA sensor that allows the direct binding of small metabolites, thus regulating the expression of various metabolic genes without the needs of protein cofactor [29, 30]. Without the protein involvement, regulation of gene expression can still occur due to the direct metabolite binding at riboswitch sequence [31]. RNA can specifically recognise and bind other molecules, including low molecular weight metabolites [32]. This includes nucleobases, cofactors, amino acids, second messenger and metal ion [33]. The metabolites are usually small, non-toxic molecule which exhibits a good cell permeability [34].

To date, there are about 15 riboswitch classes reported as shown in **Table 1**, and more of it is still unknown [35]. Among all classes of riboswitches, TPP riboswitches are the most ubiquitous in three life domains [36]. Thiamine pyrophosphate (TPP) is the most abundant riboswitch and is known to be present even in eukaryotes [37]. It has an intermediate level of sequence conservation [38]. So in many organisms (prokaryotes, algae, plants and fungi), riboswitch has been found to play the role of regulating thiamine biosynthesis [39].

In all plant taxa, the TPP riboswitch is present in the *ThiC* gene, and some of the TPP riboswitches that are lost during the gymnosperm evolution are present in the *Thi1* gene of ancient plants [40]. Studies by Cheah and co-worker testified that *Thi4* and N-myristoyltransferase (*NMT*) genes in *Neurospora crassa* are controlled by TPP riboswitch by splicing mechanism of an intron located in the 5′ untranslated region (UTR) [39].

From the perspective of evolution, the presence of TPP riboswitch in ancient plant taxa suggests that this mechanism is active 400 million years ago, in early emergence of vascular plants [40]. The ancient plant taxa including ancient land plants consist of supplementary TPP riboswitch which ought to be found in the *Thi1* gene and no longer found, suggesting that during gymnosperm evolution, this sequence might be lost from this family gene [40]. Apart from that, the alternative splicing of 3′ UTR gene also found in lycophytes, which are an ancient vascular plant family that existed around 150–200 million years before angiosperm (i.e. *Arabidopsis* and rice) [40]. **Table 2** shows the list of the discovered TPP riboswitches in various organisms.

Generally, riboswitches in bacteria can be found on the upstream 5′ region of the non-coding region of mRNA, while in plant and fungi, this regulatory element resides at the 3′ end of the untranslated region of a gene [20, 40, 41]. Although the location of TPP riboswitch in prokaryotes and eukaryotes might differ, its structure reveals a high similarity. This difference in location suggests a unique mode of action for the plant riboswitch [40].

Type	Riboswitches class	Gene	Reference
Amino acid derivatives	Purine	*ydhL*	[48]
	Lysine	*Asd*	[41]
	Glycine		[49]
Carbohydrates	Glucosamine-6-phosphate	*glmS*	[50]
Enzyme cofactor	Flavin mononucleotide	*ThiC*	[29]
	Thiamine pyrophosphate	*BtuB*	[51]
	Cobalamin (B_{12})	*S-box*	[52]
	Tetrahydrofolate (THF)		[53]
	S-adenosyl methionine		
	S-adenosyl homocysteine		
Nucleotide precursor	Adenine, guanine	*pbuE*	[54]
	c-di-GMP	*tfoX*	[55]
	pre-queuosine (preQ1)	*ykv*	[56]

Table 1. Riboswitch classes reported across all kingdom of life.

Gene	Location	Organism	Reference
ThiC	3′ UTR	*Arabidopsis thaliana*	[41]
	5′ UTR	*Chlamydomonas reinhardtii*	[21]
	3′ UTR	*Oryza sativa*	[49]
		Poa secunda	[49]
		Solanum lycopersicon	[26]
		Thalassiosira pseudonana	[37]
		Phaeodactylum tricornutum	[37]
		Alishewanella sp.	[36]
		Flowering plant	[58]
Thi4	5′ UTR	*Neurospora crassa*	[39]
		Volvox carteri	[21]
		Fusarium oxysporum	[41]
ThiA		*Aspergillus oryzae*	[52]
ThiM	5′ UTR	*Escherichia coli*	[49]
Thi-box		*Bacillus subtilis*	[29]
		Rhizobium sp.	[60]
Thi1	3′ UTR	Ancient plant (bryophytes, lycophytes)	[40]
ThiR	5′ UTR	*Haloferax volcanii*	[61]

Table 2. The list of RNA regulatory element involved in thiamine biosynthesis pathway, TPP riboswitch, in various organisms.

The biosynthesis of thiamine is uncommon from other vitamins. This is because previous study by Guan et al. revealed that the energy cost of thiamine synthesis is higher as compared to other vitamin cofactors [42]. Therefore, the location of riboswitch at the initial pathway strongly suggests that a novel riboswitch regulates the regulation of thiamine.

6. Thiamine biofortification in plants

As previously mentioned, thiamine has shown to act as cofactor and activator for plant stress and disease resistance. Furthermore, supplementation and accumulation of thiamine in plants showed no evidence of toxicity towards the plants as supported by the feeding studies [3]. However, a review by Goyer in 2010 suggested that thiamine production will be regulated in order to perfectly match the production to the demand of the cofactor. The study also stated that thiamine biosynthesis is regulated via (1) riboswitch-dependent gene regulation and (2) tissue specificity, stress dependence and post-translational regulation. Tissue-specific transcription factors have been found in THI1 gene, and the regulation has been widely studied [43] at the promoter level. The promoter activity in the roots is not due to light regulation but rather to promoter tissue specificity. On the other hand, stress dependence can be seen in maize seedlings where under osmotic and oxidative stresses, TPK enzyme activity increased [44] but exhibited a decrease under normal condition [45]. Furthermore, post-translational regulation or feedback inhibition has been identified in TH1 where excess of HMP-PP and ATP has shown to inhibit TH1 activity.

Gene transcript/ enzymes/thiamine derivatives	Stress	Outcomes	References
THIC	Oxidative, osmotic, temperature (cold), biotic (colonisation by endophyte)	Increase in expression	[15, 18, 25, 62, 63]
	Exogenous thiamine	Decrease in expression	[64]
THI4	Light, oxidative, biotic (colonisation by endophyte)	Increase in expression	[18, 25, 65, 66]
	Dark	Decrease in expression	
TH1	Oxidative, biotic (colonisation by endophyte)	Increase in expression	[18, 25, 45]
TPK	Osmotic, salinity, oxidative, biotic (colonisation by endophyte)	Enzyme activity increase	[18, 44, 45]
Total thiamine	Osmotic, salinity, oxidative, biotic (colonisation by endophyte)	Increase in concentration	[18, 25, 45]
	Exogenous thiamine	Decrease in expression	[64]

Table 3. Effects of stress towards thiamine biosynthesis in plants.

Total thiamine content in wild-type plants is mainly composed of thiamine, thiamine monophosphate (ThMP) and thiamine diphosphate (ThDP) [27]. Overexpression of THIC and THI4 simultaneously has shown to increased thiamine levels up to sixfold and ThDP levels twofold compared to single overexpression of either THIC or THI4 which showed no elevation of total thiamine content [11]. This shows the relationship between thiamine biosynthesis genes and thiamine production. Elevation of thiamine content and also the thiamine biosynthesis gene transcripts in plants have been demonstrated quite extensively via the application of biotic and abiotic stresses. Utilisation of these stresses may aid in the fortification of thiamine in crops. **Table 3** shows the studies done in understanding the effects of the application of stress towards thiamine production in plants.

Apart from that, higher possibilities of thiamine fortification in plants could be achieved via genetic manipulation. Genetic engineering via mutation of riboswitch coding sequence in plant model organism, *Arabidopsis*, has produce an organism with deficiency in TPP riboswitch activity and enhanced accumulation of total thiamine esters [20]. However, due to increasing TPP concentrations, this condition has led to an increase of metabolic flux into the TCA cycle and pentose phosphate pathway which causes a significant increase in the organism respiratory rate, hence more CO_2 production [20]. Genetic manipulation in *Arabidopsis* and rice by overexpression of THIC and THI4 has shown to increase thiamine levels up to sixfold and ThDP levels twofold in *Arabidopsis* and increased total thiamine level up by fivefold in *Oryza sativa* [11, 46]. Furthermore, genetic manipulation of TPK via promoter enhancement in *Arabidopsis* has led to an increased expression of TPK up to 30-fold and transketolase enzyme activity by 2.5-fold [47]. The mutant plant also resulted in chlorotic and slow-growth characteristics. However, levels of total thiamine of mutant plants were significantly lower compared to control.

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7. Conclusion

Overall, based on the extensive studies done, thiamine fortification in plants could be achieve via both abiotic and biotic stress and genetic engineering [20, 24, 25, 45]. Manipulation by the knowledge available on the riboswitch associated with THIC could likely be an effective strategy to manipulate thiamine levels in plants, especially in terms of biofortification. However, it is well agreed that the process on enhancing thiamine levels in plants is not as straightforward and as easy as it seems. Further understanding of the two key precursors (HMP and HET) will be required as this will lead to the accumulation of thiamine, with hopefully least side effects. These two intermediates have been shown to be not toxic to plants, and plant tolerance towards stress is expected to increase when the levels of these two intermediates are enhanced. However, the modification of this will still come with its own challenges since it involves highly complex enzymes which are regulated very tightly and there have not been much studies on the understanding of the mechanisms just yet.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Section 3

Folate

Chapter 4

Nutritional Guidance in Sakado Folate Project

Notification of the C677T Genotype of Methylenetetrahydrofolate Reductase Increased both Serum Folate and the Intake of Green Vegetables

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Additional information is available at the end of the chapter

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Abstract

Background: Serum folate levels are lower in TT homozygotes of the single-nucleotide polymorphism (rs1801133) of methylenetetrahydrofolate reductase (MTHFR) than in CC homozygotes and CT heterozygotes.

Objective: To improve folate status, the genotype was notified to each subject to motivate them to eat more green-yellow vegetables.

Design: Genotype, dietary folate intake, and blood biochemistry were determined and statistically analyzed for 404 subjects (109 males, mean 58.9 years; 295 females, mean 61.8 years). Their serum folate and total homocysteine (tHcy) concentrations were measured before and after receiving nutritional guidance and genotype notification.

Results: The frequencies of the CC, CT, and TT MTHFR genotypes were 35.4, 49.7, and 14.8%, respectively. TT homozygote participants significantly increased their intake of green-yellow vegetables ($p < 0.01$) and of food-derived folate ($p < 0.05$) following nutritional guidance. The increase in serum folate ($p < 0.001$) and the decrease in tHcy ($p < 0.001$) in TT homozygotes following nutritional guidance were more than twice that of the CC homozygote and CT heterozygote participants. An increase in broccoli, spinach and Komatsuna intake was observed following nutritional guidance, irrespective of the season.

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Conclusion: Genotype notification was effective in increasing the intake of green-yellow vegetables and in improving folate status in TT homozygote participants.

Keywords: folate, homocysteine, genome notification, polymorphism, vegetable

1. Introduction

Widespread commercial genotyping direct to consumers (DTC) is anticipated to be applied to personalized dietary recommendations, but it remains unclear if providing individuals with their personal genetic information changes dietary behavior. The authors of this current chapter have tried for 11 years to increase the intake of green-yellow vegetables by the general Japanese population by providing personalized nutritional guidance with genotype notification to improve folate intake. The international standard or recommended dietary allowance (RDA) of folic acid is 400 μg/day to prevent diseases such as spina bifida, stroke, and dementia. The single-nucleotide polymorphism C677T (rs1801133) of methylenetetrahydrofolate reductase (MTHFR) has the effect that TT homozygotes require a higher folate intake than do CT heterozygotes and CC homozygotes. For this reason, Sakado City and Kagawa Nutrition University, Department of Medical Chemistry, cooperated to implement the "Sakado Folate Project" by genotyping the MTHFR polymorphism of subjects and notified participants of their genotype and urged participants to increase their green-yellow vegetable intake to prevent stroke, dementia, and to reduce medical costs [1]. Here we show that genotype notification of the risky TT homozygote is effective.

1.1. RDA of folate in Japan and by the WHO

The RDA of folic acid for adults is only 240 μg/day in Japan [2], but the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) recommend a folic acid intake of 400 μg/day worldwide [3, 4]. Folic acid is a common name for the compound pteroyl monoglutamate. On the other hand, "folate" is an umbrella term that refers to many compounds derived from pteroyl monoglutamate that are not chemically well-characterized, including other derivatives of pteroylglutamates. Folates are differentiated by the reduced state of the pteridine ring, one carbon substitution at the N5 and/or N10 positions (formyl, methyl, methylene, and methenyl), and the length of the γ-polyglutamyl residues [3, 4]. Polyglutamyl 5-methyltetrahydrofolate species are the most abundant naturally occurring folates in vegetables [3, 4]. The bioavailability of synthetic folic acid (monoglutamyl pteridine) is approximately 70% better than that of dietary sources of folate (mainly polyglutamyl pteridine), and therefore, dietary folate equivalent (DFE) is generally used [3, 4]. Thus, the nutritional value of any chemical form of "folate" is expressed as folic acid in the Japanese RDA [2]. Dietary reference intake (DRI) for the Japanese population is based on the estimated average requirement (EAR), defined as satisfying the required amount of a nutrient for 50% of the population [2]. Based on the distribution of the required amount measured in a target group, RDA is defined as the amount satisfying the dietary requirements of most people (97–98%) in a specific population. Thus, RDA is calculated using EAR (EAR +2 × standard

deviations of EAR) [2]. Most folate in food is present as pteroyl polyglutamate substituted with a one carbon unit, and it is tightly bound to MTHFR as coenzyme. The Japanese RDA of folate is insufficient for persons with polymorphisms in genes involved in folate metabolism and in elderly persons with decreased folate bioavailability [1].

The average folate intake in Japan in 2016 was 277 μg/day, which is higher than the Japanese RDA of 240 μg/day for adults. However, the folate intake of women aged 20–29 years was only 236 μg/day in 2016 [5], which is much less than the RDA of 480 μg/day for pregnant women [2]. One reason for this folate deficiency is the low intake of green-yellow vegetables. The daily Japanese average intake of vegetables and green-yellow vegetables was 265.9 and 84.5 g [5], considerably less than the 350 g (76%) and 120 g (70%), respectively, recommended in the report entitled "Ministry of Health, Labor and Welfare Healthy People 21 Japan" [6]. Folate intake higher than these recommended levels by Japanese was quite effective in preventing cardiovascular diseases, as established by the Japan Collaborative Cohort Study on a total of 23,119 men and 35,611 women [7]. Increased dietary folate intake, from <272 to >536 μg/day, resulted in a 51% reduction in mortality for men from heart failure ($P < 0.01$) [7]. Therefore, a folate intake of 243–253 μg/day by the general Japanese population is insufficient to prevent cardiovascular diseases, and thus higher folate intake will help reduce medical costs [1].

1.2. Folate metabolism and homocysteine

In the United States, folate in food is expressed as pteroyl monoglutamate, which is 1.7 fold more effective than pteroyl polyglutamate in food [4] because most coenzyme-type folate (polyglutamyl tetrahydrofolate and its C-1 derivatives) is liberated from MTHFR during the cooking and processing of foods and by protein digestion in the stomach. The liberated pteroyl polyglutamate is digested into pteroyl monoglutamate by the intestinal microvillous enzyme conjugase [8] and is absorbed from the epithelial cells of the upper part of the small intestine by reduced folate transporter [9]. Therefore, the absorption rate of pteroyl monoglutamate is estimated to be about 50% [2, 4]. On the other hand, synthetic folate contained in supplements is a monoglutamyl folate, and 90% is estimated to be absorbed [4]. These relative bioavailabilities of folate were confirmed using deuterium-labeled monoglutamyl tetrahydrofolates and folate in human subjects [10]. The most accurate dietary folate metabolism technique is the dual isotope method using [^{13}C5] folate and [$^{2}H_2$] folate [11]. The metabolism of [$^{13}C_5$] folic acid in fortified white and whole-wheat bread, rice, pasta, or in solution was evaluated in human subjects injected with [$^{2}H_2$] folate [11]. The results indicated no significant differences in bioavailability among the various fortified foods and the control ($p = 0.607$). However, there are personal differences in folate bioavailability partly caused by polymorphism of MTHFR that can lead to cardiovascular diseases [12].

Homocysteine (tHcy), a factor that causes vascular endothelial damage common to cardiovascular diseases, is an amino acid produced by the metabolism of the essential amino acid methionine [13]. Homocysteine produces reactive oxygen species that impair many cell components. Folate, vitamin B_{12} (VB_{12}) and vitamin B_6 (VB_6) are involved in the metabolism of tHcy and decrease the serum tHcy concentration [13]. Homocysteine metabolism occurs mainly by two pathways. One is a remethylation pathway that converts tHcy into methionine. Vitamin B_{12} acts as a coenzyme in methionine synthase (MS), which catalyzes the methylation

of tHcy using 5-methyltetrahydrofolate as a methyl group donor. MTHFR is responsible for the production of 5-methyltetrahydrofolate from methylenetetrahydrofolate [13]. The other pathway for metabolizing tHcy is a sulfur transfer pathway that converts tHcy to cysteine via cystathionine by cystathionine β-synthase, which uses vitamin B_6 as a coenzyme. Therefore, a deficiency in folate, vitamin B_{12}, or vitamin B_6 increases tHcy in the blood [13].

1.3. Genetic polymorphisms related to folate metabolism

The C677T [Ala222Val] mutation (rs1801133) of MTHFR is the single-nucleotide polymorphism of a gene involved in folate metabolism that has the greatest effect on cardiovascular diseases [12, 14] and fetal development [15]. MTHFR (E.C.1.1.1.68) is the enzyme that reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [4]. MTHFR TT homozygote individuals have high serum tHcy [16] and low serum folate levels [16] and have increased risk for cardiovascular diseases [14, 17]. MTHFR encoded by the TT genotype is a homozygote of a point mutation of C677T. This mutant protein is heat-sensitive because one alanine residue is mutated to valine. The enzyme activity of the CT type (heterozygote) is 65% that of the CC genotype (wild-type), and the activity of the TT type (homozygote) is 30% that of the wild type [18]. The mean residual enzyme activities after heat treatment (46°C, 5 min) were 37.0% (34.1–42.6%) in the controls and 15.2 and 15.1% in the two TT homozygotes [18]. The low activity and thermolability of the MTHFR TT homozygote results in decreased production of 5-methyltetrahydrofolate: the pathway from homocysteine to methionine is inhibited, and the tHcy level in the blood rises [18]. A healthy subject has tHcy levels of between 3 and 15 μmol/L, and a tHcy level of 15 μmol/L or higher is referred to as hyperhomocysteinemia [4]. Hyperhomocysteinemia promotes arteriosclerosis via vascular endothelial cell disorder and promotes blood coagulation and smooth muscle cell proliferation [17].

1.4. MTHFR C677T gene polymorphism and dietary intake of folate

The bioavailability of folate is low in TT homozygotes and CT heterozygotes [16]. Approximately 15% of the Japanese population is C677T MTHFR TT homozygotes [16]. TT homozygotes require 400 μg/day of folic acid to increase the serum folate level to that of CC homozygotes and CT heterozygotes [16]. Folate deficiency among persons with dementia was confirmed by meta-analysis of 31 studies [19] and in particular of studies of Japanese with dementia who are TT homozygotes [20]. The risk of brain infarction is 3.4-fold higher in TT homozygotes compared to that in CC homozygotes [21]. A randomized controlled double blind test was performed to confirm the exact folate requirement of Japanese [22]. There are ethnic differences in the prevalence of MTHFR polymorphism [23].

Although the RDA of folate in Japan is 240 μg/day [2], MTHFR TT homozygotes are folate deficient [16]. It is reported that serum folate levels are significantly lower in the TT type than the CC and CT types, and tHcy is significantly higher even if the intake of folate is 240 μg/day [2] as judged by the tHcy level not exceeding 14 μmol/L [2]. We have conducted intervention studies on folate intake by young Japanese women [1, 16, 22] and shown that an intake of 400 μg/day of folic acid causes differences in serum folate between genotypes of the MTHFR C677T gene polymorphism to disappear, even in the case of TT homozygotes [16].

1.5. The significance of vegetable intake

Compared with the intake of folate alone (e.g., as a supplement), the increase in the intake of vegetables targeted in this study is accompanied by an increase in the intake of various vitamins, minerals, and dietary fiber, thereby greatly improving nutrition overall. Reports from the WHO/FAO [24] and WCRF/AICR [25] summarized the relationship between vegetable intake and disease and showed that increased vegetable intake is effective against obesity, cardiovascular disease, type 2 diabetes, and several types of cancer (mouth, pharynx, larynx, esophagus, stomach). Increased fruit intake has been assessed as "probable decreasing risk" or more, and increased ingestion of vegetables and fruits has been proposed for maintaining and promoting health [24, 25].

1.6. Outline of the Sakado Folate Project

To prevent the above-described diseases caused by folate deficiency, and especially in MTHFR TT homozygotes, Sakado City made an agreement called the "Sakado Folate Project" in 2006 with Kagawa Nutrition University [1]. The project includes lectures, genotyping, blood analysis, nutrition surveys, genotype notification, and guidance on increasing the intake of green-yellow vegetables and folate, based on data from the subjects [1]. The lectures provide the subjects with an overview of available biomarkers (serum folate and homocysteine concentrations) and the interpretation of the significance of these biomarkers across a range of clinical and population-based uses. In the same lecture, we explain the genetic polymorphisms and obtain written informed consent in accordance with the instructions of the Declaration of Helsinki. The study procedures were approved by the Kagawa Nutrition University, Human Subjects and Genome Ethics Committee (approval number; no. 134, 300 G).

One month after taking blood samples from the participants, the genotype was announced by the medical doctor to the subjects who agreed to know their genotype. Furthermore, nutritional and exercise guidance was provided by the registered dietitian. To supply adequate folate easily, we developed "folate-fortified rice" containing (per 100 g rice) folate 26.7 mg, thiamin 187 mg, vitamin B_6 66.7 mg, and vitamin B_{12} 320 μg. This rice was developed in collaboration with House Wellness Foods Corporation Company. Specified amounts of this "folate-fortified rice" were mixed with typical rice and boiled before eating [1]. We also developed a folate-fortified bread called Sakado Folate Bread (folic acid 340 ± 21 μg/100 g; 215 ± 14.7 μg/slice/64.0 g bread) [1].

More important aspects of the project included the health education of 101,513 citizens through volunteers, and wider consumption of folate-fortified food, especially folate-fortified rice. According to the official report issued by Sakado City on the nutritional behavior of the participants, after the start of this project, 80% of the participants were aware of the importance of folic acid, 90% tried to eat more vegetables, and 73% wanted to obtain advice on improving their health. Moreover, both "Folate-fortified rice" and "Sakado Folate Bread" are commercially available, and after participating in this program, citizens can continue to improve their folate status by eating these staples fortified with folate rather than by increasing their intake of green-yellow vegetables [1].

Here, we report the effects of genotype notification on increased intake of green-yellow vegetables and the effect on increased serum folate and decreased serum homocysteine levels.

2. Methods

2.1. Subjects and survey method

The total number of participants was 1008 (mean age 62.82 years): 266 males (mean age 63.56 years) and 742 females (mean age 62.55 years). Of these, 396 subjects (104 males with a mean age of 59.87 and 292 females with a mean age of 61.77 years) who were assessed before and after enrolling in the program were selected by excluding 111 participants who had taken vitamin supplements prior to enrollment. From the 404 subjects, a detailed survey was conducted on 249 subjects (78 males; mean age 58.2 years and 171 females; mean age 60.4 years) regarding their green vegetable intake. The data were collected from the beginning of the project from 2006 to 2012.

