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Advances in Biochemistry & Applications in Medicine

Chapter 1

Transdermal delivery of Drugs using bio-compatible hydrogels and microneedles

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1. Introduction

Transdermal delivery represents an attractive alternative to oral delivery of drugs and is poised to provide a substitute to hypodermic injection too. People have practiced transdermal delivery for thousands of years by placing topical drugs or formulations on the skin for remedial effects. This practice is still in use in current era and large number of topical formulations are available for local curative effects [1].

Transdermal drug delivery systems (TDDS) are also named also as "Transdermal patches" or "Skin patches". Transdermal drug delivery system was first introduced more than 20 years ago. The technology generated tremendous excitement and interest amongst major pharmaceutical companies in the 1980s and 90s. By mid to late 1990s, the trend of Transdermal drug delivery system companies merging into larger organizations [2]. Transdermal drug technology specialists are performing extensive research for newer methods that can effectively and painlessly deliver larger molecules in therapeutic quantities to overcome the difficulties associated with the oral route.

Transdermal route of drug delivery provides various advantages such as improved patient acceptability, easy to use, by-passing the hepatic metabolism, non-invasive or minimally invasive.

Transdermal delivery has a variety of advantages compared with the oral route and hypodermal injections. For example:

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- It is used when there is a significant first-pass effect of the liver that can prematurely metabolize drugs. Many orally delivered drugs irritate the gastrointestinal mucosa and a large number undergo extensive 'first-pass' inactivation by liver. Transdermal drug delivery can by-pass the liver inactivation of drugs.

- Improved patient compliance is big advantage in comparison to oral and hypodermic injections. It is of great advantage in patients who are nauseated or unconscious. Patients have difficulty in swallowing tablets and capsules and some patients are tempted to crush tablets to assist in swallowing which destroys any controlled release characteristics of the tablets.

- A controlled delivery of drugs through skin can provide less fluctuation in the circulating levels of drugs and reduce the drug spike concentrations observed after orally delivered drugs.

- The drug release is such that there is a predictable and extended duration of activity. Greater flexibility of dosage in that dosing can be easily terminated by removal of the skin patch which is not possible in case of oral or hypodermal injections.

- Transdermal delivery also has advantages over hypodermic injections, which are painful, and generate lesser or no dangerous medical waste and does not pose the risk of disease transmission by needle re-use, especially in developing countries [5].

- In addition, transdermal systems are non-invasive and can be self-administered hence does not require any expert training for application.

- They can provide release for long periods of time (up to one week).

- They also improve patient compliance and can be of utmost importance in diseases where continuous drug administration is required.

In 1981, first transdermal patch approved was of Socopolamine, a drug used to treat motion sickness leading to nausea, and vomiting. As per the literature there are now more than 35 transdermal products, containing at least 13 approved molecules [3]. The value of global market for transdermal delivery as reported by Jain PharmaBiotech, was $12.7 billion in the year 2005 and is predicted to increase to $21.5 billion in the year 2010 and $31.5 billion in the year 2015. New technology, in form of adjuvants that boost the transfer across the skin barrier, as well as 'active' delivery that uses some form of energy to convey the ingredient, are poised to accelerate this growth. Creams, ointments, and lotions, the original transdermal delivery vehicles, mostly treat localized skin diseases, although that is now changing [4]. Non-medicated patches include thermal and cold patches, weight loss patches, nutrient patches, skin care patches (therapeutic and cosmetic), aroma patches, and patches that measure sunlight exposure.

2. First Transdermal Patch

The first transdermal system drug delivery system for systemic delivery was a scopolamine patch. It was a three-day patch that delivers scopolamine to treat motion sickness was approved for use in the United States in 1979.