As outcome measures, we obtained data such as serum folate and homocysteine levels and MTHFR genotype and also analyzed the results of a questionnaire that included food intake, and particularly green vegetable (folate rich vegetables, not green-yellow vegetables) consumption. Following the Sakado Folate Project, we collected the number of green vegetable dishes taken each week in the morning, afternoon, and evening in seven areas of Sakado City using a self-administered questionnaire of monthly intake of green vegetables.

2.2. Blood biochemistry

Venous blood samples were collected in plain and EDTA-containing Venoject tubes from the cubital vein of each participant before breakfast at the beginning and at the end of the Sakado Folate Project [1]. Whole blood was subjected to genomic DNA extraction as described in the next section. The serum was isolated and stored at −80°C until analysis. Serum folate and vitamin B_{12} concentrations were measured at an external laboratory (SRL, Inc., Tokyo, Japan) using a chemiluminescence enzyme immunoassay (Access 2, Beckman Coulter, Inc., CA, USA). Serum total homocysteine concentration was determined by enzyme assay using an Alfressa Auto Hcy kit (Alfressa Pharma, Inc., Osaka, Japan) [26]. In addition to serum folate and vitamin B_{12}, measurements, 28 general biochemistry/hematology parameters were analyzed by SRL Corporation; however, only serum folate and serum homocysteine levels were applicable to this study.

2.3. Genotyping

DNA was extracted from whole blood using a Magtration System (Precision Systems Science Co. Ltd., Chiba, Japan) and magnetic beads [27]. To rapidly and inexpensively genotype a large number of blood samples, we developed an automated genotyping machine using a bead array in a capillary tube [28]. This BIST method specifically genotypes the C677T

single-nucleotide polymorphism (rs1801133) in MTHFR using beads in a straw tip [28]. If necessary, DNA was amplified by polymerase chain reaction and analyzed by electrophoresis in a 10% polyacrylamide gel.

2.4. Evaluation of the intake of nutrients/food groups by FFQ, DHQL, and BDHQ questionnaires

Three questionnaires were previously used to collect data regarding meals. These questionnaires were similar and evaluated vegetable and nutrient intake: FFQ (Food Frequency Questionnaire), DHQL (a larger version of a self-administered diet history method questionnaire), and BDHQ (a brief self-administered diet history questionnaire) [29, 30]. We evaluated the data in the three questionnaires in the same manner. The BDHQ and DHQL questionnaires were obtained from EBN Tokyo, Japan, and we requested automatic counting [29, 30]. Use of a calculation program allowed the food intake records of approximately 40 nutrients and 150 food types to be calculated and to output an individualized document for each subject. The BDHQ questionnaire uses a simplified structure, as well as simplified replies and data processing, while maintaining the characteristics of the DHQ questionnaire [29, 30]. The results of a validity study on BDHQ have been reported in a research report. The calculated nutrients were folate, retinol equivalent (vitamin A), vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, niacin, vitamin B_6, vitamin B_{12}, pantothenic acid, vitamin C, n-3 type fatty acid, and n-6 fatty acid. In addition, the food group could be selected from the following groups: cereals, potatoes, sugar, sweeteners, pulses, green-yellow vegetables, other vegetables, fruits, fish and shellfish, meat, eggs, milk, fats and oils, confections, and seasonings/spices.

2.5. Statistical analysis

Statistical analysis of the current data was conducted using IBM SPSS statistics version 21, and past data were analyzed using programs such as Stat view. The average value and standard deviation were calculated for age and nutrient/food group intake. Multiple regression analysis was used for vegetable intake, continuous variable independent variables (such as folate intake), and continuous variables (such as serum folate). Logistic regression analysis was used as a qualitative dependent variable: for example, in the case of gender, male is 0 and female is 1, and in the case of genetic polymorphism, TT type is 1, CT type is 2, and CC type is 3 as dummy variable calculated.

3. Results

3.1. Genotype distributions of MTHFR polymorphisms

Of the initial 404 subjects, 399 people provided complete data, allowing the frequencies of the CC, CT, and TT MTHFR genotypes to be calculated: 35.4, 49.7, and 14.8%, respectively. Of the 249 people whose vegetable intake was surveyed in detail, the frequencies of the CC, CT, and TT MTHFR genotypes were 36.5, 49.8, and 13.6%, respectively. Prior to obtaining nutritional

guidance, the serum folate levels of the subjects increased according to the number of C alleles, and folate utilization was high (correlation coefficient r = 0.375, p < 0.001), and conversely, homocysteine levels decreased according to the number of C alleles (r = − 0.520, p < 0.001).

Of all the participants (n = 395), only the subjects with TT type polymorphism (n = 57) increased their intake of green-yellow vegetables significantly, from 110.14 ± 4.65 to 139.29 ± 75.74 g after genotype notification and completing the nutritional guidance program (**Figure 1**, p < 0.01).

Intake was increased by 29.15 g (+26.5%) by the TT group, exceeding the national target of 120 g [6], while the increase was 10.44 g (+7.9%) for the TC group and there was no increase by the CC group. Moreover, the intake of food-derived folate also increased only in subjects with the TT type genotype, from 321.22 ± 101.94 to 348.71 ± 100.23 μg after genotype notification and nutritional guidance (**Figure 2**, p < 0.05). The increment in the increase in folate intake by subjects with the TT type genotype was 29.15 μg (+8.6%), exceeding the Japanese RDA of 240 μg [2] but not the WHO RDA of 400 μg [3, 4].

Previous studies [6, 16] reported that subjects with the TT genotype showed lower serum folate levels than subjects with the CT and CC genotypes prior to nutritional guidance (**Figure 3**). However, following genotype notification and nutritional guidance, there was a significant increase in serum folate (**Figure 3**, p < 0.001 in the TT, p < 0.01 in the TC, and p < 0.01 in the CC genotypes) due to increased green vegetable intake by all subjects, regardless of genotype (n = 399).

In contrast to the low serum folate level of subjects with the TT genotype prior to nutritional guidance, subjects with the TT genotype showed the highest serum folate levels, ranging from 6.75 ± 4.40 to 11.80 ± 7.88 ng/ml among three genotypes following nutritional guidance

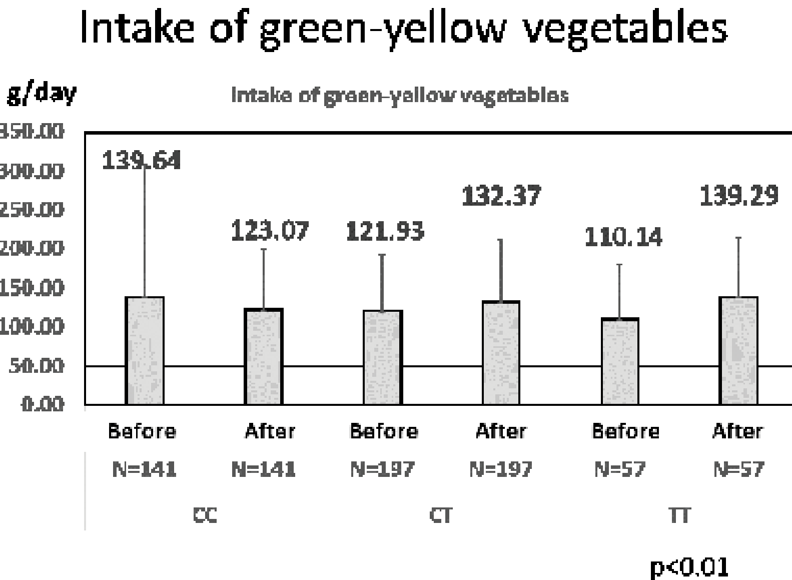

Figure 1. Effect of genotype notification on the intake of green-yellow vegetables by individuals with three MTHFR genotypes: Before: before nutritional guidance. After: after nutritional guidance.

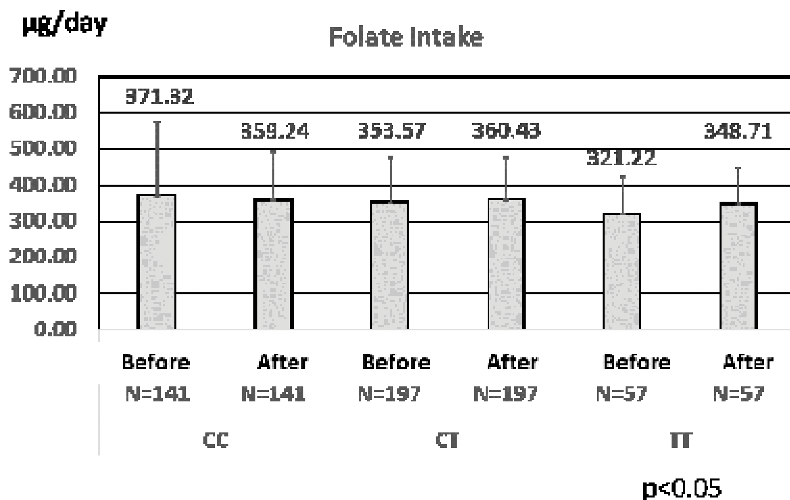

Figure 2. Effect of genotype notification on the intake of dietary folate by individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

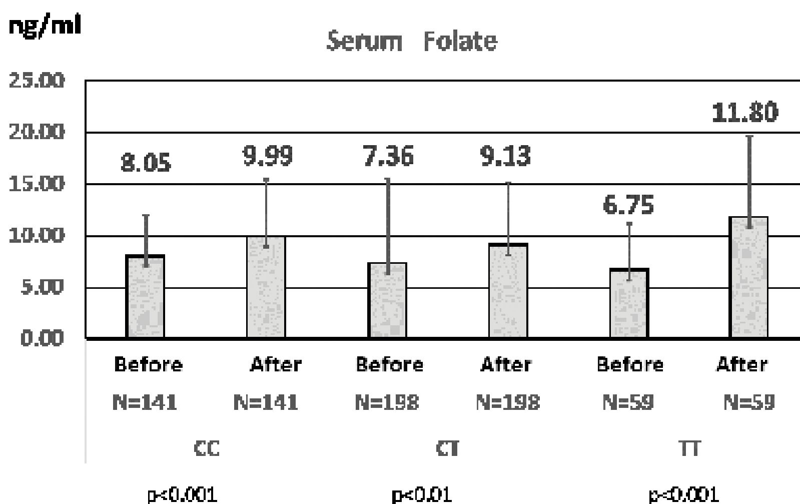

Figure 3. Effect of genotype notification on serum folate levels in individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

(**Figure 3**). Serum folate levels in subjects with the TT genotype showed an increase in serum folate of 5 ng/ml or more in both males and females, more than double that observed in the CC type and CT type groups (**Figure 3**, $p < 0.001$). The decrease in serum homocysteine (**Figure 4**, $p < 0.001$) following genotype notification and nutritional guidance regarding

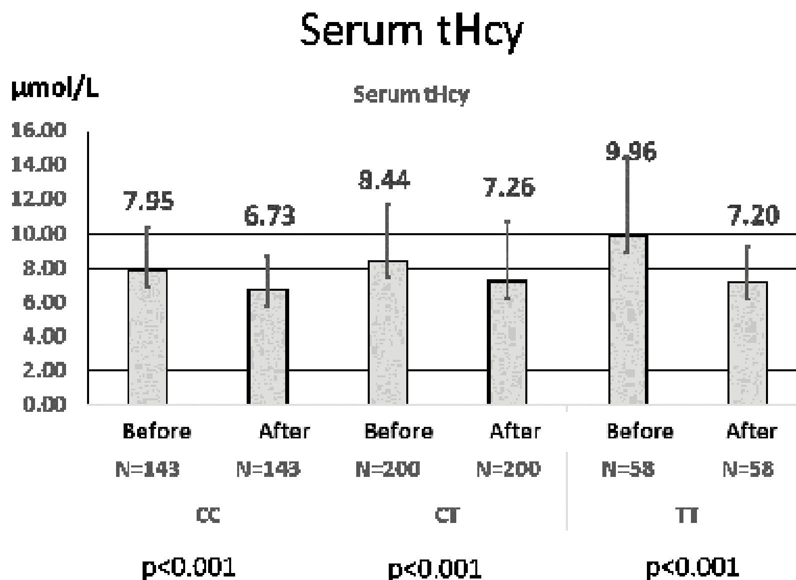

Figure 4. Effect of genotype notification on serum homocysteine levels in individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

green vegetable intake was significant in all three genotype groups. Subjects with the TT type genotype showed the largest reduction in homocysteine levels, from 9.96 ± 4.65 to 7.20 ± 2.11 μmole/L (**Figure 4**, $p < 0.001$). Males showed lower serum folate concentrations and higher serum homocysteine levels than females.

Total vegetable intake was highest in the TT homozygotes, from 244.85 ± 134.00 to 273.21 ± 107.29 g, but the increase did not reach significance levels.

There was a significant inverse correlation between serum folate and homocysteine levels prior to nutritional guidance, as demonstrated by the correlation coefficient ($r = -0.37$), and this correlation has a definite genetic polymorphism influence. However, the number of times green vegetables were taken in the morning, afternoon, and evening did not reach significance levels, and no change was seen in serum folate or serum homocysteine levels.

The frequency of green vegetable intake during the morning, afternoon, and evening showed an increasing trend in all areas of the city following nutritional guidance. The frequency of green vegetable intake was about 7.5–11 times per week for males and females prior to nutritional guidance, corresponding to about 126.4 g green-yellow vegetables per serving. The total average frequency of green vegetable intake in seven city districts by TT homozygotes increased from 9.0 to 12.0 in males ($p < 0.05$) but only from 2.8 to 3.9 in females (not significant). The intake of broccoli, Komatsuna, spinach, and vegetable juice increased the most following nutritional guidance. The type of vegetables consumed differed slightly depending on the season and location (farmlands and cities). We confirmed that the major source of vegetables was supermarkets, regardless of season and location. Since the number of men participating in the study was small, we confirmed the increase in green vegetable intake following

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nutritional guidance by totaling the vegetable intake in each of the three daily meals (breakfast, lunch, and dinner). In women, a significant increase was observed throughout the day and at dinner, but the TT type group was as small as nine subjects and the genotype-specific increase did not reach significance levels.

4. Discussion

According to the National Health and Nutrition Survey of 2016 [5], the average daily vegetable intake in Japan was 265.9 g (males: 272.3 g, females: 260.4 g). In the present study, the daily vegetable intake prior to nutritional guidance was 282.2 ± 202.0 g, which is similar to the national average [5]. However, the daily intake of green-yellow vegetables in Japan was on average 84.5 g (83.4 g for males, 85.4 g for females) [5] and for the 60–69 year age group, the average intake reported in the National Health and Nutrition Survey of 2016 was 97.5 g (94.7 g for males, 99.9 g for females) [5]. Prior to nutritional guidance, the folate intake was 126.4 g ± 116.2 g by subjects in the entire city of Sakado. We believe that there is a growing interest in vegetable consumption. As mentioned in the previous section, after the start of this project 11 years ago, 80% of Sakado citizens were aware of the importance of folic acid, 90% tried to eat more vegetables, and 73% wanted to obtain advice on improving their health. The average Japanese daily folate intake was 277 μg (283 μg for males, 272 μg for females) and 322 μg (328 μg for males, 317 μg for females) for Japanese in the 60–69 year age group as reported in the National Health and Nutrition Survey of 2016 [5]. The daily intake of green-yellow vegetables by the residents of Sakado City was already 355.4 ± 154.4 g prior to nutritional guidance because Sakado residents had received nutritional guidance for 11 years during the Sakado Folate Project [1]. The frequency of green vegetable intake was about 7.5–11 times per week for males and females prior to participants attending our lectures, corresponding to about 126.4 g of green-yellow vegetables per serving. Since there is no proportional relationship between the number of dishes served at a meal and the amount of vegetables taken, it is difficult to compare accurately, but the number of intake of green vegetables can be judged to be simple and useful as well as this previous study. In addition, the self-descriptive simple survey table for vegetable intake of the kind used in this study is widely used in the United States and is called "a rapid food screener" [31]. This screener is a useful tool for quickly monitoring patients' diets and the health care provider can use it as a prelude to brief counseling or as the first stage of triage.

Unfortunately, Japan's National Health and Nutrition Survey does not quantify serum folate or serum homocysteine, in contrast to surveys in other countries, so it cannot be compared with this study. However, the accurate blood analysis values obtained in this study showed that both serum folate and serum homocysteine levels were significantly improved following nutritional guidance for all genetic polymorphism groups.

This conclusion is supported by the finding that folate rice was taken more frequently in the previous study compared with other folate sources, including vegetables. In addition, the intake of green-yellow vegetables increased significantly in both male and female TT homozygote subjects following nutritional guidance (**Figure 1**). The correlation coefficient between

each indicator is high (r = 0.358) according to the number of C alleles with high folate-utilizing ability, as evidenced by the previous study by our laboratory [16], and serum folate increases and homocysteine decreases according to the number of C alleles (r = −0.52). In addition, serum folate concentration and serum homocysteine inversely correlate (r = −0.37) since folate deficiency accompanies an increase in homocysteine. However, there was no significant correlation with green vegetable intake in the morning, afternoon, and evening (r = −0.04). Rice folate, green tea, laver, and other foods may be additional sources of folate.

Although genotype notification in the Sakado Folate Project was effective in motivating folate intake, especially by those with the TT genotype, the increase in serum folate (from 17.4 to 22.5 nmol/L, 129%, averaged data from 2006 to 2012) was less than that observed following compulsory folate fortification in the United States (from 12.1 to 30.2 nmol/L, 149.6%) [1].

In general, even following nutritional guidance, it is difficult to change behavior such as increasing the number of green vegetable dishes, but among all subjects, both males and females with the TT polymorphism most significantly improved their green-yellow vegetable intake following gene notification (**Figures 1** and **2**). Although the total folate intake exceeded 400 μg, it is necessary to supplement various vitamins, minerals, dietary fiber, and antioxidant compounds found in green vegetables. It is therefore desirable to improve nutritional guidance to further increase green-yellow vegetable intake.

4.1. Efficacy of genotype notification for promoting healthy habits

The widespread commercial application of genotype notification, called direct to consumer (DTC), may be effective for encouraging healthy lifestyles. A meta-analysis of eight papers from seven different studies revealed a significant impact of genetic notification on smoking cessation in comparison to controls (clinical risk notification or no intervention) in short-term follow-ups of less than 6 months (RR = 1.55, 95% CI 1.09–2.21) [32]. In addition, genotype notification was associated with short-term increased depression and anxiety [32]. However, genotype notification is not always effective [33]. The effects of genotype notification of an oncogene (L-myc) genotype to smokers on their ability to quit smoking were tested [33]. Some smokers were allocated to the genotype notification group (intervention group) and the rest served as controls. Twenty-two of the 276 smokers in the control group stated that they quit smoking (8.0%) and 15 (5.8%) in the 257 genotype-notified group quit, providing an odds ratio (OR) of cessation for the intervention of 0.64 (95% confidence interval, 0.32–1.28). It was concluded that more smokers might quit if better methods explaining the need to quit and for notifying participants of their genotypes were employed [33].

Genotype notification of the risky mutant homozygote of the fatty acid Δ5 desaturase 1 (FADS1) gene resulted in increased intake of eicosapentaenoic acid (EPA) ($p = 1.0 \times 10^{-4}$) [34]. Red blood cell content of EPA also increased. The notified group showed increased awareness of EPA by the end of the study, but during the 12-week genotype notification period notification did not appear to influence intake [34].

The prevention of Alzheimer's disease and cardiovascular diseases might be influenced by genotype notification. According to the report of Hietaranta-Luoma et al. [35], subjects notified of the ApoE Ɛ4+ genotype and of the Ɛ4- genotype were compared with a control group with

regard to their changes in diet (e.g., fat quality, vegetables), alcohol consumption, and exercise. Dietary fat quality improved more in the Ɛ4+ group than in the Ɛ4- and control groups after obtaining genotype-based health advice, but only for a short time [35].

These unsuccessful examples of nutritional guidance with genotype notification highlight five reasons for the success of the Sakado Folate Project [1].

1. Effective organization of volunteers commended by the government for their effectiveness in promoting health.
2. Invention of a rapid and inexpensive genetic polymorphism analyzer.
3. Involvement of a well-trained registered dietician for overseeing the nutritional survey, providing advice on promoting folate intake, and for addressing anxiety.
4. Cooperation of the local government and the mayor of Sakado City.
5. Development of two folate-fortified foods: Sakado Folate Bread and Folate Rice.

To date, in addition to the successful increase in folate status, there have been no reports of depression or anxiety by the participants because of the well-trained staff providing *nutrigenomic* guidance.

5. Conclusion

The effectiveness of genotype notification was demonstrated in the case of MTHFR polymorphism. Of all the participants following nutritional guidance, only subjects with the TT genotype significantly increased their intake of both green-yellow vegetables (**Figure 1**, $p < 0.01$) and food-derived folate (**Figure 2**, $p < 0.05$). The increase in serum folate (**Figure 3**) and decrease in homocysteine (**Figure 4**) levels were greatest in subjects with the TT genotype, and these changes were confirmed in subjects with the other genotypes.

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the data, and supervised the acquisition of blood samples as a medical doctor. Thanks are also due to Dr. Takeshi Yoshizawa for his statistical analysis, and Ms. Konomi Tanaka, Ms. Wakana Ohkawa, Ms. Shuri Akiyama, and Ms. Emi Yokoyama of Kagawa Nutrition University for their collection and calculation of the data.

Disclosures

The authors report no conflicts of interest with respect to this study.

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Folate in Dentistry

Aysan Lektemur Alpan and Nebi Cansin Karakan

Additional information is available at the end of the chapter

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Abstract

Balanced nutrition is the key point of a healthy life includes intake of vitamins and minerals. Vitamins such as folate (B9) have an important role in system homeostasis. Vitamin B derivatives, also folate are water-soluble vitamin class which plays a key role in cell metabolism. Folate is necessary to produce new cells via stimulating DNA and RNA methylation. Folate has positive effect on recurrent aphthous stomatitis, gingival hyperplasia, preventing early childhood caries and periodontal diseases. Alveolar bone and periodontal ligament development are related to sufficient concentrations of folate. Folate reduces gum bleeding, and increases osteoblastic activity and bone mineral density, also decreases osteoclastic activity. Effect on DNA and RNA metabolism causes the reduction of reactive oxygen species. In early stages of pregnancy, folate deficiency may cause birth anomalies due to neural tube defects such as lip, alveolar and palatal clefts. Folate deficiency effects on DNA and RNA metabolism negatively. DNA and RNA repair, production and methylation system is being interrupted. Therefore chromosal abnormalities occur and that situation may cause cancer and leukemia. Folate is mainly provides systemic homeostasis and important for maintaining chromosomal activities. Consequently adequate concentrations of folate must be taken regularly.

Keywords: gum disease, dentistry, folate

1. Introduction

Factors such as nutrition, heredity and environmental conditions are affecting human health. Poor diet and sedentary lifestyle are among the main causes of morbidity and mortality worldwide. Recent developments in nutritional science show that diet may have an important role not only in maintaining optimum health, but also in reducing the risk of some diseases. It is necessary for people to have a healthy and balanced diet for healthy life, body growth, renewal, development and work. Otherwise, the nutrients needed for the body are not taken

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in time and in sufficient quantities, so the resistance to the diseases decreases and the treatment of the diseases becomes long, difficult and expensive. The importance of vitamins and minerals in balanced nutrition is better understood in the 20th century. The evaluation of food is made according to the chemical composition they contain. In this way, the needs of a human body can be determined by biochemical concepts. The ingredients of the foods include carbohydrates, proteins, fats, minerals, vitamins and water from the basic compound ingredients. Studies showed that; those who consume fruits and vegetables regularly are found to be at a lower risk than those who consume less in terms of the risk of developing cancer [1]. Adequate nutrition is an integral part for maintaining good oral health. There is a constant synergy between nutrition and the integrity of the health and ill mouth cavity. There is also an interdependent relationship between them: nutrition affects oral health, and oral health affects nutrition [2]. It has been reported that the consumption of fruits and vegetables reduces mouth, esophagus, lung, stomach, colorectum, larynx, pancreas, breast and prostate cancer [3]. In addition to their liquid and pulp content, fruits and vegetables are important for the high levels of vitamins and minerals they contain. In particular, the antioxidant properties of vitamin A, E, vitamin C and β-carotene for fruits and vegetables are the best sources and most studied vitamins for oral health. Besides these vitamins, vitamins B6, folate, vitamin K, vitamin E and niacin content are also important.

Vitamin B complex is a water-soluble vitamin class that plays an important role in cell metabolism. Eight vitamins which are different from each other in terms of their chemical composition and pharmacological properties create this family [4]. Folate which is called "folium" means leaf, is one of the vitamins of group B, dissolved in water and was first separated from natural foods in 1943. Folate is involved in single carbon metabolism in the body, providing single carbon unit for purine and thymidylate synthesis and essential biologic product for deoxyribonucleic acid (DNA) and neurotransmitters methylation such as phospholipids, proteins. Thus, the construction of nucleic acids and the conversion of some amino acids to each other (conversion of serine, glycine and homocysteine to methionine, glutamic acid catabolism of histidine) are achieved [5]. Folate stands out as a molecule having biological importance in recent years. Folate is a water-soluble vitamin in the structure of pteroylglutamic acid composed of pteridine, p-aminobenzoic acid and glutamic acid. Folate is mainly involved in important biochemical events such as the metabolism of purines and pyrimidine homocysteine and methionine amino acids. Folate is essential to produce and maintenance of new cells and DNA, **ribonucleic acid** (RNA) synthesis through methylation [6]. It is a carrier of 1-carbon parts (methyl and formyl groups) in the cells, and acts role for the synthesis of human macromolecules for example methionine, deoxythymidylate monophosphate, and purines [7].

Lentil, green vegetables, citrus fruits, sparrowgrass, dried beans, broccoli, sunflower seeds, cereal, avocado and tomato juice contain high amounts of folate. In case of gastrointestinal, kidney or liver function deficiency, as well as an urinary problem, folate excretion may increase and folate deficiency occurs. Also an inflammation due to any disease can reduce folate concentration. Cancer and anemia negatively effect on folate metabolism. Forgetfulness, dizziness, overstrain and shortness of breath can be the symptoms of folate deficiency. Regular clinical visits and blood test assessments are important to diagnose the deficiency. The estimated

average requirement for folate is 320 μg/day and the recommended dietary allowance value is 400 μg/day. It can be accelerated for pregnant women up to 600 μg/day as well as lactation 500 μg/day [8].

Alcoholics, elder people, and those who take drugs such as methotrexate and phenytoin are high risk groups in term of folate deficiency [9]. Some disease such as ulcerative colitis, Crohn's disease may alter the absorption of the folate resulting delayed healing and increased risk of oral infections [9]. Deficiency can lead in microcytic anemia (iron deficiency) or macrocytic anemia (B12 or folate deficiency) associated with some oral pathologies such as red/swollen tongue, burning of tongue/oral mucosa and angular cheilitis [9]. Folate deficiency may result in increased oxidative stress, endothelial dysfunction, genetic instability, deterioration of DNA repair, and cell apoptosis as well as periodontal disease [7]. Inadequate folate uptake or lowering of some medicines decreased folate levels in body caused by some medications, uncover some side effects, especially in oral mucosa. Folate was investigated for many aspects in dentistry especially in mucosal lesions.

2. Folate in dentistry

2.1. Folate in dental caries

Early childhood caries are identified as one or more decayed missing or filled tooth surfaces in primary dentition between 0 and 71 months. Balanced nutrition and vitamin containing consumption such as folic acid is necessary for preventing early childhood dental caries [10]. Tooth caries is the microbiological infectious disease of the teeth which results in the destruction and locally dissolution of calcified tissues. It occurs with impaired physiological balance between tooth mineral and dental plaque. Caries lesions occur when a large number of bacteria with the ability to produce acidic environments thus demineralize the tooth structure. At the onset of caries lesion, the causal relationship between caries and organisms in the mouth flora is not well understood. Calcium and phosphate ions in high concentration in saliva play an important role in remineralization.

Homocysteine is a sulfur-containing amino acid formed during the metabolism of methionine and has a central role in the metabolic pathways of thiol compounds [11]. Vitamin B12, vitamin B6 and folate deficiency, which are necessary for homocysteine metabolism, can cause hyperhomocysteinemia. There is a negative correlation between serum vitamin B12, folate, vitamin B6 concentrations and plasma homocysteine concentration in healthy subjects [12]. There are many mechanisms that effect hyperhomocysteinemia, such as the induction of smooth muscle cell proliferation in the vascular intima layer, the increase in lipid accumulation in the vessel wall, the difficulty of endothelial cell breakdown, the activation of platelets and leukocytes, the increase of low density lipoprotein oxidation, the activation of platelet thromboxane synthesis, increasing oxidative stress [13, 14]. Saliva has a protective role in developing caries with its protein, hormone, antibody, antibacterial and antioxidant contents. Lower folate intake causes a rise in homocysteine levels, resulting in an increase in salivary

oxidative markers and thus an increase in caries activity [15]. In a cohort study, insufficient folate consuming in pregnancy (<6 ng/mL) increases early childhood caries in toddlers. This study defines folate deficiency as a risk factor for developing early childhood caries [16].

2.2. Folate in periodontal diseases

Periodontitis is a disease caused by specific microorganisms and causing periodontal ligament and alveolar bone loss by affecting supporting tissues of teeth [17]. Microbial dental plaque is required to start the periodontal destruction but it is not sufficient to exacerbate the periodontitis. Host inflammatory response takes an important place to modulate the disease course. Genetics, smoking, general health, diet, social variables etc. may affect the host immune response and periodontal destruction [18].

In recent years, macronutrients and micronutrients, which modulate proinflammatory and anti-inflammatory mechanisms affecting host immune response to combat with periodontitis, are gaining importance [19]. Folate takes an important place for preserving the integrity of the periodontal tissues. Gingival necrosis, periodontal ligament and alveolar bone loss can develop when the folate deficiency in body exists [20]. Folic acid deficiency reduces lymphocyte production, decreases cytotoxic T cell activity and phagocytic function of neutrophils leading the rapid development and progression of periodontal tissue destruction. High turnover of squamous epithelium process which is essential for repair of periodontal tissues is damaged when the folate levels are reduced [20].

Akpinar et al. [4] investigated effects of different B vitamins on alveolar bone loss in rats. 64 male Wistar rats were used and riboflavin, nicotinamide and folate were applied to doses 50–100 mg/kg. Serum IL-1 beta and IL-10 levels were measured by using ELISA. Alveolar bone loss, osteoclast, osteoblast number and inflammatory cell infiltration were examined histopathologically. 100 mg/kg folate group was revealed more 1 L-1 beta reduction and bone loss in all B vitamins were similar comparing the control group. They concluded that systemic administration of riboflavin, nicotinamide and folate increased osteoblast activity, decreased osteoclast numbers, and reduced alveolar bone loss in rat model.

Esaki et al. [21] studied the relationship between folate levels and gingival bleeding in 497 patients who were nonsmokers. According to the multiple regression analysis results, dietary folic acid was significantly correlated with gingival bleeding but it is not correlated with Community Periodontal Index scores.

In another rat study preventive effects of folate supplements on cyclosporine-associated bone loss; 40 male rats were divided into 5 groups. Folate were given 20 mg/kg daily via gastric gavage for 6 weeks. In cyclosporine group, mean homocysteine level was significantly higher than the other groups. Folate revealed more total mandibular volume, absolute bone volume and volume of cavities comparing with cyclosporine group [22].

Erdemir and Bergstrom [23] investigated smoking, folate and vitamin B12 levels in chronic periodontitis patients. As a result; a negative influence on the response to nonsurgical periodontal therapy in smokers and folate levels of smokers gradually decreased 8.0 ng/ml at

baseline to 7.2 ng/ml at 6 months. However, these levels increased in nonsmokers. They concluded that consuming folate and B12 rich foods provides beneficial effects for smoker periodontitis patients.

Yu et al. [7] investigated the age-related periodontal disease and folate levels in a cross sectional study, based on the data of the National Health and Nutrition Examination Survey (NHANES) 2001/02. Periodontal examination and analysis of serum folate level were performed, according to study results, low serum folate levels were correlated with periodontal disease. Authors concluded that, folate has a preventive role for development of periodontal disease and nutritional status was a messenger for oral health.

2.3. Folate in recurrent aphthous stomatitis

Recurrent aphthous stomatitis (RAS) is an oral disease which characterized recurrent/painful ulcerations on oral mucosa such as labial, buccal, alveolar and ventral tongue (**Figure 1**). Many etiological factors such as immune disorders, hematologic diseases, hypovitaminosis, nutritional deficiencies, allergy, psychological disorders have been discussed in terms of disease etiology but exact causes of RAS remains unclear [24]. Although some studies showed that multiple nutritional deficiencies including B1, B2, B6 and B12, folate, iron and ferritin may be possible etiologies of RAS [25].

Sun et al. [25]. performed a study with 273 healthy and 273 patients with RAS. Blood iron, hemoglobin, homocysteine, B12 and folate levels were determined. RAS patients showed significantly lower mean hemoglobin and iron levels comparing the healthy subjects. In terms of mean B12, folate and homocysteine levels, RAS patients did not show any significant difference to healthy subjects.

A study was carried out with 60 patient had RAS in 6 months. Analysis was performed to determine serum ferritin and serum B12 and red blood cell (RBC) folate. RAS group had low serum folate 51.7% as well as serum ferritin, serum B12 levels in comparison, healthy subjects [26]. However, Aynali and colleagues have indicated that B12 deficiency may play a role in

Figure 1. A major aphthous stomatitis on labial mucosa.

etiology underlying RAS [27]. In their study folate and hemoglobin levels were not statistically different with that of healthy group.

Burgan et al. [28] investigated the hematinic deficiency prevalence in 286 individual (143 RAS patient and 143 control group). Hemoglobin, ferritin, vitamin B12 and folate levels were determined in serum. 54 RAS (37.8%) patient indicated low ferritin, folate or vitamin B12 compared with 26 Control (18.2%) group with a significant difference. Although male patients with RAS did not show any folate deficiency, females were deficient to folate at 9.2% rate. When hematinic deficiencies are listed, vitamin B12 (26.6%) is the first, followed by iron (16.8%) and folate (4.9%). The authors concluded that RAS can be controlled by controlling the ferritin, folate and vitamin B12 levels of patients. However Barnadas et al. concluded that replacing these elements did not make statistical difference in reducing the frequency of RAS [29]. In another study that agrees with this study, patients received daily multivitamins (including A, B1, B2, B3, B5, B6, B9, B12, C, D and E) in addition their diets showed no significant changes as for reduction in the number or duration of RAS episodes [30].

2.4. Folate in gingival hyperplasia

Gingival hyperplasia (GH) refers to the changes in gingival size and increase in gingival contour (**Figure 2**). The cause of this increase is sometimes an inflammation or sometimes an increase in gum fibrillation due to chronic irritation. Inflammatory cells reaching the inflamed area increase the size of the gingiva. As the event becomes chronic, the number of collagen fibrils in the gingiva increases and the gingival size grows. Although gingival hyperplasia is more likely to be caused by the inflammation which developed from dental plaque, some medications such as antiepileptic drugs, anticonvulsants and immunosuppressants have also been associated with GH [31]. Antiepileptic agent phenytoin, anticonvulsant agents valproic acid, carbamazepine, phenobarbital and vigabatrin, immunosuppressant cyclosporin A and calcium channel blocker dihydropyridines, diltiazem and verapamil have GH as an adverse effect [32–35]. The exact mechanisms that induce the GH have not been clearly understood, although there are a lot of studies about this topic, contradictory results remain. Some of

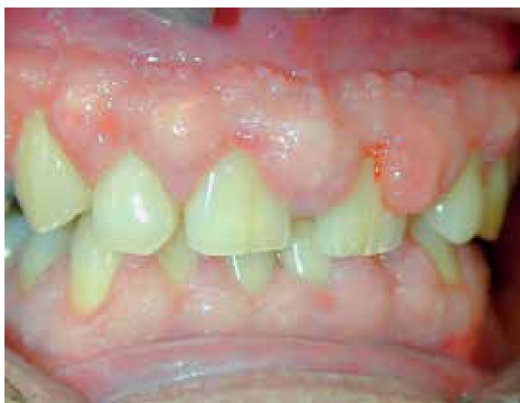

Figure 2. Typical gingival hyperplasia image.

the studies concluded that phenytoin and cyclosporine A are capable to inhibit extracellular matrix (ECM) production by gingival fibroblast and cell proliferation in vitro [36].

On the other hand, some studies indicated that the accumulation of proteins such as collagen in ECM may be caused by an imbalance between the synthesis and the degradation of ECM, became a possible explanation of the development of GH [37]. Collagen fibrils are degraded via two ways: secretion of collagenases which is named extracellular way; and the intracellular way, by collagen phagocytosis by fibroblasts [38].

Antiepileptic agents, immunosuppressants and calcium channel blockers induce a decrease in the Ca2+ cell influx by implementing differences in the sodium-calcium exchange in the result of a reduction in the uptake of folate; all these changes limit the production of active collagenase [39]. A number of studies have been conducted that point to severe folate deficiencies resulting from long-term use of phenytoin. Folate, on the other hand, were held responsible for the significant decrease in serum concentration of phenytoin, as much as to accelerate seizures [40–42] (**Figure 3**).

Vogel [43] suggested that drug induced GH may be a secondary to a local folate deficiency. Heimburger [44] noted that some tissues need greater folate for maintaining its function than other tissues which may lead to localized deficiencies, in spite of the serum folate is detected normal ranges although such localized folate deficiencies may result from reduced tissue intake due to a congenital malfunction. Opladen et al. investigated the reaction of anticonvulsant drugs on the folate receptor 1 (FOLR1)-dependent 5-methyltetrahydrofolate (MTHF) which is primary biologically active form of folate transport. The authors have dedicated reactive oxygen species (ROS) production were accelerated via metabolic cleavage caused by some anticonvulsants (valproate, carbamazepine and phenytoin). Side effect of drugs and ROS development on FOLR1-dependent 5-MTHF uptake were investigated and it was concluded that MTHF uptake was connected on the time and dosage of medication. At normal ranges of MTHF concentrations, the high-affinity FOLR1 serves the main mechanism for cellular uptake, however phenytoin increased MTHF uptake but ROS damages this physiologic condition leading to inhibition in folate transport and decrease in folate uptake in gingival fibroblasts [45].

Inoue and Harrison [46] stated that taking folate supplement with phenytoin may reduce or prevent the GH. In some studies, it was determined that recurrence of GH following surgical intervention decreased when the patient received folate [47, 48].

Based on the results obtained from various studies related to reduced plasma and tissue folate levels induced by phenytoin, folic acid was tested both topically and systemically to prevent the inevitable adverse effects of long-term phenytoin therapy [49]. However conflicting results available in literature about treatment with folate would have a therapeutic effect on phenytoin induced GH [50].

Arya et al. [51] investigated the effect of folate on phenytoin induced GH in 120 patients with epilepsy aged 6–15 years on phenytoin monotherapy for 6 months were 62 and 58, respectively, in folate and placebo arms. 0.5 mg/day of folate were given for 6 months. After 4 months, 21% of the folate arm and 83% of the placebo arm had developed phenytoin-induced GH. At

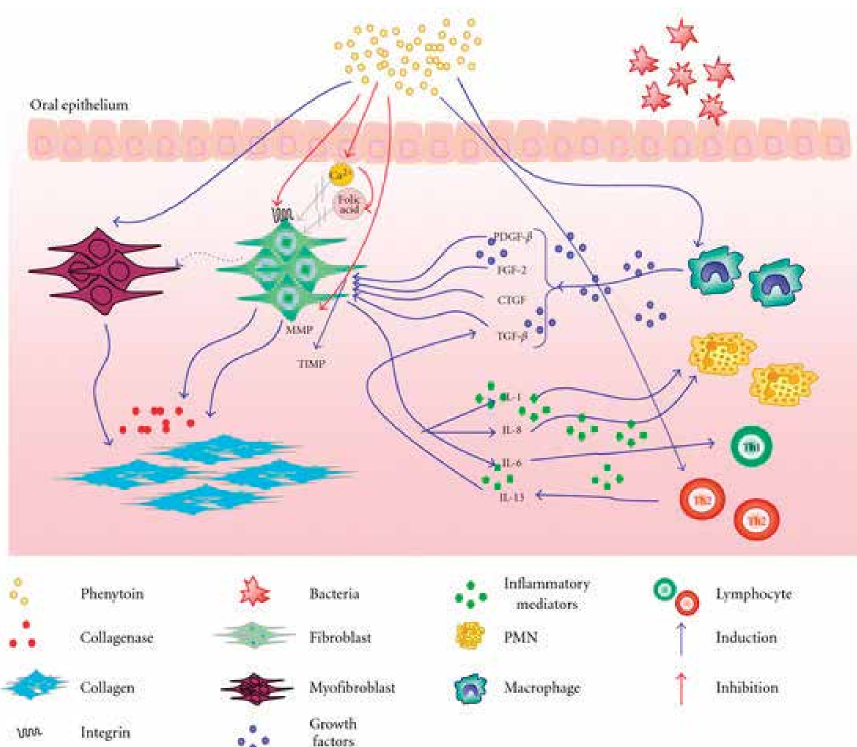

Figure 3. Mechanism of gingival hyperplasia development. Phenytoin induces a decrease in the Ca2+ cell influx that leads a reduction in the uptake of folic acid; this action limits the production of active collagenase. Phenytoin decreases collagen endocytosis via induction of a lower expression of α 2 β 1-integrin by fibroblasts and it also stimulates myofibroblasts. Cytokines is also responsible in gingival overgrowth. IL-6, IL-1, and IL-8 are produced by fibroblasts that activated by phenytoin. These mediators are responsible T cell activation and allowing the neutrophils to become active in the connective tissue. This interaction seems to be associated with fibrotic diseases at a high rate. Microbial dental plaque induce a local inflammatory response which plays important role to develop GH. CTGF, PDGF, FGF and TGF-β are growth factors which are found in fibrotic tissue and takes place in phenytoin induced GH. Th2 cell activated IL-13 production can be affected by phenytoin, furthermore; the drug may activate to macrophages releasing different growth factors such as TGF-β and CTGF. Fibroblast proliferation, collagen biosynthesis, activation of TIMPs, inhibition of MMPs and ECM synthesis which are essential to develop GH occur based on all of these biological events. PDGF-β: platelet derived growth factor; FGF-2: fibroblast growth factor-2; TGF-β: transforming growth factor-β; CTGF: connective tissue growth factor; MMP: matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase [31].

the end of the study the occurrence of phenytoin induced GH was found to be significantly decreased with folate supplementation from 88% in the control population to only 21% of those receiving folate.

In a study a total of 100 patients between the ages 18 and 50 years, who were clinically diagnosed with epilepsy treated with phenytoin participated. Assessment of serum folate level was carried out by chemiluminescent method using immulite kit at the start of and after 1 year of phenytoin therapy. The mean difference between the start and after 1 year folate level was calculated as −7.530. The authors concluded that, the use of folate as an adjuvant to phenytoin therapy in the prevention of phenytoin-induced gingival enlargement can be considered but attention is required drug interactions between the two [52].

Dogan et al. [53]. performed a study to determine the role of folate on phenytoin induced human gingival fibroblasts overgrowth by investigating its effect on IL-1beta which has been stated to accelerate the ECM production in fibroblasts induced depending tumor necrosis factor alpha (TNFalpha) in vitro. The IL-1beta level in cells in the phenytoin treated group was found 1 pg/ml. 20 or 40 ng/ml folate treated samples achieved 0.8 and 0.7 pg/ml, respectively near the control group value that was 0.7 pg/ml. Folate application decreased IL-1beta level as nearly control group. However, in the double-blind randomized controlled trial, authors compared folic supplement (3 mg/day for 16 weeks) to prevent GH. They concluded that the folate is an inadequate therapy for preventing GH [54]. Using folate (1 mg/ml mouthwash) was considered to be more effective than systemic application [48]. In one study, the authors noted that topical folate may bind to exogenous endotoxin, leading to the reduction in GH and reduce gingival inflammation. Patients who had low baseline plasma and RBC folate responded good results to topical folate than normal people [55].

2.5. Folate in birth anomalies

Neural tube defects, cleft lip, alveolar and palate are the most common malformations in humans. In spite of more advanced therapeutic measures taken in recent years, such anomalies are still being encountered and individuals born with such anomalies are exposed to severe physical and psychological difficulties.

The etiologic factors that constitute cleft lip and palate are not known exactly and are accepted as a multifactorial anomaly. In the etiology of cleft lip and palate role of both genetic and environmental factors thought to cause [56].

During the pregnancy of the mother, especially in first trimester of pregnancy exposure to chemical substances and/or the use of medicines (benzodiazepines, phenobarbital, diphenylhydantoin, diazepam, cortisone, salicylates etc.), the use of alcohol or cigarettes, infectious disease (rubella etc.), diabetes, folate deficiency, stress, exposure to radiation, inadequate or excessive vitamin A intake can affect these facial anomalies [56]. Studies that have performed during the last 20 years on the etiology of cleft lip and palate shows that there is a small but significant link between the risk of having a child with cleft lip and palate and smoking in the first 3 months of pregnancy. Researches have shown that smoking mothers have lower folate values than nonsmokers and therefore have a higher risk of having child with cleft lip and palate. It has been reported that alcohol use during pregnancy also increases the risk of developing cleft lip and palate [56–59].

Most of the face development occurs at 4–8 weeks in pregnancy. At the end of the 10th week a clear face appearance emerges. In the process of facial development, medial nasal processes, lateral nasal processes and maxillary processes combine to form the normal nose, upper palate and lip anatomy. The result of the combination of the medial nasal and maxillary process, oral and nasal cavities are separated. The mandibular process forms the lower jaw, lower lip and lower part of the cheek. The junctions of facial processes are weak and they are affected very quickly from any pause in this phase. Development and merging inability of these processes result in lip or palatal clefts [60].

The classification used today is the Kernahan classification based on the embryonic formation theory. In this class, the limit used for separating deformities is foramen incisivum. The structures in front (premaxilla and nose) are called "primary palate" and the structures behind it (hard and soft palate) are called "secondary palate." Accordingly, Kernahan has divided the lip and palate cleft into three main groups: 1. Only primer palate (lip and premaxilla) clefts 2. Only the secondary palate clefts 3. Co-clefts of primer and secondary palate [61] (**Figure 4**). With a simple classification, oral clefts can be separated into two main categories: cleft palate only and cleft lip with or without cleft palate. Causal mechanisms of these categories may be different. Most of the clefts are defined as isolated, which means that there are no accompanying birth defects (**Figure 5**). In non-isolated clefts different severe anomalies may develop, congenital heart defects and neural tube defects are more frequently accompany with oral clefts [62].