Michaels et al. [5] reported that scopolamine can cross through human skin as found in exvivo permeation studies had a substantial flux through excised human skin. This led to the quest for first ever transdermal patch. This study progressed to the further investigation of the mechanism of percutaneous delivery of scopolamine through stratum corneum into the systemic circulation [6]. All these studies led to development of transdermal therapeutic system (TTS) by the Alza Corporation. Patch was capable of controlled administration of scopolamine through the surface of the skin to the systemic circulation [7,8]. Further extensive studies included the analysis of skin site where patch can provide maximum permeation into the system. It was found that Zaffaroni design of the patch applied behind the ear is best for permeation. Zaffaroni design of the patch constitutes a drug reservoir containing drug and a microporous membrane that commands slow and controlled the delivery of scopolamine [9]. The device was tested with Alza employees sailing in a large sailboat through a rough stretch of water close to the Golden Gate Bridge known as the 'potato patch'. Employees wearing the placebo patch were sick, whereas most of those wearing the scopolamine patch did not feel any sickness (Hoffman, 2008). In 1979, a 2.5 cm2 -TTS (which is still one of the smallest patches on the market) designed to deliver 1.5 mg of scopolamine over 3 days (TransdermSco¯p®; Novartis Consumer Health, Parsippany, NJ, USA) was the first transdermal patch to reach the US market. Transdermal scopolamine was very effective against motion sickness but also associated with minimal side effects [11].

A nitroglycerin ointment was the only transdermal product on the market until the marketing of the transdermal scopolamine patch. Whereas the nitroglycerin ointment led to more sustained serum levels than sublingual and p.o. sustained release capsule dose forms [13].

In 1981, patches for nitroglycerin were approved, and currently there exists a number of patches for drugs such as clonidine, fentanyl, lidocaine, nicotine, nitroglycerin, oestradiol, oxybutinin, scopolamine, and testosterone. There are also combination patches for contraception, as well as hormone replacement. Depending on the drug being administered, the patches may deliver drug from one to seven days [12].

This list below includes transdermal patches and delivery systems approved by the US Food and Drug Administration (FDA [1]. Topical creams, ointments, gels and sprays are not included.

Table 1: List of FDA approved transdermal patches and delivery systems.

Approval year	Drug/Product name	Indication	Marketing company
1979	Scopolamine/TransdermScop	Motion sickness	Novartis Consumer Health (Parsippany, NJ, USA)
1981	Nitroglycerin/TransdermNitro	Angina pectoris	Novartis (East Hannover, NJ, USA)
1984	Clonidine/Catapres-TTS	Hypertension	Boehringer Ingelheim (Ridgefield, CT, USA)
1986	Estradiol/Estraderm	Menopausal symptoms	Novartis
1990	Fentanyl/Duragesic	Chronic pain	Janssen Pharmaceutica (Titusville, NJ, USA)
1991	Nicotine/Nicoderm, Habitrol, ProStep	Smoking cessation	GlaxoSmithKline (Philadelphia), Novartis Consumer Health, Elan (Gainesville, GA, USA)
1993	Testosterone/Testoderm	Testosterone deficiency	Alza (Mountain View, CA, USA)
1995	Lidocaine with epinephrine (iontophoresis)/Iontocaine	Local dermal analgesia	Iomed (Salt Lake City, UT, USA)
1998	Estradiol with norethidrone/Combipatch	Menopausal symptoms	Novartis
1999	Lidocaine/Lidoderm	Post-herpetic neuralgia pain	Endo Pharmaceuticals (Chadds Ford, PA, USA)
2001	Ethinyl estradiol with norelgestromin/ Ortho Evra	Contraception	Ortho-McNeil Pharmaceutical (Raritan, NJ, USA)
2003	Estradiol with levonorgestrel/Climara Pro	Menopausal symptoms	Bayer Healthcare Pharmaceuticals (Wayne, NJ, USA)
2003	Oxybutynin/Oxytrol	Overactive bladder	Watson Pharma (Corona, CA, USA)
2004	Lidocaine (ultrasound)/SonoPrep	Local dermal anesthesia	Echo Therapeutics (Franklin, MA, USA)

2005	Lidocaine with tetracaine/Synera	Local dermal analgesia	Endo Pharmaceuticals
2006	Fentanyl HCl (iontophoresis)/Ionsys	Acute postoperative pain	Alza
2006	Methylphenidate/Daytrana	Attention deficit hyperactivity disorder	Shire (Wayne, PA, USA)
2006	Selegiline/Emsam	Major depressive disorder	Bristol-Myers Squibb (Princeton, NJ, USA)
2007	Rotigotine/Neupro	Parkinson's disease	Schwarz Pharma (Mequon, WI, USA)
2007	Rivastigmine/Exelon	Dementia	Novartis

3. Transdermal Patch

A transdermal patch is defined as adhesive medicated patch that is applied on to skin and it delivers an exact dose of drug through the skin into the bloodstream with a predetermined rate of release to reach in the body. Today the most common transdermal systems present in the market mainly are based on semi permeable membranes which were called as patches. A skin patch uses a special membrane to control the rate at which the liquid drug contained in the reservoir within the patch can pass through the skin and into the bloodstream.