Oral cleft defects are the most frequent newborn defects that can be observed 1/500 approximately in worldwide which are related to folate deficiency. Palatal and lip clefts are responsible for these oral clefts [63]. Oral cleft occurrence in early life period is an important health problem in which different surgical procedures must be frequently performed. Also dental treatment, physiological support, speech correction are the secondary common problems [64]. In an animal study on the relationship between folic acid and cleft lip and palate, more cleft lip and palate was found in animals fed with folate deficient diet. In another study in which folic acid supplementation was given to pregnant mothers using anticonvulsant medication, none of the 33 mothers who received folic acid supplementation were reported to have cleft lip and palate and/or developmental defects [59]. Although the mechanism of action of folic acid is not fully understood, it is suggested that women who are planning to have children in order to prevent cleft lip and palate and neural tube defects present with 0.4 mg of folic acid daily before the 12th week of pregnancy and before getting pregnant [58, 59, 65]. Folic acid and reducing risk for neural tube defects is well recognized. Facial and tooth tissues develop from neural crest cells which originate from the dorsolateral aspect of the developing neural tube thus neural tube defects and oral clefts are embryologically related to each other [66]. Folate is also an important vitamin which is required for synthesis of DNA and RNA. There is a wide investigation about maternal consumption of vitamins and especially folate that reduces oral cleft recurrence and occurrence [67]. Both 0.4 and 4 mg doses of folate intake significantly reduced oral cleft prevalence during pregnancy may play an important role on preventing oral clefts [64, 67]. In contrast, a population based study revealed that supplementary

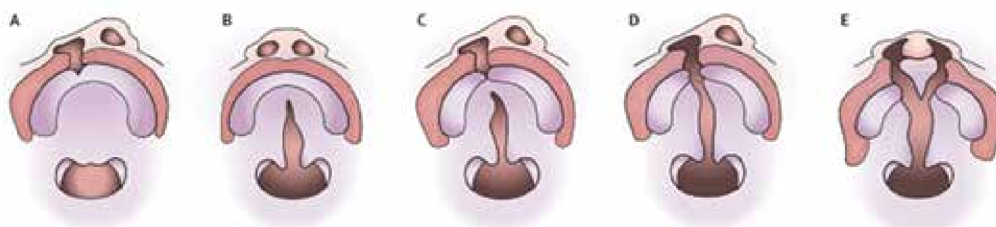

Figure 4. (A) Cleft lip and alveolus. (B) Cleft palate. (C) Incomplete unilateral cleft lip and palate. (D) Complete unilateral cleft lip and palate. (E) Complete bilateral cleft lip and palate [63].

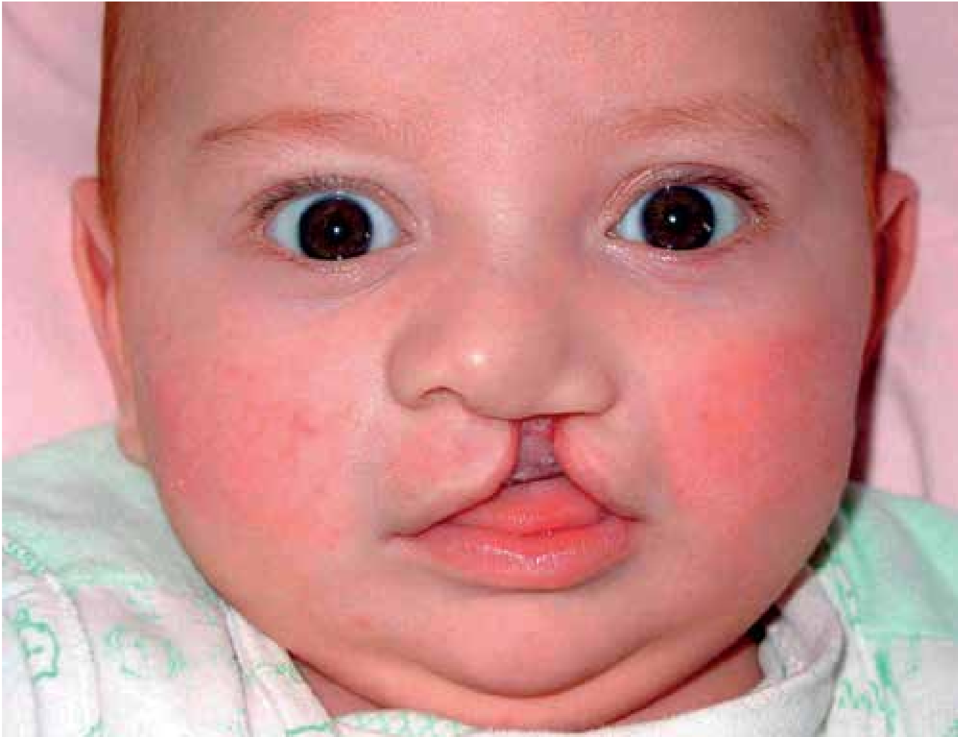

Figure 5. A baby born with cleft lip and palate.

folate intake has no effect on the prevalence of oral clefts [62]. In another recent study folate intake 4.36 fold reduced palatal and lip or combined clefts when used in early pregnancy (4–12 weeks) 400 μg daily [68]. In a population-based study 896,674 live births which 1623 had oral clefts (isolated oral clefts, *n* 1311; non-isolated oral clefts, *n* 312) were investigated. 21.5% women used vitamin supplements before getting pregnant. Vitamin use provided no additional benefit to prevent the isolated oral clefts [62].

2.6. Folate in cancers

Folate is necessary for maintaining proper body functions also for the preservation of genomic integrity. Folate joins in two groups of biological reactions, one of them is biosynthesis of nucleotides and the other is methylation reactions. These events are required in the basic biological mechanism of DNA synthesis, repair and methylation. Also it is needed for mitochondria to function correctly and preservation of mitochondrial DNA. Specifically, DNA damage caused by the deficiency of folate may result in the formation of chromosomal abnormalities, these abnormalities are considered as one of the main results of cancer and leukemia [69].

Folate deficiency in rats has demonstrated increased sensitivity to carcinogenicity. Although the mechanism of anticarcinogenic action of folate is not fully known, it is thought to be related to DNA methylation. It is thought that folate can decrease carcinogenesis because of

its role in the maintenance of the level of SAM (S-adenosylmethionine) and the production of deoxythymidine monophosphate necessary for DNA synthesis. In the case of hypomethylation in cytosine-guanine chains, the expression of specific oncogenes may increase. It has been reported that defective or incomplete methylation DNA associated with dietary folate deficiency can develop a mechanism to cause cancer and aging [70].

There are recommendations about that folate intake may decrease oral cavity and pharyngeal cancers (OPC). Therefore many studies have limited sample size and the common problem is the main source of dietary folate [71]. OPC is seventh most common cancer worldwide. Tobacco and alcohol may be identified as the main risk factors for OPC; additionally, dietary risk factors are also responsible for OPC. Fruit and vegetable rich diet which highly include folate can reduce risk of OPC, [72, 73]. Recently authors have found that folate intake may significantly reduce overall OPC risk. Folate intake by consumption revealed weaker association between OPC risks. Using alcohol increased risk of OPC about 11% when compared to never/light drinkers [71]. In another study significant difference was found according to aldehyde dehydrogenase 2 gene polymorphism that related to alcohol consumption. Also folate intake has reduced OPC risk in this patients [74]. Interestingly authors stated that dietary folate intake may contribute to the proliferation in early-stage colon cancer. Folate is strongly related to DNA and RNA replication and tumor suppressor gene expression. It is recommended that daily 400 micrograms of folate is necessary for the homeostasis. Also HPV is responsible for initiating OPC, and folate has an important role in suppressing carcinogenic cell production via mediating methyl groups for CpG-specific DNA methylation [75]. Using alcohol reduces gastrointestinal absorption of folate, and it has been shown that high alcohol intake causes a higher acetaldehyde plasma concentration resulting reduced folate plasma levels [76]. In a more recent study folate-alcohol intake association in women's oral cancer revealed that high alcohol intake with low folate intake increases cancer risk. Also high alcohol intake with higher folate intake decreases the risk [77]. Alcohol dehydrogenase and cytochrome P450 2E1 enzymes convert ethanol to acetaldehyde which plays key role on cariogenic effect of alcohol. Acetaldehyde has effects on DNA methylation and DNA repair systems [78]. It is hypothesized that increased levels of aldehyde dehydrogenase 1 enzyme also increases acetate concentrations which is an end product of acetaldehyde. So acetaldehyde concentration which has a negative effect on folate metabolism reduces. We can conclude that acetaldehyde may play an important role as a key factor in OPC. At this point we can explain how the carcinogenic effect of alcohol reacts on human body. As a result people who drink high alcohol and oppositely have low folate intake are in the most risky class [79].

3. Conclusion

Socioeconomic factors are the most important factors leading to nutritional disorders. Folate deficiency prevents the organism from maintaining its important metabolic activities and causes various disorders. Especially to participate in DNA synthesis, in the early stages of pregnancy and the baby is very important for the development of children to adolescence. Among the daily diet of constantly consumed nutrients folate content is very important in

terms of combating folate deficiency. We have to be vigilant and take into consideration as an etiological factor the clinical table which develops from the deficiency of these vitamins. Controlled studies have shown that the use of periconceptional folate reduces the frequency of urinary system, cardiovascular and extremity anomalies, as well as the frequency of cleft lip and palate [80]. In addition, correction of the folate status of the person reduces vascular diseases and the incidences of certain cancers [81]. Taking all of these into consideration, for the primary protection of various diseases, enrichment of foods with folate, promotion of the use of folate tablets by risky people and education of consumption of foods rich in folate should be considered.

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Deficiency of Folate in Pregnancy on Diverse Subjects Using FTIR Spectroscopy

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Additional information is available at the end of the chapter

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Abstract

This study is an attempt to assess, evaluate and compare the spectral difference in saliva and serum between healthy and anomalies pregnant women because of deficiency of folate by utilizing Fourier Transform Infrared Spectroscopy. Folate is required for the development of healthy embryo and plays vital role in the fetus spinal cord and brain development. The present work is to study the folate deficiency in pregnancy-Anomalies (open neural defect) and contrast the outcome of the result with normal healthy pregnant women. The outcome of the results showed that there is a significant difference or contrast between the folate of healthy pregnant and anomalies (open neural defect) in pregnant women, both in the sample of saliva and serum. From the spectral analysis, the intensity ratio parameters have been computed and introduced. The result of the outcomes shows that for both qualitative and quantitative investigation of biological fluids and to distinguish between the sample sets from healthy and anomalies-diseased groups, FTIR is utilized. The internal standard method is described in characterizing the samples quantitatively.

Keywords: saliva, serum, folate deficiency, FTIR, pregnant women

1. Introduction

Historically, investigations of saliva of female sex hormones were utilized for fertility and pregnancy monitoring [1–3]. Changes in salivary pattern in each trimester of normal pregnant women have been compared by utilizing FTIR spectroscopy both qualitatively and quantitatively [4]. However, a recent finding shows that these assays might be helpful and useful

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beyond the investigation of reproductive concerns. There is a clear picture that during pregnancy there are changes and hormone level fluctuations in normal pregnancy. In specific issues with the fetus during pregnancy complications, frequent serum sampling testing for hormone analysis is invasive, inconvenient, and requires skilled personal to draw samples. However, whole saliva provides an excellent sample to observe the hormone levels. Fourier Transform Infrared Spectroscopy (FTIR) is utilized to characterize the structural and chemical composition of human saliva of pregnant women. Each and every molecule excited to higher states of vibration using light at a particular wavelengths which corresponds to the excited vibration modes of frequency. This information or data can be utilized to outline or map the absorption positions and help to recognize in identifying the chemical properties of the tissue. FT-IR spectroscopy can provide unique infrared chemical fingerprints of specimens, highly sensitive which can yield new insights into positional salivary changes in pregnant women. A key advantage of FT-IR is that it is a non-manipulative, quick and non-invasive collection method, simple transport of the material, easy and no additional need for media which can identify a wide range of chemical targets (**Figure 1**).

Saliva, similar to blood, contains protein and nucleic acid molecules which are vast, large and complex that reflects the physiological status. Essential folate consumption, during the period of preconception, is the perfect time when a woman becomes pregnant and it helps to protect against numeral congenital deformities, including neural tube defects which are the most prominent birth defects that happen from deficiency of folate [5]. Neural tube defects which

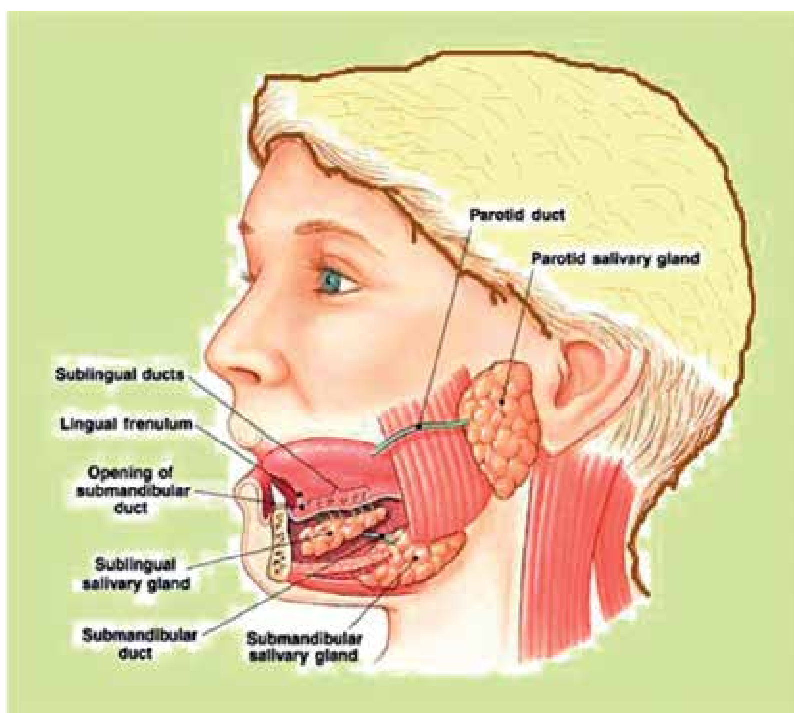

Figure 1. The salivary gland.

cause critical abnormalities of the central nervous system that procure in babies during the initial stage or first few weeks of pregnancy, terminating in malformations of skull, spine and brain. The normal defects in neural tube are anencephaly and spina bifida. The risk of neural tube defects is reduced in a critical way when additive folate is devouring into a healthy diet before conception and during the 1st month after conception [6, 7]. Folate supplements has additionally been appeared to lessen the risk of congenital heart defects, limb defects, cleft lips [8] and urinary tract irregularities [9]. Folate deficiency during pregnancy may likewise raise the risk of preterm delivery, spontaneous premature birth, baby low birth weight and retardation of fetal growth and complications in pregnancy, as abruption placental and pre-eclampsia [10]. Supplementation with Folate may likewise ensure the fetus against disease when the mother is battling disease or taking prescriptions or smoking during pregnancy [11]. It includes oocyte development, implantation, placentation, including the general impacts of folate and pregnancy. Consequently, it is important to get adequate and sufficient amounts through the routine diet to avoid from subfertility [12]. There is an improvement through worldwide that pre-birth high folate in perspective of low vitamin B_{12} resulting in epigenetic changes in the unborn predisposing them to grown-up onset of fetal cause infection or disease in particular metabolic disorders, central adiposity and adult illnesses, as Type 2 diabetes [13]. Moreover, another dynamic area of research and concern is that either substantially more or too little folate in utero affects epigenetic changes to the brain bringing about a mental imbalance as autism spectrum disorders [14, 15]. A salivary test is more secure and safer than utilizing serum. The noninvasive collection approach incredibly increases their readiness or willingness to experience health inspections, wellbeing reviews, it reduces uneasiness, tension and monitors their general health wellbeing over time and helps in diagonose morbidities in the beginning period that is the early stage. Utilizing an effective assay and successful measure, salivary diagnostics assumes a vital role in routine monitoring the health and observing the early disease detection [16]. In mid IR spectroscopy, the pure folate is described with hydroxyl stretching and stretching of NH vibrations, the bond of C=O for stretching vibration of carboxyl group, and the bond of C=O stretching vibration of $–CONH_2$ group and bending of NH vibration [17]. A connection has additionally been confirmed between neural tube defects in human and defective metabolism of folate [18, 19]. Furthermore, an association has been described between neural tube defects.

2. Pregnancy

Pregnancy is the development and fertilization of one or more offspring, known as fetus or an embryo, in a woman's uterus. In a pregnancy, there can be various multiple gestations, as on account of triplets or twins. Childbirth generally occurs around 38 weeks after origination [20]; in women who have a menstrual cycle length of a month, this is roughly 40 weeks from the begin of the last normal ordinary menstrual period (LMP). Human pregnancy is the most studied of every single mammalian pregnancy.

The term embryo is utilized to describe the developing offspring during the initial 2 months following conception. The term fetus is utilized from 2 months until birth.

In many societies' medical or legal definitions, human pregnancy is somewhat arbitrarily divided into three trimester periods as a means to simplify the different stages of prenatal development. The first trimester carries the highest risk of miscarriage (natural death of embryo or fetus). During the second trimester, the development of the fetus can be more easily monitored and diagnosed. The beginning of the third trimester often approximates the point of viability, or the ability of the fetus to survive, with or without medical help, outside of the uterus.

3. Serum

In blood, serum does not contain white or red blood cells and it is the serum component which is neither a blood cell nor a clotting factor; it is the blood plasma with the removed fibrinogens. Serum which includes all proteins not utilized as a part of blood clotting that is coagulation and all electrolytes, antibodies, hormones, antigens, and any exogenous substances such as microorganisms and drugs. An investigation of serum is serology, and may likewise include proteomics. Serum is utilized as a part of numerous diagnostic tests, and also in addition blood typing (**Figure 2**).

The blood is centrifuged to remove cellular components. Anti-coagulated blood which yields plasma containing clotting factors and fibrinogen. Coagulated blood (thickened or clotted blood) yields serum without fibrinogen, and remain some clotting factors.

Serum is a basic and essential factor for the self-renewal of embryonic stem cells which is in combination with the cytokine leukemia inhibitory factor. The clear fluid that can be isolated from coagulated blood. Serum differs or contrasts from plasma, the portion of liquid of normal unclotted blood containing the white and red cells and platelets. The coagulation which makes the difference between plasma and serum. The "Serum" which includes maternal serum, serum glutamic oxaloacetic transaminase (SGOT), alpha-fetoprotein (MSAFP), serum glutamic pyruvic transaminase (SGPT), and serum hepatitis.

The term "serum" is also used to designate any normal or pathological fluid that resembles serum as, for example, the fluid in a blister.

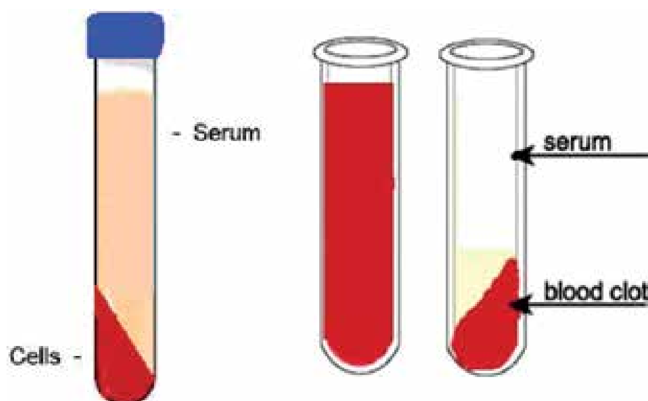

Figure 2. Serum.

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4. Deficiency of folate

Folic acid is called as vitamin B-9 or folate. In the B-complex family, it is a water-solvent vitamin. Everybody needs a diet which includes folate, regardless of whether or not they are pregnant, as inadequacy of folate leads to medical or health issues [20]. However, adequate intake of folate is considered particularly during pregnancy.

4.1. Folate and neural tube defects

The most effective argument for pregnant women requiring folate supplements originates from the link between adequate intake of folate and reduced risk of having a child with defects in neural tube. Defects in Neural tube are a classification of congenital defects in birth which is affecting the spinal cord and the brain, the most widely common being is anencephaly and spina bifida. Defects in neural tube can be seriously disabling or fatal for a baby development.

In addition, there has been some clear evidence that folate may decrease the risk of other birth defects too, and that a mother with low folate may likewise have a higher risk of miscarriage, placental abruption, preclampsia and preterm delivery due to the connection between low folate and homocysteine levels [10, 20–24].

5. Fourier transform infrared spectroscopy (FTIR)

The preferred method of infrared spectroscopy is FT-IR, and it stands for Fourier Transform infrared, in Infrared Spectroscopy, in which the infrared radiation is passed through a sample. A portion of the infrared radiation is absorbed by the sample and some of it is transmitted. The spectrum resulting, represents the transmission and molecular absorption which makes a molecular finger print sample. Like a unique mark of fingerprint no two one of a kind molecular structures which produces the similar infrared range of spectrum. This makes infrared spectroscopy valuable for various investigation. Information provided by FTIR,

- It can identify unknown materials,
- It can determine the quality or consistency of a sample and
- It can determine the amount of components in a mixture

The term Fourier transform infrared spectroscopy originates from the fact that a Fourier transform (a mathematical algorithm) is required to convert the raw data into the actual spectrum.

5.1. Developmental background

In 1957, the first low cost spectrophotometer capable for recording an infrared range of spectrum was the Perkin Elmer Infrared. This instrument covered the range of wavelength from 2.5 to 15 μm (range of wave number 4000–660 cm^{-1}). Later instruments utilized potassium bromide crystals to stretch out or extend the range to 25 μm (400 cm^{-1}) and cesium

iodide 50 μm (200 cm^{-1}). Beyond the region 50 μm (200 cm^{-1}) prominently known as the region of far-infrared, at long wavelengths it converges into the region of microwave.

5.2. Fourier transform infrared (FTIR) spectrometers

Fourier change spectrometers have replaced recently dispersive instruments for most applications because of their predominant speed and sensitivity or affectability. They have extraordinarily expanded the capacities of infrared spectroscopy and have been applied to various or many areas that are exceptionally difficult or about difficult to analyze by dispersive instruments. Rather than viewing each and every component frequency sequentially, as in a dispersive Infrared spectrometer, all frequencies are inspected and examined simultaneously in Fourier Transform Infrared (FTIR) Spectroscopy. FTIR depends on the basic or fundamental principles of molecular spectroscopy. The multitude of experimental techniques some of which are found in other oil tests analysis, and others that are sophisticated to the point that they are of significance just in look into research facilities.

The essential rule or the basis principle behind molecular spectroscopy is that the molecules which are specific, absorbs light energy at particular wavelengths, known as their resonance frequencies. For instance, the molecules of water resonates around the 3450 wavenumber (given the symbol cm^{-1}), in the region infrared of the electromagnetic range of spectrum.

A FTIR spectrometer works by taking a sample of small quantity and introduce it with the infrared cell, and it is subjected to light source of infrared scanned from 4000 cm^{-1} to around 600 cm^{-1}. The light intensity transmitted through the sample is measured at each wavenumber which allows the amount of light absorbed by the sample to be resolved as the contrast between the light intensity after and before the sample cell. This is known is the infrared range of spectrum of the sample.

A wavenumber, given the symbol of cm^{-1}, is just the reverse or inverse of the wavelength of the light. For instance, 3450 cm^{-1}, the resonance frequency of water which corresponds to the wavelength of light of 0.00000290 or 2.9×10^{-6} m, in the region of infrared of the electromagnetic spectrum. As opposed to utilizing the cumbersome unit of 10^{-6} m, spectroscopists basically take the inverse to give a number which is easier and more helpful to utilize.

In the infrared region of the range of spectrum, the molecule of resonance frequencies of an atom due to the presence of functional group molecule which is specific to the molecule. A functional group is a group of two or more atoms bonded together in a particular way.

6. Research design and data collection

6.1. Spectrum One FT-IR spectrometer (Perkin-Elmer)

In infrared Spectroscopy, the PerkinElmer Spectrum 100 Series FT-IR spectrometers are the highest quality level in testing materials, academia and in applications of research. The new Spectrum 100S version exhibits or demonstrates the highest sensitivity in its class which is

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enabling much faster measurement of even the most samples demanding such as absorbing highly or poorly reflecting materials.

For the more cost-conscious laboratory, the Spectrum 100R combines ease of use, reliability, performance at a cost typically observed among instruments with a small amount of the 100R's systematic power.

Characterizing the standard for the technology of FTIR, for more than 60 years, PerkinElmer is an accomplished and knowledgeable supplier and experience of FTIR spectrometers for research facilities and laboratories around the world. By adopting a complete quality strategy - from design of product, development and assembling through client or customer service and support - PerkinElmer gives the most highest quality FTIR system of frameworks, alongside the most exact, accurate and reproducible outcomes in the industry.

The Perkin Elmer Spectrum 100 Series spectrometers are bench top instruments that provides all the following in one self-contained unit as shown in **Figure 3**. The sample compartment of a large, purgeable, the instruments which can operate in ratio, interferogram mode or in a single beam. An optical system that gives data collection over a total range of 7800–370 cm^{-1} (220 cm^{-1} with CsI beam splitter) with a best resolution of 0.5 cm^{-1} for the spectrum 10 FT-IR, a mid-infrared detector-either DTGS or $LiTaO_3$ (lithium tantalate) as standard and the using MCT (Mercury Cadmium Telluride) or PAS (a photoacoustic detector) option for the spectrum 100 FT-IR.

6.1.1. Software

Generally a single software platform incorporates all the functions required for infrared analyses; instrument control, data manipulation and analysis, and flexible report utilities. A suite of optional software packages provide advanced capabilities or functions designed for specific application areas.

Figure 3. The spectrum 100 series spectrometer.

6.1.2. Applications

FTIR can be used in all applications where a dispersive spectrometer was used in the past. In addition, the multiplex and throughput advantages have opened up new areas of application. These include: Micro-examples. The forensic analysis of tiny samples which can be investigated in the sample chamber with the aid of an infrared microscope Minor examples, the surface image can be analyzed with the scanning magnifying lens. A surface picture can be obtained by scanning [25]. Another case is the utilization of FTIR which is used to describe creative or artistic materials in old-master paintings [26].

In Emission spectra, rather than recording the range of light spectrum transmitted through the sample, FTIR spectrometer can be utilized to obtain range of light produced by the sample. Such emission or outflow could be incited by different processes, and the most widely recognized ones are Raman scattering and luminescence. Little change is required to an absorption of spectrometer of FTIR to record spectra of emission and along these numerous commercial FTIR spectrometers combine both emission/Raman modes and absorption [27].

In Photocurrent spectra, the mode utilizes a standard FTIR spectrometer absorption. The examined or studied sample is set rather than the FTIR detector, and its photocurrent, prompted by the spectrometer's broadband source, which is utilized to record the interferrogram, which is then changed over into the photoconductivity sample spectrum [28].

6.2. Data collection

The FTIR spectroscopic features of saliva and serum of normal and anomalies pregnant women – deficiency of folate, changes in the salivary hormones of normal and anomalies are discussed. Informed consent were obtained from all subjects as approved by local ethics committee. The saliva samples were collected from normal and anomalies pregnant women from Saveetha Hospital, at Chennai. Saliva samples were collected from 20 volunteers in each set. 5 mL saliva samples were obtained in a tube from each individual and then used for the spectral analysis. All the procedures and methods of sampling were performed between 12 p.m. and 1 p.m. The measurement of FTIR spectra were totally completed at Sophisticated Analytical Instrumentation Facility, IIT, Madras, Chennai-36, using range one PerkinElmer FTIR spectrophotometer. The spectra were recorded in the region of mid infrared of 4000–400 cm^{-1} in the absorption mode. 50 μL of each solution was spread evenly and uniformly on the window of crystal of thallium bromide. The samples were air dried for water evaporation to isolate out the stray out the bands of absorption due to water. The spectrometer is furnished or equipped with a globar source and DTGS cooled locator. The sampling window is scanned as the background and 32 scans are co-included with a spectral determination resolution of 1 cm^{-1}. All the spectra were corrected with baseline and it has been standardized to achieve the identical area under the curve.

Intensity ratio parameters are computed and it shows that the FTIR spectroscopy has been successfully applied in the study of analysis of saliva and serum of normal and anomalies pregnant women.

7. Vibrational analysis FTIR

The FT-IR spectra of saliva and the serum samples of anomalies and normal pregnant women demonstrates the corresponding absorption bands in their particular regions qualitatively. In any case of quantitatively, there is a considerable spectral distinction between the saliva of normal and anomalies pregnant women and the serum of normal and anomalies pregnant women. The absorbance is specifically or directly proportional to the concentration. Thus the serum and saliva sample of normal and anomalies pregnant women are investigated and analyzed quantitatively by calculating the intensity ratio among the peaks of absorption. The other region of 3600–3000 cm^{-1} includes C-H, O-H and N-H vibrations of stretching of proteins. Intermolecular hydrogen bond increases and the concentration increases as decreases in frequency and also the additional bands begin to appear and the region of 3550–3200 cm^{-1} is at the expense of the “free” hydroxyl band [29]. It is seen in the most part of the spectra that the vibrations across from stretching of N-H vibration and stretching of O-H vibrations got merged and demonstrated a single and broad curve in this region.

The band around the region 3500–2800 cm^{-1} is due to cholesterol, Phospholipids and creatine and vibrations of stretching of CH_2 and CH_3 of phospholipids, cholesterol and creatine [30]. The stetching of asymmetric and symmetric C-H vibrations of methyl and group of methylene are observed to be around 2930–2875 cm^{-1} [31]. The band at (2933–2923 cm^{-1}) is due to the stretching of C-H bands in malignant tissue [32]. The absorption band near (1820–1670 cm^{-1}) is due to stretch of C=O which is strong and it is the mode of stretching of lipids [33] and the band at 1636 cm^{-1} is due to C=C stretching of aromatic (vibrational mode). The prominent peak observed at 1697 cm^{-1} for stretching of C=O vibration of group of carboxyl, due to formamide [34]. The aborption peak at 1604 cm^{-1} is expected to the stretching of – C=O of ring of aromatic skeletal vibrations [35].

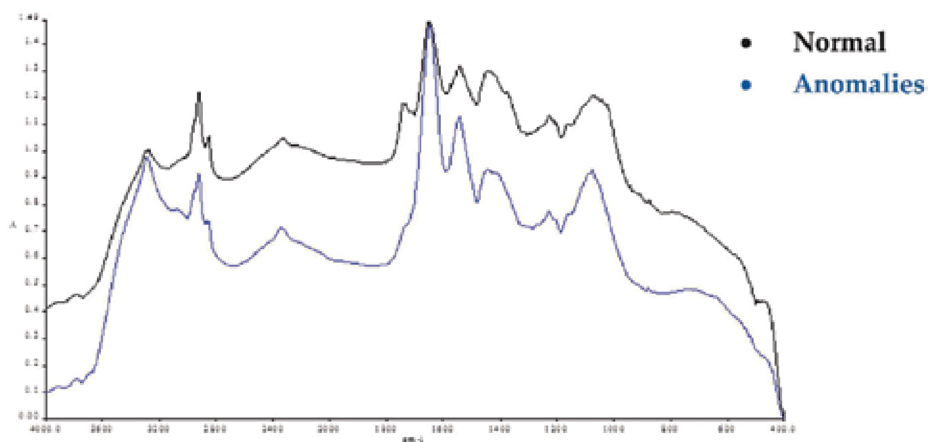

Figure 4. Comparison of saliva of normal and anomalies pregnant women.

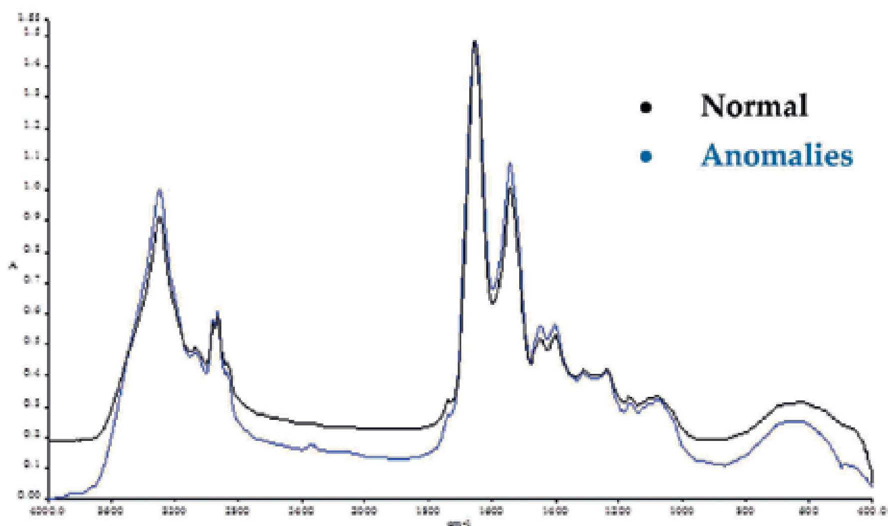

Figure 5. Comparison of serum of normal and anomalies pregnant women.

Wavenumber (cm^{-1})	Saliva		Serum	
	Normal Pregnant women	Anomalies Pregnant women	Normal Pregnant women	Anomalies Pregnant women
$R_1(I_{1338}/I_{3415})$	1.28	0.96	0.65	0.59
$R_2(I_{3546}/I_{1636})$	0.46	0.34	0.31	0.26
$R_3(I_{2854}/I_{3415})$	1.23	0.96	0.69	0.58
$R_4(I_{1743}/I_{3546})$	1.77	1.48	0.81	0.76
$R_5(I_{1482}/I_{1511})$	0.95	0.85	0.62	0.57
R_6 (I_{1482}/I_{1696})	1.01	0.91	0.73	0.68
$R_7(I_{1135}/I_{1607})$	0.89	0.81	0.47	0.40
$R_8(I_{2927}/I_{1607})$	0.97	0.92	0.90	0.85
$R_9(I_{2927}/I_{1604})$	0.98	0.94	0.92	0.87
$R_{10}(I_{1492}/I_{1511})$	0.96	0.89	0.70	0.66

Table 1. Comparative analysis of saliva and serum of normal and anomalies pregnant women.

The prominent absorption band around (1300–1000 cm^{-1}) i.e., (1150–1070 cm^{-1}) is because of stretch of C-O [29]. The peak of absorption in the region of 1480–600 cm^{-1} is relating to the band of amide II in tissue proteins. Amide II essentially comes from the stretching of C-N and bending of C-N-H vibrations feebly or weakly coupled to the stretching of bond of C=O mode. Aromatic amines shows strong stretching of C-N absorption in the region 1342–1266 cm^{-1}. The absorption shows up at higher frequencies than the corresponding absorption of aliphatic amines due to force constant of bond of C-N which is increased by resonance with a ring. Results demonstrated that there is a significant contrasts between the level of serum and saliva samples of normal and anomalies pregnant women of mid IR spectroscopy to all studied groups.

The comparison graph between normal and anomalies samples of saliva of pregnant women is shown in **Figure 4**. Samples of Serum of normal and anomalies pregnant women which are shown in **Figure 5**. A striking spectral difference observed between the samples of serum and saliva. A precise and systematic approach has been made by utilizing FTIR spectroscopic technique to study the spectral difference between healthy normal and anomalies pregnant women by utilizing saliva and serum samples and furthermore to find the efficacy of anomalies (open neural tube) defects in the embryo or fetus and absence of folate present in the pregnant women. The internal standard among the absorption peaks can be computed. In order to quantify the spectral difference, and ten intensity ratio parameters R_1 (I_{1338}/I_{3415}) R_2 (I_{3546}/I_{1636}) R_3 (I_{2854}/I_{3415}) R_4 (I_{1743}/I_{3546}) R_5 (I_{1482}/I_{1511}) R_6 (I_{1482}/I_{1696}) R_7 (I_{1135}/I_{1607}) R_8 (I_{2927}/I_{1607}) R_9 (I_{2927}/I_{1604}) R_{10} (I_{1492}/I_{1511}) have been introduced and calculated as shown in **Table 1**.

8. Statistical analysis

Variance analysis was implemented to recognize the spectral variations that were statistically significant. The t-test is one of the most rapid techniques for grouping or classifying of biological data. In the current study, the t-tests were utilized to separate certain regions of the FT-IR spectra analyzed or examined normal saliva and serum samples of healthy normal pregnant women and saliva, serum samples of anomalies pregnant women. For whole range of spectrum from 400 to 4000 cm^{-1}, analysis of statistical was performed by t-test and it shows the full effective classification to recognize or distinguish healthy normal and anomalies saliva, serum samples of pregnant women. In the analysis of t-test, considering the analysis of mean difference variance of the analysis, the t-test was carried out suggesting that the analysis of saliva investigation is better contrasted or compared with the analysis of serum. The absorbance values observed which gives a macroscopic value difference as contrasted to the minute variance observed in the analysis of serum utilized by FTIR. The intensity ratio parameters of saliva and serum samples of healthy and anomalies pregnant women are as follows: R_1 (I_{1338}/I_{3415}), R_2 (I_{3546}/I_{1636}), R_3 (I_{2854}/I_{3415}), R_4 (I_{1743}/I_{3546}), R_5 (I_{1482}/I_{1511}), R_6 (I_{1482}/I_{1696}), R_7 (I_{1135}/I_{1607}), R_8 (I_{2927}/I_{1607}), R_9 (I_{2927}/I_{1604}), R_{10} (I_{1492}/I_{1511}).

Utilizing FT-IR, saliva and serum samples were analyzed and the outcome of the results were statistically analyzed and compared using the t-tests. The standard deviation and the mean

Variance was identified and showed that the intensity ratio of saliva which predicts a good result analysis as compared with the analysis of Serum samples by utilizing FT-IR Spectrum (**Table 2**).

The t-test result outcomes were analyzed and prove that from the two samples of variances normal and anomalies pregnant women, the Saliva test gives an easier variance analysis for identification of anomalies as reviewed or explored through the measurable or statistical t-test (**Tables 3–8**).

The mean value is observed through the distribution of 't' at confidence interval of 95% which shows that the mean of saliva is more suitable for analysis of anomalies pregnant women as compared to analysis of serum through the correlation or comparison of intensity ratio parameters. The confidence intervals calculation and tests significance, the values of df which is associated with the unequal condition of variance are adjusted and rounded off to the closest integer.

This test is utilized for correlating the means for two samples, regardless of whether they have unequal replicate numbers. In basic terms, contrast between the actual difference between two means in connection to the variation in the data which is expressed as the standard deviation of the distinction between the methods and means utilizing the t-test.

Statistical tests take into account making statements with a higher degree level of exactness, however can't really proving or disproving anything. Significant outcome of the result at the 95% probability level make perfect data, which is adequate to help a conclusion with 95% confidence (however there is a 1 of every 20 chance of being wrong). In Biological work, to maintain and acknowledges and accepts this level of significance as being reasonable.

Saliva Analysis		Serum Analysis	
Mean difference of absorbance values	0.568	Mean Difference of absorbance values	0.054
Standard Deviation	0.19458	Standard Deviation	0.02319
Standard Error	0.06153	Standard Error	0.00733

Table 2. Statistical analysis-I.

	Saliva analysis	Serum analysis	Total
n	10	10	20
$\sum X$	5.6800000	0.5399999	6.2200000
$\sum X^2$	3.5669999	0.0340000	3.6009999
SS	0.3408	0.0048	1.6666
Mean	0.568	0.054	0.311

Table 3. Statistical analysis-II.

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t-test assuming equal Sample Variances					
$Mean_a$-$Mean_b$	t	Df	p	one-tailed	<.0001
0.514	+8.29	18		two-tailed	<.0001

Table 4. Statistical analysis-III.

F-Test for the significance of the difference between the variance of the two samples			
df_1	df_2	F	P
9	9	70.4	<.0001

Table 5. Statistical analysis-IV.

t-test Assuming Unequal Sample Variances					
$Mean_a$-$Mean_b$	t	Df	p	one-tailed	<.0001
0.514	+8.29	9.26		two-tailed	<.0001

Table 6. Statistical analysis-V.

Confidence Intervals			
	Observed	0.95	0.99
$Mean_a$	0.568	0.1391	0.2
$Mean_b$	0.054	0.0166	0.0238
$Mean_a$-$Mean_b$ [Assuming equal sample variances]	0.514	0.1301	0.1785
$Mean_a$-$Mean_b$ [Assuming unequal sample variances]	0.514	0.14	0.2014
Independent Samples			

Table 7. Statistical analysis-VI.

Options consider:	
One sample analysis	Two numbers of cases given.
Equal variance	Mean1 eq: 0.568; mean2 eq: 0.054
Confidence intervals	Population standard deviation
Mean 1: 0.568	estimated using sample
Mean 2: 0.054	T-distribution used
Std Dev.1: 0.19458	Difference between means:
Std Dev.2: 0.00733	0.514se=0.0024
	95% CI of difference:
	0.5092 <0.514< 0.5188 (Wald)
	t= 210.4; df= 9; p= 1

Table 8. Statistical analysis-VII.

9. Analysis with histogram

The bar diagram which is shown in **Figures 6** and **7** between the intensity ratio parameters and the values of absorbance were obtained from the spectra of FT-IR. The histogram clearly

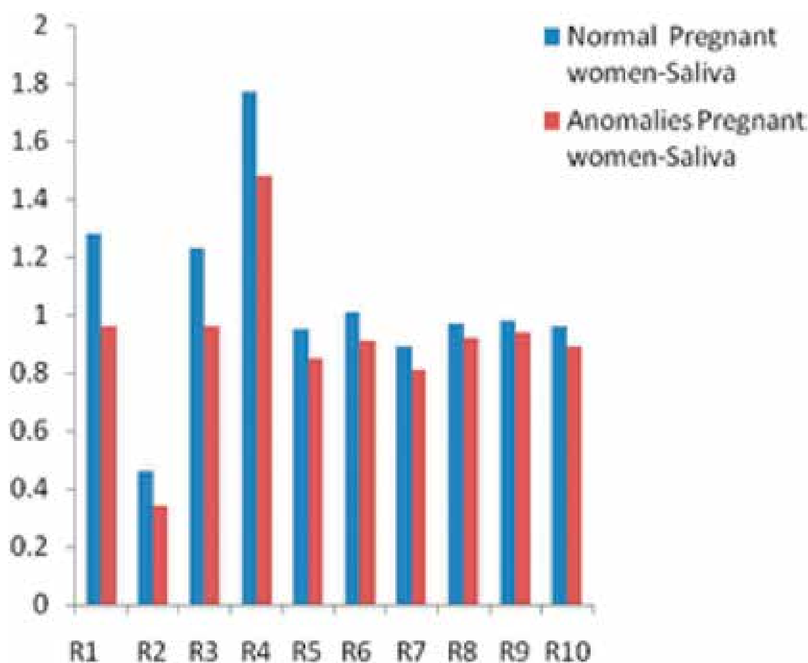

Figure 6. Comparison of intensity ratio parameters of saliva normal pregnant women and anomalies pregnant women.

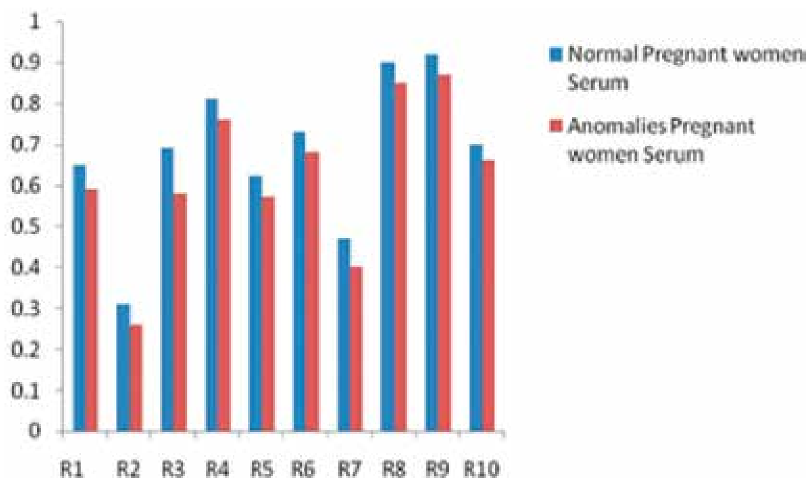

Figure 7. Comparison of intensity ratio parameters of serum normal pregnant women and anomalies pregnant women.

picture out and shows a striking contrast between the normal and anomalies pregnant women for both saliva and serum sample.

10. Conclusion

With FT-IR spectroscopy, biochemical changes and the spectral difference of both serum and saliva of normal healthy pregnant women and anomalies (open neural defect) in pregnant women are compared and detected. It is concluded that the diagnostics of saliva have a high potential to revolutionize the generation next, and offer a simple, inexpensive, and noninvasive approach for diseased detection.

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Abbreviations

FT Fourier transform

FTIR Fourier transform infrared spectroscopy

IR infrared spectroscopy

LMP last menstrual period

MSAF maternal serum alpha fetoprotein

SGOT serum glutamic oxaloacetic transaminase

SGPT serum glutamic pyruvic transaminase

ZPD zero path difference

A. Appendix and Nomenclature

This appendix presents the definition of terms/notation used throughout the chapter.

Notation	Definition
FTIR	Fourier-transform infrared spectrometer simultaneously collects high-spectral-resolution data over a wide spectral range. It is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas
LMP	The last menstrual period (LMP) refers to the start date of a woman's most recent menstruation, or period as indicated by the first day of bleeding
DTGS	Deuterated triglycine sulfate detector (DTGS) is a very sensitive room-temperature detector for mid-infrared range measurements that employs temperature-sensitive ferroelectric crystals of deuterated triglycine sulfate
R1	Intensity ratio parameter 1
t-test	A t-test is an investigation of two population means which implies using statistical examination, a t-test with two samples is commonly utilized with size of a small sample, testing the contrast or difference between the variance of two normal distribution are not known.
F-test	An F-test is any statistical test in which the statistic test has an F-distribution under the null hypothesis.
Std. Dev1	Standard deviation 1 is a quantity expressing by how much the members of a group differ from the mean value for the group.

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Section 4

Other B vitamins

Chapter 7

Niacin, Metabolic Stress and Insulin Resistance in Dairy Cows

Marko Cincović, Talija Hristovska and
Branislava Belić

Additional information is available at the end of the chapter

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Abstract

The periparturient period in cows is associated with metabolic stress and a state of negative energy balance, which are characterized by increased lipolysis, ketogenesis, hepatic steatosis, oxidative stress and insulin resistance. Such metabolic changes may exert adverse effects on the health and milk yield of lactating cows. The pharmacokinetics of niacin in ruminants is specific as rumen microorganisms facilitate both the synthesis of tryptophan and the degradation of niacin. Niacin administration to cows leads to an increase in the coenzyme activity, encompassing the activity of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes are actively involved in the metabolism of lipids and carbohydrates, whereas NAD protects the organism from oxidative stress. In periparturient cows, the supplementation of niacin has been found to induce depressed lipolysis and a limited impact of nonesterified fatty acids on all metabolic processes. It also results in decreased lipid peroxidation regardless of the magnitude of lipolysis in the periparturient period. Furthermore, niacin reduces the concentration of ketone bodies, thus preventing the development of fatty lever disease and ketosis in cows. The anti-inflammatory effect of niacin is manifested in stimulating the secretion of adiponectin and inhibiting immune cells.