Recently, the use of transdermal patch technology is restricted to only a few drugs. To persuade a drug to penetrate the skin and reach the systemic circulation in sufficient quantities, in the right time frame to exert a desired pharmaco-therapeutic effect is no small task. In order to engineer the drug for passage through skin it is important to take into consideration the basic skin histology so as to comprehend possible percutaneous delivery routes and challenges associated with the quest.

4. Anatomy and Physiology of Skin [14,15]

Human skin comprises of three distinct but mutually dependent tissues (Figure 1): A) Epidermis: Constituted of two parts:

1. The stratified stratum corneum and underlying
2. vascular, cellular, viable epidermis

B) Underlying dermis of connective tissues and

C) Hypodermis.

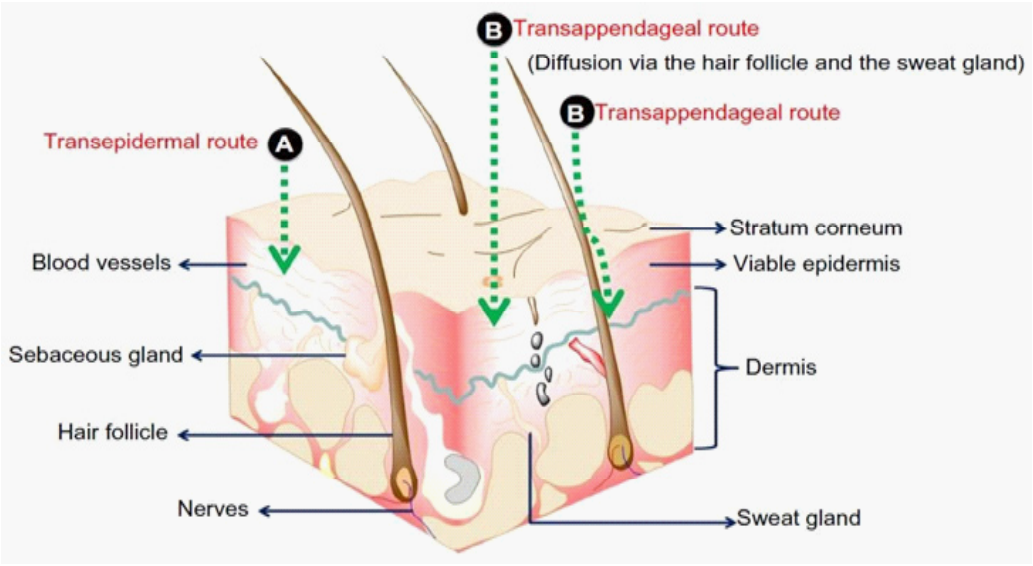

Figure 1: Structure of skin [16].

A. Epidermis: The uppermost multilayered layer of the skin which varies in thickness, because of changes in cell size and number of cell layers, ranging from 0.8 mm on palms and soles down to 0.06 mm on the eyelids. It consists outer stratum corneum and viable epidermis underneath.

1) Stratum corneum (Horney layer): This is the outermost layer of skin and the major barrier to transdermal delivery of drugs. It is approximately 10 µm thick when dry, but swells to several times this thickness when fully hydrated. It has 10 to 30 layers of dead, keratinized cells called corneocytes.

2) Viable epidermis: This is situated beneath the outermost layer and varies in thickness ranging from 0.06 mm on the eyelids sole upto 0.8 mm on the palms. Going inwards, it consists of various layers as stratum granulosum, stratum lucidum, stratum spinosum and the stratum basal. In the basal layer, mitosis divisions of the cells constantly reproduce the epidermis and this proliferation compensates the loss of dead horney cells from the skin surface.

B. Dermis: Dermis is made up of a mash network of connective tissue 3 to 5mm thick layer containing blood vessels, lymph vessels and nerves. Hence it provides nutrients and oxygen to the skin while removing toxins and waste products. Capillaries reach to within 0.2 mm of skin surface and provide sink conditions for most molecules penetrating the skin barrier. The blood supply thus keeps the dermal concentration of a permeant very low which is responsible for concentration gradient across the epidermis essential for transdermal permeation through diffusion.