Keywords: transition period, nonesterified fatty acid, insulin, glucose, oxidative stress

1. Introduction

Metabolic stress in dairy cows occurs around the time of parturition as a consequence of heightened milk production requirements, accompanied by depressed feed intake and

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a negative energy balance. Accordingly, the organism enters a state of increased lipolysis, oxidative stress and insulin resistance. Niacin is a well-known antilipolytic vitamin which enhances gluconeogenesis and insulin concentrations in the blood [1]. A review of the literature has revealed that the effect of niacin is dependent upon the dosage administered, pharmaceutical form, duration of administration and biological features of cows [2, 3]. There is a limited body of information on the relationship between niacin and insulin productions and efficiency in cows, as well as on the NAD and NADP response to niacin administration. This chapter will elucidate the mechanism of metabolic stress in cows, the pharmacokinetics of niacin, the physiology of the niacin-containing NAD and NADP coenzymes, as well as the biological effect of niacin on lipolysis, lipolysis-dependent metabolic adaptations and insulin resistance in dairy cows.

2. Metabolic stress in cows

In addition to a number of metabolic and physiological adaptations, the periparturient period in cows is associated with a dramatic increase in the nutritional requirements essential for foetal growth and milk synthesis. The nutritional requirements of the placenta and foetus are highest in the last 3 weeks of pregnancy, whereas the dry matter intake (DMI) is reduced by 10–30% relative to the DMI in the early dry period. As milk production surges from the onset of lactation to the yield required to sustain the calf, the ongoing adaptations occur rapidly, resulting in a marked discrepancy between the varying nutritional requirements and concomitant adaptations. The peak of lactation is expected to be reached in weeks 4–8 postpartum, whereas the highest dry matter intake is achieved in weeks 10–22 after parturition [1, 2]. The greatest negative energy balance in dairy cows is recorded around day 14 of lactation, continuing even to day 72 of lactation (as reported by the same author) [4]. During early lactation, the energy requirements for milk production and proper tissue function exceed the amount of energy ingested. To compensate for a negative energy balance, energy and protein body reserves are mobilized, expending to approximately 600 g/d of fat and 40 g/d of protein in the first 8 weeks after parturition [5]. The failure of these adaptive mechanisms has been implicated in the occurrence of common metabolic disorders in early lactation. Postpartum metabolic disorders are interrelated and concurrent, greatly influencing the fertility of cows. Fatty liver syndrome and degeneration, ketosis, parturient paresis, mastitis, hypomagnesaemia, rumen acidosis, displaced abomasum, laminitis, postpartum infections and fertility problems are the predominant diseases of dairy cows in the periparturient period [6]. A glucose mass of 72 g is required to produce 1 kg of milk [7]. In ruminants, the largest amount of carbohydrates ingested is fermented in the rumen, whereas very little glucose is absorbed from the digestive tract. Consequently, glucose requirements of dairy cows are, for the most part, met by gluconeogenesis, i.e. the synthesis of glucose from propionates, amino acids, glycerol and liver lactates. A postpartum increase in the expression of key enzymes, i.e. pyruvate carboxylase and phosphoenolpyruvate carboxylase, enhances the magnitude of gluconeogenesis in the liver. Substantial discrepancies between the depressed feed intake and elevated energy requirements of the mammary gland in this period incite the organism to provide sufficient energy to

the mammary gland in order to maintain the persistence of lactation. This is achieved through a number of metabolic adaptations induced by the hormonal changes and associated tissue responses. Increased liver gluconeogenesis, liver glycogen depletion, increased lipolysis, protein catabolism and limited glucose utilization by all tissues other than the mammary gland represent some of the alternative means by which the organism meets the energy requirements of the mammary gland. Growth hormone levels increase around parturition, resulting in the increased responsiveness of adipose tissue to lipolytic signals such as norepinephrine. An increased release of NEFAs from adipose tissue subsequently ensues, which are converted by the liver to ketone bodies and used as alternate fuels for extramammary tissues. The ketones serve as alternate fuels which can replace glucose in many tissues, thus conserving glucose for milk synthesis. Elevated somatotropin levels also enhance gluconeogenesis [8]. An increase in corticosteroid concentrations around parturition enhances the responsiveness of adipocytes to the action of catecholamines and stimulates glycogenolysis as well as gluconeogenesis [9]. Depressed plasma insulin concentrations and decreased insulin sensitivity enable the insulin-independent uptake of nutrients by the mammary gland, whereas insulin-dependent tissues increase the oxidation of fatty acids and reduce the utilization of glucose.

Adipose tissue has a pivotal role in homeorhesis and metabolic stress in cows. In the dry period and late lactation, anabolic processes predominate as the cow's body stores triglycerides in adipose tissue, which is thereafter sensitive to insulin (the key antilipolytic hormone reducing the degradation of triglycerides in adipose tissue cells and facilitating the synthesis of fatty acids and glycerol). A negative energy balance in early lactation is associated with a number of metabolic changes, leading to increased lipid catabolism in adipose tissue and the mobilization of body fat stores. The degradation of triglycerides stored in adipocytes also ensues, accompanied by the release of NEFAs and glycerol. The mobilization of adipose tissue fat is mediated by the following similarly functioning enzymes: monoglyceride lipase (MGL), hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) [10]. ATGL initiates lipolysis followed by the actions of HSL on diacylglycerol and MGL on monoacylglycerol. An increase in the action of triglyceride lipase is recorded at low insulin levels in the blood. The name of hormone-sensitive lipase itself suggests that hormones such as catecholamines, adrenocorticotropic hormone (ACTH) and glucagon stimulate the action of this intracellular neutral lipase [11]. The mobilization of fatty acids from body stores is induced by both energy deficits and changes in neuroendocrine regulation. Hormonal changes such as low insulin and glucagon concentrations significantly contribute to initiating and maintaining the mobilization of depot fat, whereas reduced insulin resistance, as an indicator of decreased insulin functional capacity, is of paramount importance [12]. Low plasma insulin concentrations enhance the action of triacylglycerol lipase and inhibit the entry of NEFAs, glycerol and glucose into adipocytes by reducing the action of lipoprotein lipase (the enzyme which hydrolyzes triacylglycerols in chylomicrons and very-low-density lipoproteins) as well as the expression/translocation of GLUT4 molecules. Lipolysis occurs in a state of reduced insulin sensitivity or low serum insulin concentrations (which is characteristic of early lactation), resulting in increased serum NEFA concentrations. In ruminants, acetate is a major substrate for the de novo synthesis of fatty acids, and adipose tissue is of overriding importance to the process. The degree of in vitro incorporation of acetate in the de novo synthesis of fatty acids

in adipose tissue is significantly reduced in late pregnancy (15 days antepartum), compared to days 120 and 240 of lactation, and completely impeded in early lactation [13]. Depressed lipogenesis is mainly attributable to hypoinsulinemia and decreased insulin sensitivity of adipose tissue, i.e. increased insulin resistance.

3. Pharmacokinetics of niacin, NAD and NADP

Niacin is a vitamin essential to energy metabolism. Physiologically, niacin is incorporated into the coenzyme nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These cofactors are involved in numerous metabolic processes: (1) anabolic pathways (NADPH/NADP) such as the syntheses of lipids and nucleic acids which require reducing equivalents provided by NADPH and (2) catabolic pathways (NADH/NAD). NAD is involved in a great many oxidation-reduction reactions as an electron carrier capable of accepting and donating electrons. NAD+, the oxidized form of NAD, can accept electrons in the reduction of NAD+ to NADH, whereas it can donate electrons in the oxidation of NADH to NAD+. Moreover, NAD is a source of adenosine diphosphate ribose (ADP-ribose) for protein modification. It is also a precursor of two second messenger molecules (cyclic ADP-ribose and nicotinamide adenine dinucleotide phosphate), which augment intracellular calcium concentrations and have a central role in a number of metabolic pathways. Another physiological effect of nicotinic acid is the potential to suppress lipolysis when administered in higher doses [14, 15]. Niacin is a generic descriptor for two vitamers: nicotinic acid and nicotinamide. Both forms of niacin are nutritionally equivalent and can be used for the synthesis of NAD. However, their biological proportions vary, and only nicotinamide can act as a reactive component [16].

In addition to ingested feed as a niacin source, niacin can also be synthesized in animals by the enzymatic conversion of tryptophan and quinolinic acid to niacin. Furthermore, rumen microorganisms synthesize niacin as well, using aspartates and dihydroxyacetone phosphate [17]. Previous research suggested that dairy cows did not require an exogenous supplementation of vitamin B due to a sufficient supply of this vitamin from the feed ingested and the synthesis of niacin in the rumen. However, milk production in high-yielding dairy cows has significantly increased, accompanied by increased vitamin B requirements. Depressed feed intake in the periparturient period, often continuing long after the onset of lactation, impedes the inflow of feed precursors (which are of immense importance to the ruminal synthesis of niacin), thus further increasing the need for niacin supplementation.

As an oral supplement, niacin can be rumen-protected or not rumen-protected. Niacin supplements which are not ruminally protected are less stable in the rumen and are readily degraded and, thus, should be administered in higher pharmacological doses [18]. The rumen-protected form of niacin is often found encapsulated and is commonly referred to as encapsulated niacin. These products are practically small pallets with niacin placed in the centre and covered by several layers of lipids. As encapsulation enhances the bioavailability

of niacin in the small intestine, the lipid layers in the pallet are relatively undegradable in the rumen and thereby prevent the degradation of niacin by rumen microorganisms [19].

The pharmacokinetics of orally administered medications in ruminants depends on the form of the medication (rumen-protected vs. not rumen-protected), whereas the pharmacokinetics of niacin is further influenced by two niacin vitamers: nicotinic acid and nicotinamide. The metabolism of nicotinic acid and nicotinamide, which are involved in the biosynthesis of NAD, may differ markedly. Unlike differences found in the concentration of each niacin vitamers [20], there is no difference in the total amount of niacin in the rumen between roughage and concentrate rations in a 40:60 to 60:40 ratio, respectively. Rumen microorganisms also synthesize niacin. The ruminal synthesis of niacin exceeds 2.2 g/d [21]. Increased amounts of nonfibrous carbohydrates facilitate the synthesis of niacin, whereas the roughage-to-concentrate ratio of the diet exerts no effect [22]. The duration of niacin administration greatly affects nicotinic acid concentrations in ruminal and intestinal fluids. One hour after administering niacin which is not rumen-protected, nicotinic acid concentrations in the rumen reach the peak by the conversion of nicotinamide to nicotinic acid or other forms (Campebell et al. [23] failed to detect nicotinamide in the ruminal fluid). The results of Santschi et al. [20] suggest that a considerable portion of both niacin vitamers are synthesized in the rumen, whereas the largest portion of nicotinic acid and the entire portion of nicotinamide are bound within the microbes.

Although direct absorption from the rumen is possible, the absorption of niacin from the small intestine appears to be the main route by which niacin is made available to the host. Only 17% of niacin administered is found in the duodenum as free nicotinic acid [23]. According to Santschi et al. [21], as much as 98.5% of niacin is degraded in the rumen of dairy cows. Nicotinic acid concentrations were found to be elevated in the duodenum of nicotinamide-supplemented cows (12 g/d) compared to cows supplemented with nicotinic acid [23]. However, this research focused solely on the analysis of duodenum fluids although niacin can also be found in solid intestinal contents. The niacin administered was not ruminally protected. Owing to the extensive ruminal degradation of niacin, considerably higher doses of niacin were administered to cows (12–36 g/d) in a number of studies [18]. When higher doses of niacin are administered, a surplus of undegraded niacin is more likely to reach the lower parts of the digestive tract. Increased niacin concentrations have been found in the duodenum of niacin-supplemented cows [23, 24]. A loss of niacin occurs even after abomasal infusion, to a lesser extent (approximately 85%), which corroborates the presence of both abomasal and duodenal niacin absorptions [21].

Duodenal niacin concentrations are essentially dependent on the pharmaceutical form of niacin (rumen-protected vs. not rumen-protected), the amount of niacin available and the roughage-to-concentrate ratio of the diet. According to Niehoff et al. [25], the total amount of niacin (nicotinic acid + nicotinamide) reaching the duodenum increases with an increase in the dietary share of concentrates and nicotinic acid supplements, whereas the amount of nicotinamide is solely dependent on nicotinic acid supplementation. Unsaturated nicotinic acid is of low rumen stability. Santschi et al. [21] reported that unsaturated nicotinic acid has a bioavailability of 5%. The administration of rumen-protected niacin in dairy cows leads to

augmented free niacin concentrations in the blood [19]. Morey et al. argue that encapsulated niacin treatments increase plasma nicotinamide concentrations (24 g/d of encapsulated niacin provides 9.6 g/d of bioavailable niacin).

The intestinal absorption of nicotinic acid and nicotinamide approximates to 73% and 94%, respectively, with an overall average niacin absorption of 84% from the duodenum [21]. Nicotinic acid is mostly absorbed from the duodenum. The intestinal mucosa is rich in niacin conversion enzymes such as NAD glycohydrolase. It is highly unlikely that nicotinic acid is directly converted to nicotinamide. However, nicotinic acid is readily converted to NAD in the intestinal mucosa, and excessive amounts of NAD are subsequently hydrolyzed to nicotinamide by NAD+ glycohydrolase [17]. NAD+ glycohydrolase is an enzyme that catalyzes the hydrolysis of NAD+ to produce ADP-ribose and nicotinamide [26]. Morey et al. [26] found that plasma nicotinamide concentrations decreased in niacin-treated cows 50 h after niacin administration but still exceeded those of control animals. Nicotinamide is the primary circulating form of niacin and is converted into its coenzyme forms (NAD and NADP) in the tissues. The transport of niacin in the blood is mainly associated with erythrocytes. Niacin rapidly leaves the blood stream and enters the kidney, liver and adipose tissue. There is a considerable dispute over the presence of nicotinic acid in the blood. Therefore, nicotinamide is considered the primary circulating form of niacin [17]. Nicotinic acid, which is not metabolized by the liver, can be transported to different tissues in the body by administering higher pharmacological doses of niacin.

Nicotinamide is a reactive part of NAD and NADP, which are involved in numerous oxidation-reduction reactions as coenzymes, i.e. cofactors. Enzymes containing NAD and NADP are important links in a series of reactions associated with carbohydrate, protein and lipid metabolism [27]. NAD and NADP act as an intermediate in most of the H+ transfers in metabolism, including more than 200 reactions in the metabolism of carbohydrates, fatty acids and amino acids. The most important metabolic reactions catalyzed by NAD and NADP are summarized as follows: carbohydrate metabolism (glycolysis, i.e. the anaerobic and aerobic oxidation of glucose, and the TCA (Krebs) cycle), lipid metabolism (the synthesis and breakdown of glycerol, the oxidation and synthesis of fatty acids and the synthesis of steroids) and protein metabolism (the degradation and synthesis of amino acids and the oxidation of carbon chains via the TCA cycle). The NAD and NADP coenzymes can be synthesized from niacin vitamers, tryptophan and quinolinic acid. The primary function of the liver is to synthesize NADP from tryptophan by hydrolysis in order to release niacin for its use in extrahepatic tissues. The brain, muscles and, to a lesser extent, testicles can take up nicotinamide from the bloodstream and utilize it without the previous deaminization. The nicotinamide nucleotide coenzymes are catabolized from four enzymes: NAD pyrophosphatase, NAD glycohydrolase, ADP-ribosyltransferase and poly (ADP-ribose) polymerase. Under normal conditions, there is little or no urinary excretion of either nicotinamide or nicotinic acid as both vitamers are actively reabsorbed from the glomerular filtrate. Such excretion only occurs when nicotinamide and/or nicotinic acid concentrations are so high that the transport mechanism is saturated. N^1-Methylnicotinamide and N-methyl-2-pyridone-5-carboxamide are the two principal urinary metabolites of nicotinamide in humans, rats and pigs. In herbivores, niacin is seemingly not metabolized by methylation but is mostly excreted unchanged.

Our results suggest [28] that blood NAD and NADP concentrations are a sensitive indicator of the niacin status of cows. The NAD concentrations obtained ranged from 860 to 895 pmol/mL in the control group in the weeks before and after calving. In niacin-supplemented cows, the following NAD concentrations were obtained: 1724.6 pmol/L in the week of calving (week 0), 1968.6 pmol/mL in the first week after calving and 1771.8 pmol/L in the second week after calving. The NADP concentrations obtained in the control group ranged from 385.09 to 425.62 pmol/mL during the entire period under consideration. In niacin-supplemented cows, the following NADP concentrations were obtained: 704.45 pmol/L in the week of calving (week 0), 778.36 pmol/L in the first week after calving and 796.18 pmol/L in the second week after calving.

4. Effects of niacin administration on lipolysis, ketogenesis and oxidative stress

NEFAs are the major component of triglycerides (the fat stores in the body), which consist of three fatty acids linked to glycerol. The hydrolysis of stored triglycerides (fat) in adipose tissue by hormone-sensitive lipase liberates NEFAs and glycerol. Plasma NEFA concentrations are elevated in periparturient dairy cows. Accordingly, cows mobilize fatty acids from adipose tissue as a means of adapting to a number of metabolic changes and a negative energy balance in the periparturient period. The large influx of NEFAs into the liver exceeds its fatty acid oxidation capacity and results in storing NEFAs as triglycerides in hepatocytes and muscles. Depressed feed intake and metabolic changes subsequently ensue.

In their review paper on the administration of niacin which is not rumen-protected, Niehoff et al. [3] argue that nicotinic acid can decrease NEFA concentrations under certain conditions, whereas nicotinamide does not exert the same effect. When the effect of nicotinic acid is minimized, a rebound of NEFAs above basal values occurs, followed by a return to normal concentrations. To induce these effects, the amount of niacin reaching the duodenum should be large, which can be achieved by high-dose niacin supplementation. High doses of nicotinic acid can suppress the release of fat from adipose tissue [14].

GPR109A (HM74A in humans and PUMA-G in mice) is a G-protein-coupled receptor for nicotinic acid, which has been shown to mediate the nicotinic acid-induced antilipolytic effects [15, 29]. The high-affinity receptor for nicotinic acid HM74A enhances the therapeutic effect of nicotinic acid by inhibiting adenylyl cyclase and reducing the intracellular level of cAMP in adipocytes. In vivo studies suggest that administering pharmacological doses of nicotinic acid decrease plasma NEFA concentrations by inhibiting lipolysis in cattle [14, 26, 30]. This antilipolytic potential of nicotinic acid is most likely realized by the activation of GPR109A [31–35]. The GPR109A antilipolytic pathway, already described in other mammal species, has only recently been shown to exist in a functioning form in bovine tissues under in vitro conditions. Conversely, nicotinamide has a low affinity for binding to GPR109A. The activation of GPR109A by nicotinic acid results in decreased cellular cAMP concentrations and the inhibition of adenyl cyclase. Decreased cAMP concentrations in adipocytes lead to the inactivation of protein kinase A and decreased phosphorylation of hormone-sensitive lipase, thus inducing

a reduction of lypolysis. The GPR109A receptors are found primarily in adipose tissue and immune cells, as well as in the brain, liver and muscles of cattle. BHB is the endogenous ligand of the human GPR109A, whereas nicotinamide acts as a very weak agonist at GPR109A producing no alterations in plasma lipoprotein profiles. Nicotinic acid, nicotinamide and BHB, as the ligands of the cattle GPR109A, exhibit different levels of efficiency in the induced antilipolysis under in vitro conditions. Nicotinic acid decreases the phosphorylation of hormone-sensitive lipase, thereby reducing the lipolytic response. However, nicotinamide does not exert a suppressing impact on the lipolytic activity in bovine tissues under in vitro conditions, whereas only extremely high BHB concentrations can significantly reduce the release of glycerol and phosphorylation of hormone-sensitive lipase.

Pires and Grummer [14] administered abomasal infusions of nicotinic acid (0, 6, 30 or 60 mg of NA/kg of body weight (BW)) to feed-restricted Holstein cows as a single bolus 48 h after the initiation of feed restriction. Plasma NEFA concentrations decreased from 546 to 208 ± 141 μEq/L at 1 h after the infusion of 6 mg of NA/kg of BW and to less than 100 ± 148 μEq/L at 3 h after the abomasal infusion of the two highest doses of NA. Upon the termination of NA infusions, a rebound occurred following the initial decrease of plasma NEFA concentrations. The rebound lasted up to 9 h for the 30 mg dose of NA and up to 6 h for the 6 mg dose. On balance, nicotinic acid was shown to be a potent antilipolytic agent in feed-restricted cattle with a negative energy balance. Sustained reductions in plasma NEFA concentrations are achieved as long as there is a constant supply of nicotinic acid to the lower parts of the digestive tract. The antilipolytic effect of nicotinic acid may be favourable to dairy cows provided that niacin is administered in optimal doses and forms, accompanied by a postruminal source of nicotinic acid. Nevertheless, the optimal dose of nicotinic acid should be determined, exerting a moderately inhibiting effect on lipolysis and NEFAs (adipose tissue NEFAs are an important energy source and precursors for the synthesis of fatty acids at the onset of lactation). In their study on the administration of rumen-protected niacin, Morey et al. [26] found that 24 g/d of encapsulated niacin (providing 9.6 g/d of bioavailable niacin) inhibited lipolysis in postpartum cows by decreasing postpartum NEFA concentrations. The treatment protocols used in this study are unequivocally associated with suppressing lipolysis in cattle, causing no rebound lipolysis. A total of 24 g/d of encapsulated niacin provides a source of bioavailable niacin which modifies lipid metabolism [36].

Notwithstanding the large influx of NEFAs into hepatocytes of early-lactation cows, decreased triglyceride concentrations were found in the liver of cows supplemented with rumen-protected niacin. As the accumulation of hepatic triglycerides is directly related to blood NEFA concentrations, reductions in blood NEFA concentrations lead to decreased triglyceride accumulation in postpartum niacin-supplemented cows [36]. In addition to fatty liver, the occurrence of ketosis is another negative consequence of elevated NEFA concentrations, i.e. the incomplete metabolism of NEFAs which are converted to ketone bodies. To prevent ketosis, cows should be supplemented with niacin alongside glycogen precursors such as propylene glycol and sodium propionate [37, 38]. The previous research suggests that niacin supplementation decreases plasma BHBA and NEFA concentrations with an increase in serum glucose [39, 40]. Erickson et al. [41] report significant effects of niacin on plasma BHB concentrations in niacin-supplemented cows compared to control animals. Relative to the control group, a

marked decrease in plasma BHB concentrations was recorded in cows supplemented with 12 g/d of niacin (in a crystal powder form), whereas a slighter decrease in plasma BHB concentrations was found in cows receiving 6 g/d of niacin [42].