C. Hypodermis: The hypodermis constitutes subcutaneous fat tissue that works to support dermis and epidermis. It serves as a fat storage area. This layer functions to regulate tempera

ture, provides nutritional support and mechanical protection. It carries principal blood vessels and nerves to skin and may contain sensory pressure organs.

5. Routes of Transdermal Drug Delivery

Percutaneous absorption involves passive diffusion of the substances through the skin occurring due to concentration gradient between epidermis and topical formulation. There can be two diffusional routes for a permeant to penetrate normal intact skin, the appendageal route and the epidermal route.

Appendageal route constitutes the transport via sweat glands and hair follicles with their associated sebaceous glands. These routes bypass the need of penetration through the stratum corneum hence also known as "shunt" routes. This route is considered tobe of minor importance because of its relatively small area, approximately 0.1 % of the total skin area.

Epidermal route: 1) Transcellular pathway uses epithelial cellular membrane for transport of molecules. This pathway involves passive transport of small molecules, active transport of ionic and polar compounds and endocytosis and transcytosis of macromolecules.

2) Paracellular: Paracellular pathway uses the space around or between the cells such as tight junctions for transport of molecules. Tight junctions or similar situations exist between the cells.

The principal pathway taken by a permeant depends upon the partition coefficient into the intracellular domains, whereas lipophilic permeants traverse the stratum corneum via the intercellular route. Most permeants permeate the stratum corneum by both routes. However, the tortuous intercellular pathway is widely considered to provide the principal route.

6. Kinetics of Transdermal Drug Delivery

Zhan et al., (2015) have postulated that drug release from a drug-in-adhesive patch follows first-order kinetics hence rate of drug release depends directly upon the drug concentration in the patch. However, reservoir-type transdermal drug delivery could be observed the zero-order kinetics [17].

One of the major advantages of the zero -order kinetics is that a zero-order input is easily achieved and the rate is apparently independent of the reactant concentration. This kind of kinetics ensures that drug levels in the blood remain relatively constant and does not cause any hypo or hyper concentration of drug as occurs with multiple oral dosing and hypodermic injections. This ensures that This can be of significant therapeutic benefit for certain conditions where constant stimulation of receptors or continuous interaction with other molecular targets is required and for drugs having a narrow therapeutic index.

However, this is type of drug delivery is associated with a slow onset of effect with lag time associated with response. Passive transport across the epidermis and the dermis and entry into the systemic circulation is a multistep process that can give rise to significant lag-times before steady state is attained. Thus, not only there was a quest to enable the delivery of different drugs across the skin but also a push to have ‘‘faster’’ transdermal delivery.

The rate controlling membrane, as a most important component in the reservoir-type transdermal patch, is responsible for controlling drug delivery. The rate-controlling membranes reported in previous publications included ethyl cellulose [18], collagen and chitosan [19], ethylene-vinyl acetate (EVA) [20].

Knowledge of skin permeation kinetics is vital to the successful development of transdermal systems. This permeation can be possible if the drug possesses certain physico-chemical properties. The rate of permeation across the skin (d_Q/ d_t) is given by [21]:

$$d_Q/ d_t = P_s(C_d-C_r) \quad \text{------- Eq. 1}$$

Where, C_d = concentration of skin penetrant in the donar compartment (e.g., on the surface of stratum corneum)

C_r = concentration in the receptor compartment (e.g., body) respectively

P_s = the overall permeability constant of the skin tissue to the penetrant

$$P_s = K_s D_{ss}/h_s \quad \text{------- Eq. 2}$$

Where, K_s is the partition coefficient for the interfacial partitioning of the penetrant molecule from a solution medium or a transdermal therapeutic system onto the stratum corneum,

D_{ss} is the apparent diffusivity for the steady state diffusion of the penetrant molecule through a thickness of skin tissues and

h_s is the overall thickness of skin tissues. A_s K_s, D_{ss} and h_s are constant under given conditions, the permeability coefficient (P_s) for a skin penetrant can be considered to be constant.

Permeability coffiecient = $K_s D_{ss}/h_s$ = 1/ resistance

Resistance has many components: Vehicle, Stratum corneum (usually most significant), Epidermis, Dermis.

The resistance occurs one after another ‘in series’:

$$R_{total} = R_{vechicle} + R_{sc} + R_{epidermis} + R_{dermis}$$

Total Permeability= $1/ R_{vechicle} + 1/ R_{sc} + 1/ R_{epidermis} + 1/ R_{dermis}$

The membrane limited flux (J) under steady state condition is described by equation:

$$J=DK_0/_wC/h$$

Where, J = Amount of drug passing through membrane system per unit area per unit time.