In addition to lipolysis and ketogenesis, niacin exerts a major effect on lipid peroxidation and oxidative stress. Oxidative stress occurs when excess prooxidants (free radicals) overwhelm the antioxidant capacity of the organism. Such a state is associated with metabolic stress in periparturient cows [43]. It most commonly occurs when there is an imbalance between the increased production of free radicals and the decreased ability of the organism to neutralize them. Oxidation is part of the biochemical regulatory processes of the organism responsible for generating the energy required to sustain life. During these processes, free radicals are formed, having positive physiological functions. However, a physiological imbalance between excess free radicals and the ability of the organism to neutralize them changes the oxidative status of the organism, which thereafter enters a state of real oxidative stress (conducive to a number of various disorders and diseases). The degree of oxidative stress is determined by measuring the concentration and activity of prooxidants and antioxidants. Prooxidants are reactive oxygen metabolites containing an unpaired electron in the outermost electron shell, thereby participating in oxidation-reduction reactions. These reactive molecules can integrate into genetic and/or anatomical cell structures, inducing significant changes in cellular function [44, 45].

The reactive molecules most essential to periparturient cows are formed in the process of increased lipid mobilization. Nonesterified fatty acids are fairly reactive molecules susceptible to oxidation and free radical reactions. Fats are considered the best indicator of oxidative stress. Malondialdehyde (MDA) results from the reaction between free radicals and polyunsaturated fatty acids. It readily reacts with thiobarbituric acid to form thiobarbituric acid reactive substances (TBARS). MDA and/or TBARS concentrations are significantly increased in dairy cows after parturition. Moreover, a positive correlation has been found between MDA and TBARS concentrations and NEFA and BHB concentrations [46, 47].

In ruminants, antioxidants are divided into three major categories: (1) intracellular antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), (2) nonenzymatic protein antioxidants in plasma such as protein thiol groups in albumin and (3) nonenzymatic low-molecular-weight antioxidants such as glutathione, alpha-tocopherol, beta-carotene, etc. The antioxidant capacity of the cow's body is greatly dependent on the energy balance of the body. Therefore, ketotic cows, due to a negative energy balance, have been found to exhibit decreased antioxidant activity and increased concentrations of reactive lipid molecules. Antioxidant protection is also influenced by the diet and milk yield of cows. Calving has a significant impact on the antioxidant system of the cow, leading to a decrease in antioxidant concentrations in early lactation [15, 17, 18]. Niacin has been shown to exert the following antioxidant effects: decreasing lipolysis and lipid peroxidation, participating in the conversion of oxidized glutathione (GSSG) to the reduced form (GSH) by glutathione reductase (GR), decreasing the NADH+H+/NADP+ ratio and increasing the NAD+ content. As previously mentioned herein, niacin administration increases NAD concentrations [27].

In the study of Hristovska et al. [48], niacin-supplemented cows were found to exhibit lower NEFA concentrations in the periparturient period. NEFA concentrations remained invariant

in the niacin group within the first 2 weeks after calving, whereas a significant increase in NEFA concentrations was recorded in the control group in the same period. Niacin administration exerted a significant effect on metabolic adaptations in early-lactation cows. Cows supplemented with niacin exhibited considerably lower BHB concentrations, higher cholesterol and triglyceride concentrations, lower MDA concentrations, higher glucose concentrations, lower total bilirubin concentrations, lower liver enzyme activity (AST, ALP and GGT), higher albumin concentrations and lower urea and phosphorus concentrations. As the magnitude of lipolysis increases, niacin administration greatly reduces ketogenesis and lipid peroxidation. Niacin also exerts a substantial impact on the relationship between NEFA concentrations and other metabolic parameters; thus, a weak regression relationship was found between NEFA values and glucose, cholesterol, triglycerides, total bilirubin, AST, albumin, urea and phosphorus values. Niacin reduces the dependence of metabolic adaptations in early-lactation cows on the degree of lipid mobilization. Furthermore, niacin administration to periparturient cows positively affects lipid metabolism in early lactation, i.e. decreased lipid mobilization (decreased NEFA concentrations), ketogenesis (decreased BHB concentrations) and liver lipidosis (higher triglyceride and cholesterol concentrations in the blood and higher cholesterol concentrations per unit NEFA) [49].

5. Effects of niacin administration on insulin resistance

Insulin resistance is a state of reduced biological effect of insulin, leading to a compensatory increase in insulin concentrations [12]. It is associated with a diminished insulin response to glucose, i.e. insulin hyporesponsiveness (reduced beta cell function) and/or insulin sensitivity (depressed insulin-regulated glucose uptake in tissues). From the receptor's perspective, insulin resistance is referred to as pre-receptor (decreased insulin secretion and/or increased insulin degradation), receptor (a decreased number of receptors and/or their affinity for binding insulin) and post-receptor (defects in cell signalling and translocating glucose transporters. Insulin resistance in periparturient cows is attributed to the primary glucose requirements essential for foetal growth, udder development and lactation. In Holstein cows, insulin resistance is further influenced by plasma NEFA concentrations.

As niacin decreases lipolysis and increases glycaemia, it can facilitate insulin production and efficiency, as well as reduce insulin resistance. Some studies have failed to show significant effects of either niacin treatments or niacin treatment duration on blood glucose in cows receiving either rumen-protected or not rumen-protected niacin [26, 36, 50, 51]. Thornton and Schultz [52] reported the following changes in the metabolism of glucose in ruminants upon administering pharmacological doses of nicotinic acid: increased plasma glucose and insulin concentrations, reduced tolerance to glucose and reduced insulin resistance. Di Costanzo et al. [18] found a significant increase in blood glucose concentrations in cows supplemented with 36 g/d of nicotinic acid. Such an increase in blood glucose concentrations can enhance the cellular gluconeogenic activity, induced by the partial suppression of lipogenesis. Feed-restricted cows, abomasally infused with pharmacological doses of nicotinic acid, were found to exhibit elevated insulin concentrations 4–8 h after the termination of NA infusions.

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Increased glucose concentrations were recorded during a rebound of plasma NEFA concentrations (upon the initial decrease), whereas insulin concentrations followed a similar pattern to that of the NEFA rebound [14]. Pires et al. [14] argue that decreased NEFA concentrations in feed-restricted Holstein cows infused with nicotinic acid enhance the insulin response and glucose uptake with an increase in insulin sensitivity (suggesting that blood NEFA concentrations are a relevant factor in the occurrence of insulin resistance in dairy cows with a negative energy balance). These results are consistent with the results obtained in a study involving human subjects infused with acipimox (a long-acting nicotinic acid analogue). Acipimox was shown to decrease blood NEFA concentrations, increase the response to the oral glucose tolerance test and enhance the insulin-stimulated glucose uptake in peripheral tissue (using the hyperinsulinemic-euglycemic clamp technique) [53, 54].

Niacin has been shown to greatly affect glucose concentrations. An increase in glucose concentrations is dependent upon the niacin dose administered and treatment duration. Pescara et al. [55] claim that the mechanism by which nicotinic acid increases plasma glucose concentrations is unelucidated, but it may be attributable to the increased hepatic production of glucose or reduced blood glucose clearance or both. Blood insulin concentrations followed a similar dynamic pattern to that of blood glucose concentrations. An increase in glucose concentrations was recorded on days 10 and 12 of nicotinic acid infusions, continuing 1 day after treatment termination, whereas blood insulin concentrations increased during the entire treatment process [30]. According to Titgemeyer et al. [30], it is inconclusive whether an increase in glucose concentrations leads to an increase in insulin concentrations or insulin resistance causes elevated glucose concentrations. Their model is at variance with those stating that increased NEFA concentrations are associated with insulin resistance during nicotinic acid treatments. Differences in the results obtained can partially be accounted for by different energy supplies and degrees of lipolysis, indicating that both insulin and glucose concentrations in the blood are affected by niacin. Titgemeyer et al. [30] also found that glucagon concentrations were not significantly altered, inferring that glucagon was of little or no significance to the effect of niacin on blood glucose concentrations.

One of our studies has hypothesized that niacin administration to dairy cows in the transition period can influence insulin responsiveness and resistance in adipose tissue by virtue of niacin-induced changes in NEFA, glucose and insulin concentrations [56]. A total of 30 clinically healthy, multiparous Holstein-Friesian cows in late gestation were enrolled in the study. Insulin resistance was calculated on the basis of the following insulin resistance indicators: the glucose-to-insulin (G:I) ratio and the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI). The formula for the glucose-to-insulin ratio is as follows: G:I = glucose (mg/dL)/insulin (μU/ml). The RQUICKI is calculated on the basis of plasma concentrations of glucose (mg/dl), insulin (μU/ml) and free fatty acids (mmol/l), using the following formula: RQUICKI = 1/[log (glucose mg/dL) + log (insulin μU/ml) + log (NEFA mmol/l)]. The RQUICKI is a good indicator of insulin resistance in dairy cows. Although lipolysis-dependent, the RQUICKI correlates with numerous metabolic parameters [57, 58]. The influence of niacin supplementation, in the week of calving and the first week after parturition, on glucose, insulin and NEFA concentrations, as well as RQUICKI values, was analyzed using the analysis of variance (ANOVA). According to the RQUICKI values obtained, niacin-supplemented and

control cows (n = 15 cow × 3 week = 45) were allocated to two groups: a more resistant group (RQUICKI < 0.5) and a less resistant group (RQUICKI ≥ 0.5). Differences in glucose, insulin and NEFA concentrations between the two groups were determined using paired t-tests. Moreover, a linear regression analysis (Y = bXi + a) was performed on the basis of all the parameter values obtained in the niacin-supplemented and control groups in order to determine differences in the slope of regression lines (differences in the b parameters). Cows in the niacin group, which were more resistant to insulin (RQUICKI < 0.5), exhibited higher concentrations of nonesterified fatty acids compared to more sensitive cows in the same group but still lower than those recorded in control animals. The regression analyses performed suggest the following characteristics of niacin-supplemented cows relative to the control group: increased insulin response to glucose, decreased antilipolytic effect of insulin and increased insulin efficiency (expressed as the glucose-to-insulin ratio) with a decrease in NEFA concentrations. Niacin was found to exert a dual influence on insulin resistance in early-lactation dairy cows: decreased NEFA concentrations led to a decrease in insulin resistance (due to an increase in insulin efficiency and the insulin sensitivity index), whereas elevated insulin and glucose concentrations most likely caused an increase in insulin resistance in dairy cows (due to the lower insulin sensitivity index and antilipolytic effect of insulin).

6. Effects of niacin administration on the inflammatory response following metabolic stress

Inflammation is the common denominator of a number of processes occurring in cows during the periparturient period. Therefore, increased lipolysis may precipitate a substantial release of proinflammatory cytokines within adipose tissue, i.e. adipokines, the most important of which is tumour necrosis factor alpha (TNF-α) [59]. Ohtuska et al. reported increased serum TNF-α activity in cows with moderate-to-severe fatty liver syndrome [60]. The organism protects itself from inflammation by secreting acute-phase proteins. Plasma haptoglobin and serum amyloid A concentrations have been found to be elevated in cows with fatty liver [61]. In addition to decreased albumin and cholesterol concentrations, Bertoni et al. recorded increased bilirubin, AST and GGT concentrations in cows with a high inflammatory index, which is indicative of the biochemical profile of fatty liver [62]. Inflammatory mediators were directly implicated in metabolic changes by Trevisi et al. upon the peroral administration of interferon-α during the last 2 weeks of gestation, which led to liver inflammation and the release of acute-phase proteins [63]. Relative to the control group, cows treated with interferon-α were found to exhibit significantly higher plasma ketone concentrations during the first 2 weeks after parturition. A number of experimental studies have shown a direct impact of NEFAs on inflammatory processes such as the regulation of peroxisome proliferator-activated receptors (PPARs). PPARs modulate the inflammatory response in many cells such as adipocytes. In monocytes, PPARs activate certain polyunsaturated fatty acids such as α-linolenic acid and docosapentaenoic acid, which can suppress the inflammatory response. Another instance of the effect of lipids on receptor binding is the activation of Toll-like receptors (TLRs), especially TLR4. In addition to the

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ascending regulation of proinflammatory cytokines, the activation of TLR4 can lead to the inflammatory response [64–67]. The activation of the innate immune response is incited by the activation of TLR receptors present on immune and non-immune cells (able to identify pathogens). TLR4 identifies lipopolysaccharides (endotoxins), which are the major component of the outer membrane of Gram-negative bacteria [68]. The typical proinflammatory response to lipopolysaccharides entails the expression of several acute-phase cytokines (TNF, IL-1 and IL-8) and leukocyte-endothelial adhesion molecules, as well as the influx and activation of neutrophils in inflamed tissues. Furthermore, increased lipid hydroperoxide concentrations, associated with oxidative stress, have been found to induce an increase in the proinflammatory phenotype of endothelial cells [69, 70]. TNF-α, IL-1, IL-6 and IL-8 have been implicated in the occurrence of coliform mastitis in periparturient cows in a state of oxidative stress [71].

Niacin reduces adipose tissue inflammation by increasing adiponectin concentrations, thereby regulating the metabolism of carbohydrates and insulin sensitivity of adipose tissue. Such results have been obtained in cows and laboratory mice [72, 73]. Nicotinic acid increases adiponectin secretion through G-protein-coupled receptor signalling in cattle.

Niacin administration reduces TNF-α and IL-6, as well as the activation of NF-κB in the lungs and kidneys of rats [74, 75]. In monocytes, niacin suppresses the NF-κB signalling pathway, thus reducing proinflammatory mediators (namely, TNF-α, IL-6 and MCP-1) [76, 77] and inhibiting monocyte chemotaxis [78]. It also decreases C-reactive protein (CRP) concentrations, as well as macrophage accumulation in the liver and hepatocyte inflammation, which results in reducing acute-phase protein production [79, 80]. The anti-inflammatory effect of niacin is associated with the activation of niacin receptors [76, 77].

7. Conclusion

Niacin should be administered to ruminants in adequate pharmacological doses and forms on account of their complex stomach. The antilipolytic effect of niacin reduces metabolic stress in periparturient cows. Moreover, metabolic adaptations in the periparturient period are significantly less dependent on the magnitude of lipolysis provided niacin is administered. Niacin reduces lipid peroxidation and the degree of oxidative stress in cows by the NAD and NADP coenzymes. The antilipolytic effect of niacin decreases insulin resistance in cows. However, its potential to elevate glucose and insulin concentrations may attenuate the antilipolytic effect of insulin due to increased insulin resistance in a state of metabolic stress. Niacin exerts its anti-inflammatory effect by stimulating the secretion of adiponectin and inhibiting immune cells.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

Vitamin B2 and Innovations in Improving Blood Safety

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Additional information is available at the end of the chapter

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Abstract

Although transfusion of blood components is becoming increasingly safe, the risk of transmission of known and unknown pathogens persists. The application of vitamin B2 (riboflavin) and UV light to pathogen inactivation has several appealing factors. Riboflavin is a naturally occurring vitamin with a well-known and well-characterized safety profile. This photochemical-based method is effective against clinically relevant pathogens and inactivates leukocytes without significantly compromising the content and the efficacy of whole blood or blood component. This chapter gives an overview of the innovative technology for pathogen inactivation, the Mirasol® pathogen reduction technology (PRT) System, based on riboflavin and UV light, summarizing the mechanism of action, toxicology profile, pathogen reduction performance and clinical efficacy of the process.

Keywords: riboflavin, pathogen reduction technology, blood transfusion, safety

1. Introduction

The collection, processing, transfusion of whole blood, red blood cells, platelets, plasma, and infusion of fractionated plasma components are essential medical practices, often required for the preservation of life and for the treatment of disease. Although the transfusion/infusion of these components is a vital therapy, transfusions are still associated with some risk for transmission of disease to the patient [1].

Worldwide measures to reduce the risk of transmission of diseases to recipients through blood have been continuously implemented and improved [2]. Blood safety improvements include donor's questionnaire, self-deferrals and donation screening methods designed to detect possible contaminating agents in blood. Serological testing and nucleic acid testing

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have become staples of modern blood banking and have greatly reduced the risk of disease transmission by blood product transfusion. Yet, growing socio-political changes of contemporary society together with environmental changes challenge the practice of blood transfusion with a continuous source of unforeseeable threats with the emergence and re-emergence of blood-borne pathogens [2, 3].

In the last two decades, several pathogen reduction/inactivation technologies (PRT) have been developed to allow treatment of blood products with the intent of reducing the levels of infectivity and eventually inactivating white blood cells that can cause immunological complications to blood recipients. PRT methods involve physicochemical disruption of pathogen structural elements, mostly applied to the production of plasma-derived fractionated products or photochemical modification of nucleic acids to prevent replication, applicable to labile blood components like platelet concentrates, therapeutic plasma and eventually red cell concentrates [4, 5].

One of these PRT technologies, the Mirasol PRT System, uses riboflavin or vitamin B2 as a photochemical sensitizer and relies on the association of riboflavin with nucleic acid and activation with UV-light to generate a photochemical reaction that modifies guanine residues and thus prevents replication processes. This method creates irreversible damage via electron transfer processes at the sites where riboflavin-guanine base chemistry occurs [6].

Flavins are present in all biologic fluids and tissues. The most common biologically important flavins are riboflavin and its nucleotides: riboflavin-5′-phosphate (flavin mono-nucleotide, FMN), and the intramolecular complex of FMN with adenosine-5′-monophosphate (flavin adenine dinucleotide, FAD) [7]. Riboflavin in its coenzyme form is a component of many oxidation-reduction reactions and of energy production. It is essential for growth and tissue repair in all animals from protozoa to man [8] unlike fat-soluble vitamins, which are stored in body fat, riboflavin is a water-soluble vitamin and excess amounts are rapidly excreted. Because there are no physiological stores of riboflavin and excretion is constant, frequent dietary intake is important to maintain sufficient concentration and in the case of excess, return to normal levels is commensurate with renal function [9].

The choice of riboflavin as photosensitizer in the Mirasol PRT System was reinforced by its well-documented safety profile, being widely used as food coloring in the United States, where it is "generally regarded as safe" by the FDA [10]. Neonates, including preterm and very low birth weight (VLBW) infants, requiring nutritional supplementation due to immature gastrointestinal and metabolic systems, commonly undergo parenteral nutrition with a multivitamin preparation which includes vitamin B12, thiamine, folate and riboflavin [11]. The FDA concluded in their review that the LD_{50} is orders of magnitude greater than the Recommended Daily Allowance (RDA); additionally, no reports on carcinogenicity, mutagenicity or teratogenicity associated with riboflavin have been reported to the agency [12]. In Europe, it has been approved by the Scientific Committee on Food [13]. Furthermore, an anti-neoplastic action of riboflavin photoproducts to hematological malignancies and solid tumors has been postulated, whereas high dose of riboflavin has been suggested for migraine prophylaxis [14–16].

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2. Vitamin B2 and UV light: the chemistry

Riboflavin (RB) has absorption maxima at 220, 265, 375, and 446 nm in water and is yellow-orange in color. When aqueous solutions containing RB are exposed to sunlight, RB is converted into lumichrome (LC) under neutral conditions, and into lumiflavin (LF) in alkaline solutions [17, 18]. LC is also a known metabolic breakdown product of RB in the human body [19]. Flavin systems are known to be photochemically active, and the products of flavin photochemistry are known [17, 19, 20].

The mechanism of pathogen reduction using RB likely involves three potential pathways: Type I Photochemistry [47, 48] Type II Photochemistry, [21] and the effects of UV light alone. The contribution of each of these three pathways to the Mirasol PRT System pathogen reduction process has been described in the literature.

The reported mode of action of RB in the reduction of pathogens is postulated to be based in part on the ability of RB to interact with nucleic acids and to undergo chemistry with those nucleic acids upon exposure to light. This chemistry is believed to involve both oxygen-dependent and oxygen-independent (electron transfer) processes. It has been described thoroughly in the chemical literature over the past several decades [22–30]. The use of UV light with platelets and plasma also affords a third contributor to pathogen kill via the direct action of light.

2.1. Action spectra and absorbance spectra

Figure 1 depicts the action spectrum of RB and lambda phage minus light alone (yellow) over-laid with the absorbance profile of RB in PBS (pink) and absorbance of DNA in PBS (blue). At wavelengths lower than 300 nm, RB acts to shield the effects on DNA due to the

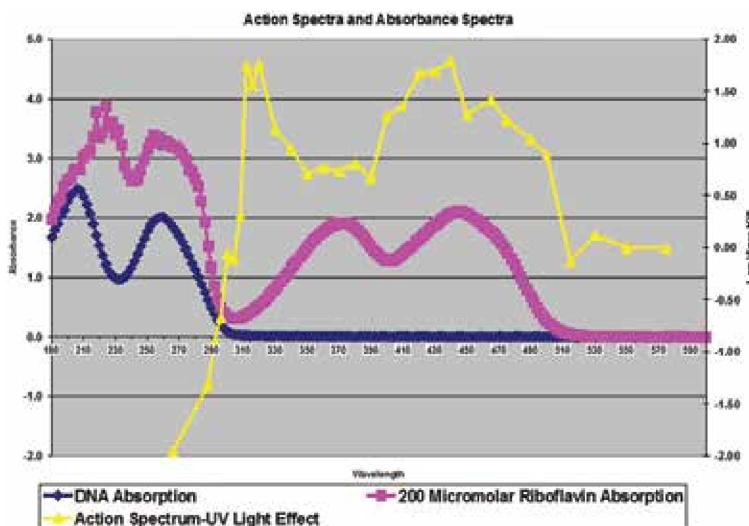

Figure 1. Action spectra and absorbance spectra of riboflavin with lambda phage.

direct action of UV light. Greater levels of inactivation in the presence of RB occur at wavelengths between 300 and 350 nm compared to the prediction due to the absorbance profile of free RB in solution. This is also observed for wavelengths higher than 500 nm. In the region of 308–575 nm, in order to achieve the same magnitude of log reduction that was observed between 266 and 304 nm, the energy required for the experiments between 308 and 575 nm was increased 50-fold from 0.1 to 5.0 J/mL. There is no inactivation of lambda phage with light of wavelength ≥330 nm in the absence of RB.

The action spectrum (AS) do not correlate perfectly with the absorption curve of either RB or LC in PBS over the entire wavelength regime. There appears to be essentially an identical amount of viral inactivation at 355 and 500 nm, although the optical densities (of solutions containing the same concentration of RB) differ by a factor of five at these two wavelengths. The phage reduction obtained at 320 nm and at 500 nm is greater than that expected based on the absorption spectrum of RB in PBS at these wavelengths. The effect is clearly seen in **Figure 1** which plots lambda phage inactivation achieved in the presence of RB at various concentrations minus that realized in its absence.

2.2. RB sensitized modification of nucleic acids

Several studies have been conducted in order to examine the ability of RB sensitization to modify nucleic acids [31]. The DNA fragmentation studies in leukocytes and bacteria utilized chemical agents that bind to portions of the DNA strand, which have been severed or broken because of chemical modification. The fragments that are produced leave regions that can be chemically tagged with a fluorescence probe and subsequently measured to provide an estimate of the extent of fragmentation that has occurred. Single-strand breaks throughout the nucleic acid sequence can be identified in this way. More complete breaks leading to denaturation of the nucleic acid can also be monitored by gel electrophoresis. In the latter case, the complete denaturation of the nucleic acid can be followed by examining migration patterns on polyacrylamide gels [31, 32]. This assay looks for much more severe and complete nucleic acid degradation than single-strand breaks.

In one set of studies, the level of DNA fragmentation occurring in white cell DNA was determined using a flow cytometric assay (Trevigen Apoptotic Cell System (TACS) assay. The level of DNA fragmentation obtained was significantly increased in the presence of RB. Similar observations were made for samples of plasmid DNA and for DNA isolated from *Escherichia coli* following treatment in the presence and absence of RB [31]. These combined studies demonstrate a sensitizing effect, with respect to nucleic acid damage, which RB imparts to samples treated with UV light. These observations are consistent with literature reports for RB.