D = Diffusion coefficient within the membrane h = Membrane thickness K = Membrane / vehicle partition coefficient C = Concentration gradient across the membrane.

Further mathematical processing [22], considering diffusion coefficient of the penetrant molecules in protein gel, H_{sc-} thickness of stratum corneum, K_{pl} – distribution coefficient of the penetrant molecules between the lipid matrix and protein gel, D_{ml} – diffusion coefficient of the penetrant molecules in lipid matrix, reveals that if the drug is applied on to the skin surface in a simple solution form, the concentration of the drug (C_b) absorbed into the body can be described by Eq. 8. If the pharmacokinetic pattern of the drug is known to follow a simple one compartment model

$$C_b=(Drug)_a/V_d X K_a/K_a.K_e (Exp^{-Ket}- Exp^{-Ket}$$

Where, $(Drug)_a$ – concentration of drug in the body, V_d – volume of drug distribution, K_a – rate constant for skin absorption, K_e - rate constant for drug elimination

If the drug is delivered to skin surface through a zero-order delivery system, then, at a steady state, a constant blood level will be achieved, which is a linear function of the rate of drug release (K_o) and is inversely proportional to the rate constant for drug elimination (K_e), and the volume of distribution (V_d).

$$C_b==K_o/K_eV_d(1-Exp^{-Ket})$$

On the other hand if the drug is administered via a Transdermal Drug Delivery System which releases the drug molecules at a first-order rate constant (K_1) the blood level of the drug will then be described by

$$C_b= K_1(Drug)_{dds}/(K_1-K_e)V_d(Exp_{-Ket}- Exp_{-K1t})$$

In this case, C_b will be dependent on the drug dose level the drug delivery system, $(Drug)_{dds}$.

The assessment of percutaneous absorption of molecules is a very important step in the evaluation of any dermal or transdermal drug delivery system. A key goal in the design and optimization of dermal or transdermal dosage forms lies in understanding the factors that determine a good in vivo performance.

7. Transdermal Drug delivery Systems [1]

7.1. First-generation of transdermal delivery systems (TDDS) utilize a topical formulation that can be a metered liquid spray, gel etc. to the skin without any transdermal patch application. Upon evaporation or absorption, these formulations can drive small lipophilic drugs into the stratum corneum. In such cases SC act as the drug reservoir for sustained release into the viable epidermis over hours [20]. For example, testosterone gels have been in use for several years and a transdermal spray has been recently approved for estradiol delivery.

7.2. The second generation of transdermal delivery systems identifies that skin permeability enhancement is mandatory to expand the scope of transdermal drugs. The ideal penetration enhancer should (i) increase skin permeability by reversibly disrupting stratum corneum structure, (ii) provide an added driving force for transport into the skin and (iii) protecting underlying living tissues. This generation employs enhancement methods such as conventional chemical enhancers, iontophoresis and sonophoresis.

7.3. The third generation of transdermal delivery systems is poised to make significant impact on drug delivery with targets being stratum corneum. This targeting enables robust disruption of the stratum corneum barrier, and thereby more effective transdermal delivery, while still protecting deeper tissues. This generation TDDS novel chemical enhancers, electroporation, cavitational ultrasound and more recently microneedles, thermal ablation and microdermabrasion [23] have been shown to deliver macromolecules, including therapeutic proteins and vaccines, across the skin in human clinical trials.

8. Techniques for Enhancement of Skin Permeabilization

It is certainly not difficult to remove the stratum corneum, as sandpaper will suffice, but the motive is to do this in a reversible and relatively painless manner that minimizes irritation, is practical for chronic conditions and with minimal risk of infection. Hence, the quest for physical methods to transiently perturb the skin barrier or to provide additional driving forces that facilitate molecular transport.

8.1. Iontophoresis

The term iontophoresis is literally means ion transfer (ionto = ion; phoresis = transfer). Iontophoresis uses an electric current to deliver a medicine or active compound or other chemical through the skin. In popular terms, it is sometimes called “an injection without the needle”.

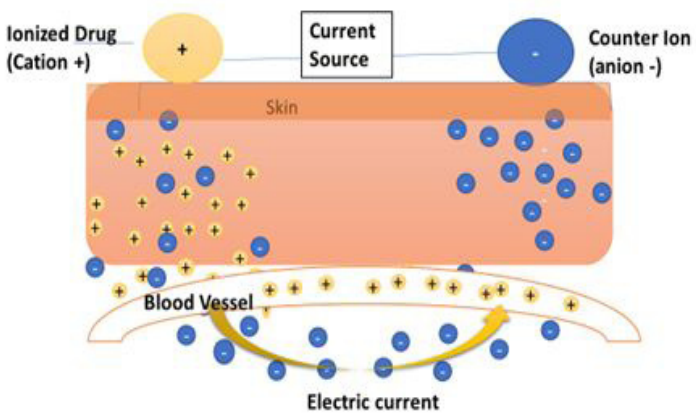

Figure 2: Diagramatic reperesentation of transdermal delivery using Iontophoresis.

This technique is not a new as it is being used since 1700's for various applications. Formally, ionotophoresis is defined as non-invasive method of thrusting high concentrations of a charged substance (a medication or bioactive agent), transdermally by repulsive electromotive force using a small electrical charge applied to an iontophoretic chamber containing a similar charged active agent and its vehicle.

Iontophoresis has advantages in pain management-as it can provide relief in response to acute pain episodes, such as post-operative pain and chronic pain, e.g. in cancer patients. The electric current controlled input kinetics allows the non-invasive administration of bolus doses as with conventional, in addition continuous current profile can be used for maintenance doses. Furthermore, iontophoresis can also be used for local pain relief or local anesthesia prior to minor surgical procedures also providing systemic pain relief [24].

8.2. Electroporation

Electroporation refers to the transient disruption of the skin using high voltage pulses [25]. This method creates temporarily aqueous pores in cell membranes, using electric pulses of high voltage and short duration. As reported by Denet *et al.,* the electrical resistance and the electrical breakdown potential of the stratum corneum (SC) is between 5 and 25kV/cm^2 and is approximately 75–100 V respectively. These along with other properties of the skin create the major barriers for permeation of drug molecules to reach systemic circulation.

Electroporation have been used successfully to facilitate the permeation of molecules through skin. It has also successfully been used to enhance skin permeability for molecules with different lipophilicities, sizes and high molecular weight biopharmaceuticals (proteins, peptides, and oligonucleotides). It has been postulated by Mori *et al.,* [26] that transdermal drug delivery enhancement using electroporation is the outcome of pore formation in the skin membrane. During electroporation, the SC is modified leading to increases in electrophoretic

mobility, molecular diffusivity, and electrical conductivity. It is well known fact that the high voltage for short duration electrical burst in transdermal electroporation can be more effective in enhancing transdermal flux of drugs in comparison with the continuous application of low voltage pulse as in the case of iontophoresis, as stated by Charoo *et al.,* [27]. Electroporation-assisted transdermal transport depends on the shape, amplitude, duration, number of electric pulses, as well as the distance between electrodes. Sugibayashi *et al.*, [28] investigated electroporation-mediated transdermal delivery of sodium benzoate. Pulse length and amplitude are also influential factors in electroporation when comparing different protocols [29]. It has been found that molecular and ionic transport across the skin exposed to a number of high voltage pulses is highly localized in sites termed local transport regions (LTRs) [29].

Transdermal electroporation is also called electropermeabilization [30]. Application of external field that exceeds the critical transmembrane potential leads to temporary electroporation of the skin [31].

Mechanism of structural changes in the skin during electroporation are still to be discovered. Backer *el al.*, [32] postulated that water pores form in the skin following application of high voltage pulses. It has been suggested that when voltage drop across the SC is more than 30 V, the skin experiences a sudden increase (up to four orders of magnitude) in permeability within 10 μs. According to this postulate, modification of SC lipid ultrastructure following application of high voltage pulses occurs due to the interaction between the water dipole and the electric field. The SC contains approximately 100 bilayer membranes in series and transient increase in permeability usually takes place when voltages of 30–100 V (100–1500 V applied voltages) are used. This is similar to the spectrum of voltages used for cell electroporation, i.e., 0.3– 1.0 V per bilayer [33]. Electroporation is a non-thermal process at the level of the cell.