Cadet and co-workers have evaluated the chemistry involved in the formation of specific lesions induced in nucleic acids by RB and light. These lesions differ from those induced by exposure to light alone in that chemically distinct oxidized species of guanine where residues are formed. This chemistry DNA fragmentation in isolated white cell DNA following exposure to light in the presence and absence of RB was evaluated because of the fact that mammalian systems do not normally contain enzymatic systems capable of repairing these types of lesions. This is in stark contrast to the predominant lesion (thymine-thymine dimers formed) upon exposure of nucleic acids or agents containing nucleic acids to light alone [33–35].

These studies identify the precise site of the lesions induced in nucleic acids treated with monochromatic 266, 308, or 355 nm light from either an excimer or Yttrium Aluminum Garnet (YAG) laser in the presence and absence of RB. The results demonstrate that in the presence of RB, the predominant modifications occur to guanine bases, as evidenced by the formation of 8-oxodGuo. The extent of the oxidized guanines formed in the presence of RB is far in excess of those observed upon exposure to light alone. These results are consistent with the literature reports of Cadet and co-workers of the mechanism of action of RB with regard to nucleic acid chemistry [24]. The results were contrasted to those using UV light alone in the absence of RB, and suggest that the addition of RB to the system specifically enhances the damage to DNA induced by UV light alone.

2.3. Phage reactivation

Virus reactivation is a phenomenon, which is known to occur through the use of host cell nucleic acid repair mechanisms. In the context of virus inactivation, the desired end target for these treatments is the prevention of virus replication. It is also desirable, in this context, to prevent repair of damaged virus particles because such repairs may render non-infectious agents capable of transmitting the disease when re-infused. This may be accomplished by generating either an extent of damage that the host system cannot repair or a type of damage that the host system does not have the capability of repairing.

Studies of the inability of bacteriophage to repair the lesions (Weigle reactivation) induced by RB and light as contrasted to the observations with light exposure alone have been conducted [31]. These studies confirm that the rescue of DNA damaged phage does not occur to the same extent when RB is present in samples exposed to light. These observations are consistent with the data suggesting that the presence of RB and UV light selectively enhances damage to the guanine bases in DNA or RNA. These data also suggest that this type and extent of damage to nucleic acids of virus in the presence of RB makes it less likely to be repaired by normal repair pathways available in host cells [36]. This result is essential for a system intended to assure the highest and most complete levels of pathogen inactivation attainable.

In summary, the Mirasol PRT process works through three independent mechanisms of action in rendering pathogens inactive. These include oxygen dependent chemistry induced by the combination of RB and light, electron transfer chemistry induced by the direct interaction of excited RB molecules with nucleic acid base pairs (primarily guanine bases) leading to oxidation products, and effects that are due to the action of UV light alone. In essence, the presence of RB in this system enhances the effects, which are due to UV light alone, creating a condition of greater sensitivity of the pathogen to the UV light to which the sample is exposed (photosensitization effect). The combination of these three modes affords broad and extended levels of pathogen inactivation with this process.

3. Toxicology and safety

Although the safety of RB has been extensively studied, there were no reports that directly supported its use in the Mirasol PRT System. Therefore a comprehensive preclinical safety

evaluation program in support of the Mirasol PRT System, designed to investigate all potential sources of concern, was conducted as part of the overall development program. In vivo animal and in vitro toxicity studies were performed using RB, lumichrome and photolyzed RB (see **Table 1**).

To obtain a consistent test article in as humane a fashion as possible for those studies, species-specific plasma was used rather than platelets. The photochemistry of RB yields equivalent photoproduct profiles in plasma products and in platelet products (which consist mainly of plasma). The absence of platelets eliminates the possibility of detecting toxic alterations to the platelet surface; however, that issue was addressed in the neoantigenicity and ^{14}C-RB binding studies.

3.1. Systemic toxicity

No toxicologically significant findings were observed in any of the studies of acute toxicity. In the repeated-dose toxicity study, the levels of RB and lumichrome in blood samples from animals receiving Mirasol PRT-treated products were below the limits of quantification, as were the levels in blood samples from animals receiving untreated plasma. These results were consistent with the observed rapid clearance of RB after IV administration, both in the literature [9] and in the pharmacokinetic study with ^{14}C-RB in Mirasol PRT-treated products. RB and its photoproducts naturally occur in human blood, see **Figure 2**. All photoproducts were found to be present in apheresis platelet products that had not undergone any photochemical treatment, although at a much lower concentration. The presence of these agents in human blood, the ubiquitous nature of RB exposure, its presence in human diets and the ability of humans to metabolize it and manage its inherent photochemistry suggests a low risk profile for this product.

3.2. Developmental toxicity and genotoxicity

No developmental toxicity was observed in the embryo-fetal development study. All fetuses were examined for malformations and developmental variations. No mutagenicity was

Test	Result
• Acute Toxicity*	Negative
• Neoantigenicity*	Negative
• Ames Mutagenicity#	Negative
• CHO Clastogenicity#	Negative
• Cytotoxicity#	Negative
• Reproductive Toxicity*	Negative
• Subchronic Toxicity*	Negative
• MMN Genotoxicity*	Negative
• Blood Compatibility#	Passed
• Leachables and Extractables#	Passed

Table 1. In vivo* and in vitro# toxicology.

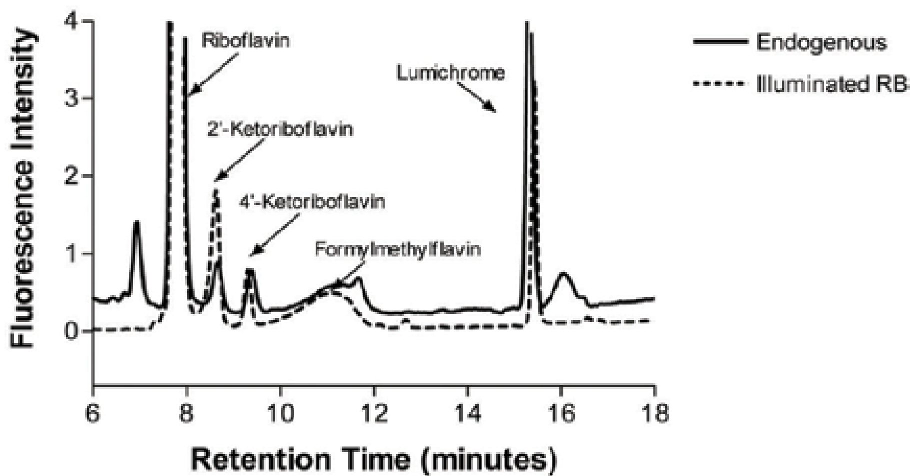

Figure 2. Riboflavin and its photoproducts are naturally present in human blood; no new compounds are formed after Mirasol treatment.

observed in the Ames test for treated or control human platelets, or for lumichrome. The in vitro and in vivo tests for clastogenicity in mammalian cells (chromosomal aberration in cultured CHO cells and micronucleus test in mouse bone marrow cells, respectively) were also performed with Mirasol PRT-treated products. Human platelets treated with the Mirasol PRT System gave negative results in all genotoxicity experiments.

3.3. Neoantigenicity and cytotoxicity

Results of studies using ^{14}C-labeled RB and exposure of platelets and plasma to UV light did not demonstrate any detectable binding of RB or its photoproducts to platelets or to plasma proteins. No evidence of neoantigenicity was observed with the Ouchterlony assay, indicating that no new antigens were formed during treatment with the Mirasol PRT System. Treatment with the Mirasol PRT System did not result in greater immunoglobulin G binding than what was observed in comparison with untreated controls, when assessed with the Capture-P assay. In the tests of lumichrome cytotoxicity, and of the cytotoxicity of Mirasol PRT-treated products, no cytotoxicity was observed.

3.4. Hemocompatibility

In tests of hemocompatibility, no hemolysis was observed. In functional assessments, when mixed with thrombocytopenic whole blood, the function of Mirasol PRT-treated platelets was well preserved, in comparison with controls [37]. Treated platelets displayed no evidence of hyperactivation or hypercoagulability.

3.5. Pharmacokinetics of photolyzed ^{14}C-RB in rats

After a single IV administration of Mirasol treated plasma containing photolyzed ^{14}C-RB, the radioactivity was well distributed from the whole blood to tissues selected for assay within

the first hour postdose. Most of the excreted urinary radioactivity was recovered by 12 h postdose, and more than half of all radioactivity was excreted in urine. Blood levels of radioactivity declined rapidly post-dose, as expected from studies of RB metabolism and excretion in humans [9]. Measurements of the radioactivity associated with the ^{14}C-RB-treated plasma indicated rapid initial apparent distribution (and/or clearance) from the systemic circulation that appeared to be complete within the first 8 to 48 h postdose.

3.6. Leachables and extractables

The leachables and extractables analyses detected no polymeric material in either test or control platelet products. The Mirasol illumination/storage bag does not contain the plasticizer di (2-ethylhexyl)phthalate (DEHP), and testing verified that this plasticizer was not present in treated and stored products. No toxicologically relevant concentrations of metals were found. These results correlate with those from the biocompatibility testing of the Mirasol illumination/storage bag elements—all elements were biocompatible.

4. Safety of blood

Blood transfusion safety is considered by the World Health Organization an integral part of each country's national health care policy and infrastructure [38]. In the last four decades safety of blood has been positively impacted by technological, economic and social improvements [2]. Improvements in blood processing and storage as per good manufacturing practices (GMP), introduction of policies discouraging paid blood donation and successive addition of screening tests for known transmissible pathogens, as Hepatitis B virus (HBV), Human Immunodeficiency viruses 1 and 2 (HIV-1/2) and Hepatitis C virus (HCV) are among the most successful measures to increase quality and safety of blood transfusion worldwide [39, 40]. From the late nineties onwards, introduction of nucleic acid testing (NAT) was able to minimize the window period of detection of these three viruses in asymptomatic blood donors to single days [41, 42].

Yet, in the last 20 years attention has been drawn to blood safety threats by recently known and/or re-emergent pathogens such as, Severe Acute Respiratory Syndrome virus (SARS), West-Nile Fever virus (WNV), Chikungunya virus (CHIKV), Dengue virus (DENV) or most recently ZIKA virus (ZIKV). Epidemics of these diseases are geographical or seasonal in nature and may not necessarily require universal reactive measures [2, 43, 44].

These unpredictable threats, as well as the long-recognized risk of bacterial transmission through platelet transfusion, may be effectively countered through the novel proactive approach with broad applicability and effectiveness, the pathogen inactivation/reduction technology (PRT) [4]. PRT has first been used to treat plasma and focused on destroying the structural elements of potential pathogens by the solvent-detergent method [45]. By the mid-nineties, the nucleic acid binding properties of Methylene Blue (MB) became exploited in a pathogen inactivation system for fresh frozen plasma using visible light [46].

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Though quite effective for the treatment of plasma, neither of these methods could be used for cellular blood products. Two newer technologies have been developed, both using UV light and two distinct chemical compounds to enable irreversible breakage of nucleic acids and blocking further replication of cells and pathogens. One technology uses amotosalen hydrochloric acid or S-59 as the photoactive-compound, which together with its photoproducts need to be removed from the blood component post-illumination due to its high toxic profile [47]. The second system uses riboflavin, a natural vitamin (vitamin B2) of which both photoproducts and catabolites are found endogenously in the normal blood and therefore do not need to be eliminated from the blood component before transfusion [6, 48, 49].

5. The Mirasol PRT process

Pathogen reduction is a proactive strategy to mitigate the risk of transfusion-transmitted infections. The Mirasol PRT System consists of an illumination/storage bag, RB solution, and an Illuminator that delivers UV light to cause permanent damage to nucleic acids of pathogens and leukocytes (see **Figure 3**). The system has been shown to be effective against clinically relevant pathogens [50, 51] and inactivates leukocytes [52] without significantly compromising the efficacy of the product [53–55] or resulting in product loss. The process involves transferring the blood component to the Illumination/Storage bag and adding 35 ± 5 mL of RB solution (500 μM). The product is then placed into the Mirasol Illuminator and exposed to UV light. After illumination, the final PRT treated product can be transfused immediately or stored without the need for additional filtration or processing. Treated plasma products are transferred to a storage bag appropriate for freezing. The Mirasol PRT System has been developed with the flexibility of treating plasma and platelet components, as well as whole blood.

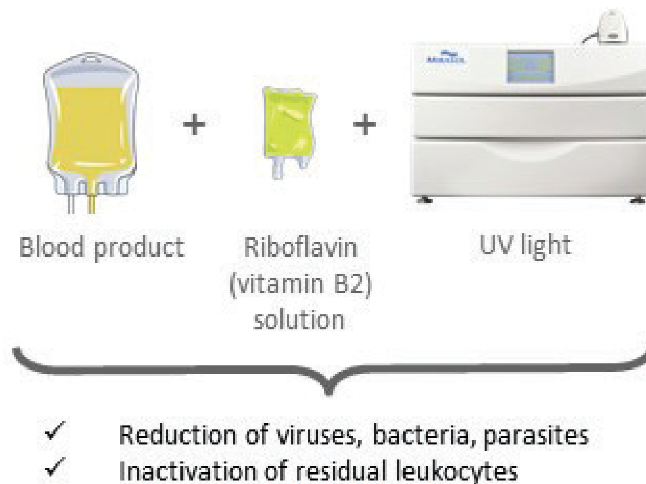

Figure 3. Mirasol PRT System.

5.1. Platelets and plasma

PRT-treated plasma products have been on the market in Europe for more than a decade and were issued a CE Mark in 2007 and 2008 for platelets and plasma respectively. FFP intended for transfusion to patients with multiple coagulation factor deficiencies (e.g. massive transfusion), emergency reversal of warfarin as well as for therapeutic plasma exchange must contain adequate functional levels of coagulation factors and other therapeutically valuable proteins. Protein levels should be as close as possible to those found in fresh plasma. Blood component processing can affect the quality of plasma products, particularly labile coagulation factors such as factors V and VIII.

Mirasol-treated FFP shows high overall protein retention under a broad range of blood banking conditions. Mirasol-treated FFP meets the European guidelines [56], showing on average factor VIIIc levels of 0.8 ± 0.2 IU/ml post treatment. Protein content meets guidelines even when whole blood is held overnight at room temperature and plasma is separated up to 18 h or frozen up to 24 h after collection. Additionally, anticoagulant factors such as protein C and protein S are well preserved after treatment with a 96% retention reported post treatment for both proteins. Extended storage of treated plasma at −30°C for up to 2 years does not significantly decrease protein quality [57].

Platelet products derived from apheresis or whole-blood can be treated with the system and products can be stored either in plasma or platelet additive solutions (PAS) for up to 7 days under standard blood banking conditions. It is critical that Mirasol-treated platelets remain viable and hemostatically effective. A series of in vitro studies have been performed to assess platelet quality after Mirasol treatment, and a correlation between in vitro parameters and in vivo performance was established [58]. In these studies pH and lactate production rate were found to be most strongly correlated with the in vivo recovery and survival of Mirasol-treated platelets. Glucose consumption rate and swirl also showed some correlation with these in vivo parameters, though to a lesser extent. P-selectin, pO_2 and pCO_2 expression in Mirasol-treated platelets, however, were poorly correlated with in vivo platelet recovery and survival. Changes in cell quality parameters do occur, cellular metabolism is up-regulated in treated platelets, and treatment induces some degree of platelet activation. However, shear-induced adhesion is maintained in Mirasol-treated platelets, and mitochondrial function is preserved [37, 59].

5.2. Whole blood: military and developing world

The Mirasol system was further developed for the treatment of whole blood, providing a single pathogen reduction and leukocyte inactivation step, followed by the use of the product as whole blood or pRBCs. The treatment of RBCs or whole blood has been more challenging due to the absorption of light by hemoglobin. Although the peak absorption of hemoglobin (400–450 nm) is outside the spectral region of the Mirasol lamp output, the UV light energy dose delivered to units of whole blood is normalized for RBC volume (J/ml_{RBC}). In vitro cell quality studies have verified that adequate quality and functionality of the RBCs and plasma components post treatment and throughout 21 days of storage is preserved [49]. In addition, crossmatch compatibility of the products is preserved. PRT treatment of whole blood has received CE marking in

2015 and is a significant step forward ensuring blood safety where whole blood transfusions are routine, such as sub-Saharan Africa and in far-forward combat situations.

6. Pathogen reduction performance

The Mirasol PRT System pathogen reduction process has been evaluated for performance against several pathogens. **Table 2** summarizes the pathogen reduction results. The data show reduction factors ranging from 2 to 6 log (99.0–99.9999% reduction) for each pathogen tested with the Mirasol PRT System. Log reduction values reported in the table were calculated by determining the number of virus particles present in infectious form prior to treatment and the number of virus particles present after treatment. The level of log reduction is reported as the starting titer expressed in units of 10× per mL minus the level after treatment expressed as the titer in 10× per mL. Because volume was constant in the samples before and after treatment, the unit of volume cancels, resulting in a reported value of log reduction.

For example, a sample containing 1,000,000 infectious virus particles per mL would of course have 10^6 virus particles per mL. If after treatment, only 100 particles per mL were measured in tissue culture infectivity assays, this would correspond to 10^2 virus particles per mL. The log reduction reported for this system would be 10^4 or 4 logs. This corresponds to a reduction in virus level of 99.99%. Because values are reported in log units, 100% reduction is never achievable.

Despite the fact that bacterial contamination of blood products poses one of the greatest risks for transfusion, there are currently no standards in place that establish a panel of species to test or a method to evaluate technology for pathogen reduction. A panel based on published hemovigilance studies incorporating those species responsible for the majority of morbidity and mortality in transfusion-associated reactions was utilized to guide study targets. Two complementary test methods were developed, as described below, to measure bacterial reduction performance.

To assess bacterial reduction efficiency of the system, two complementary test methods, known as "High Spike Bacterial Titer" and "Low Spike Bacterial Titer" tests, for bacterial reduction have been developed to measure the Mirasol PRT System performance. Both methods involve inoculation of known titers of bacteria (a "spiking" study) into platelet products followed by PRT treatment and subsequent measurement of the presence of bacteria. The objective of the High Spike Bacterial Titer experiments is to determine the overall bacterial reduction ability of the system against a severely contaminated platelet product. These studies may not, however,

Pathogen type	Typical performance
Viruses (enveloped, non-enveloped; intracellular, extracellular)	~2 to 6 log (99.0–99.9999%)
Parasites (Malaria, Chagas, Babesiosis, Leishmaniasis, Scub typhus)	≥ 3.0 to ≥ 5.0 (≥ 99.9% to ≥ 99.999%)
Bacteria (Gram +, Gram −)	~2 to 5 log (99.0–99.999%)

Table 2. Pathogen reduction performance.

represent a clinically relevant finding in that viable bacteria remaining after treatment may grow to high titers through the storage period. The objective of the "Low Spike Bacterial Titer Experiments" is to spike a platelet product with a more clinically relevant bacterial titer, treat the product using the Mirasol PRT System, and evaluate the platelet product using a standard culture system through a 7-day storage period to determine if it has remained culture negative, indicating that the platelet product meets release criteria for transfusion. The system demonstrated 98% effectiveness in these studies against a broad range of bacteria [60]. The combined data from these studies demonstrates the bacterial reduction capability of the system under conditions that are still substantially higher challenges than may be anticipated in an actual clinical setting.

7. Clinical performance

There have been 11 completed clinical studies with the Mirasol PRT System for Platelets stored in 100% plasma or platelet additive solution (PAS). There are two ongoing clinical studies in the United States, one study with platelets and one with RBCs derived from Mirasol-Treated Whole Blood. Primary outcome measure in most clinical studies has been levels of circulating platelets in thrombocytopenic patient's blood after prophylactic transfusion. Both CI (count increment) and CCI (corrected count increment) are accepted as surrogate markers of platelet transfusion efficacy, but they do not necessarily account for platelet function or bleeding outcomes in patients and they rely upon the assumption that a sufficient number of circulating, intact platelets will provide protection against bleeding. Patient factors and platelet product variability have been shown in published studies to affect increments, limiting the sensitivity of the CCI as a clinical efficacy measure. The CCI at one and 24 h after transfusion is decreased in patients receiving PRT treated products, compared to patients receiving control products. In two recent clinical trials Grade 2 or higher bleeding was the primary endpoint [55, 66]. Although lower CCIs were observed in these 2 studies, no difference in clinically meaningful bleeding in thrombocytopenic patients was observed.

The clinical evaluation of the Mirasol Whole Blood system includes a clinical trial in patients assessing the incidence of transfusion transmitted *Plasmodium* spp. infection that was conducted in Kumasi, Ghana [61]. Treatment of whole blood reduced significantly the incidence of transfusion-transmitted malaria. The safety profile and clinical outcomes were similar between test and control groups.

8. Current adoption for routine use

Since 2007, when the Canadian Consensus Conference on Pathogen Inactivation (PI) concluded that a proactive approach in accordance to the precautionary principle would reduce the theoretical risk and help sustain public confidence in the blood supply, many national and international committees, such as the Advisory Committee on Blood Safety and Availability (ACBSA), USA and the European Committee on Blood Transfusion of the Council of Europe discussed the accumulating evidence about the efficacy and safety of PRT [62, 63].

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PRT treatment of blood components is regarded as the next step to increase blood safety and support the credibility of blood institutions and health policy makers. However, there is a lack of consistency in the decision-making criteria used by regulatory bodies and blood operators regarding PRT implementation. The European Directorate for the Quality of Medicines & Healthcare of the Council of Europe in its Guide to the Preparation, Use and Quality Assurance of Blood Components, 19th Edition defines properties and requirements for therapeutic plasma, platelet concentrates and cryoprecipitate treated with PRT [56], yet PRT treatment of blood components is mandated in very few countries in Europe. Belgium, Switzerland and France have made the use of pathogen-inactivation mandatory for the treatment of platelet concentrates. Plasma treated by PRT is mandatory in Belgium, whereas the use of solvent/detergent treated plasma is more widespread in Europe but not mandated by national agencies.

The Mirasol PRT system has been gradually adopted in Europe, Asia and Latin-America. A hemovigilance program, based on the collection of passive hemovigilance data of Mirasol-treated components in multiple blood transfusion centers in Europe started in 2010. By 2015 data about 94,509 transfused platelet concentrates and 96,115 plasma transfusions were recorded in the program [64]. By 2017 over 750,000 disposable treatment sets have been distributed worldwide and 225,000 transfusion data have been recorded in the hemovigilance program.

9. Future

It is reasonable to envisage a future when all labile blood components will be PR treated to ensure a safe and sustainable blood supply in accordance with regional transfusion best practices. PR treatment of WB represents the most efficient implementation path to achieve this goal. It has been recently demonstrated through a clinical trial in a malaria-endemic country that a WB PR technology based upon riboflavin and UV light does reduce the risk of transfusion-transmitted malaria [61]. RBCs derived from PR-treated WB have shown good quality and recovery in health subjects and are currently being evaluated in a pivotal clinical trial [65].

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Conflict of interest

Marcia Cardoso and Susanne Marschner are employees of Terumo BCT, the manufacturer of the Mirasol PRT System.

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B-group vitamins are involved in numerous metabolic reactions and their widespread deficiency can cause a large series of health problems. The aim of this book is to provide an update on the current use and perspectives of B-group vitamins. Novel methods to detect folates in pregnant women, the use and role of folate dentistry, the use of genotype notification to modify food intake behavior, thiamin metabolism in Archaea and its role in plants and in crop improvement, the use of riboflavin in blood safety and niacin in metabolic stress and resistance in dairy cows are some of the subjects that are described in this multitopic book written by authors from seven different countries.

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