8.3. Sonophoresis

Sonophoresis is a physical technique which employs the ultrasound waves onto the skin surface which to enhance skin permeability. Sonophoresis has been used successfully, to effectively deliver various types of drugs regardless of their electrical characteristics and coupled with other TDD methods to enhance drug delivery rates. These drugs have included hydrophilic and large molecular weight drugs [34]. Fellinger and Schmidt were first to bring the concept of ultrasound for TDD in 1950 for the successful treatment of polyarthritis using hydrocortisone ointment combined with sonophoresis [35,36,37]. However, the first ultrasound device for transdermal application was approved in 2004 by the FDA for the delivery of local dermal anesthesia by the Sontra Medical, SonoPrep®. Since that time, ultrasound has been widely used as a TDD system in the treatment of many other diseases including bone joint diseases and bursitis [38].

There are two main mechanisms currently known for skin permeation by sonophoresis:

thermal and cavitation effects. Between these two effects, cavitation is believed to be the predominant mechanism responsible for sonophoresis [39,40,41].

8.3.1. Thermal effect

When ultrasound passes through a medium, energy is partially absorbed in the form of heat energy [42]. In the human body, ultrasound energy absorbed by tissue causes a local temperature increase that is dependent upon ultrasound frequency, intensity, area of the ultrasound beam, duration of exposure, and the rate of heat removal by blood flow or conduction [43]. Merino *et al*. reported enhanced transdermal permeability caused by this temperature increase [44]. The skin temperature was increased by 20°C with low frequency ultrasound (20 kHz), and the delivery of mannitol was enhanced 35-fold. The resultant temperature increase of the skin may enhance permeability due to an increase in diffusivity of the skin.

8.3.2. Cavitation

Cavitation is defined as creation of cavities as well as expansion, contraction, and distortion of pre-existing gaseous bubbles in a liquid medium [45]. The likelihood of cavitation occurrence is closely related to ultrasound frequency as well as bubble characteristics such as size and shape. Since cavitation nuclei in biologic environments are random in size, type, and shape, the likelihood of cavitation is unpredictable.

8.4. Chemical Enhancement

The skin is meant to prevent excessive water loss from the internal organs and to limit the ability of xenobiotics and hazardous substances to enter the body. Recent studies have suggested that suitably designed combinations of chemical enhancers can balance trade-offs between enhancement and irritation based on the hypothesis that certain enhancer combinations are especially potent when present at specific, narrow compositions. This approach enables a strategy to target effects that enhance skin permeability in the stratum corneum, but avoids irritation in deeper tissues where the formulation composition becomes diluted or otherwise altered.

A study was carried out, examining close to 500 different pairs of chemical enhancers formulated to have more than 5000 compositions [46]. Finally a combination of sodium laureth sulfate (an anionic surfactant) and phenyl piperazine (a compound with aromatic nitrogen) at concentrations of 0.35 and 0.15 wt%, in a 1:1 mixture of ethanol and phosphate buffered saline dramatically increased enhancement with low skin irritation. *In vitro* screening results were validated with *in vivo* delivery of a peptide (leuprolide acetate) to hairless rats. These results suggest that combinations of chemical enhancers may succeed the delivery of macromolecules where individual enhancers have generally failed. Work on this approach continues in industry

[47].

Numerous compounds have been evaluated for penetration enhancing activity [48], including sulphoxides (such as dimethylsulphoxide, DMSO), Azones (e.g. laurocapram), pyrrolidones (for example 2-pyrrolidone, 2P), alcohols and alkanols (ethanol, or decanol), glycols (for example propylene glycol, PG, a common excipient in topically applied dosage forms), surfactants (also common in dosage forms) and terpenes.

8.5. Microneedles

Microneedles are fabricated to permeate the skin non-invasively to deliver drugs into through skin. Solid microneedles have been shown to painlessly pierce the skin to increase skin permeability for a variety of small molecules. Microneedles can be dip coated with a variety of compounds such as small molecules, DNA, proteins, and virus particles. In a recent study, naltrex one was administered to healthy volunteers whose skin was pre-treated with micro needles. After applying the naltrex one patch, therapeutic levels of naltrex one were achieved [2]. Transdermal patches with microscopic projections called microneedles were used to facilitate transdermal drug transport. Needles ranging from approximately 10-100 μm in length are arranged in arrays. When pressed into the skin, the arrays make microscopic punctures that are large enough to deliver macromolecules, but small enough for painless adminstration. The drug is surface coated on the microneedles to aid in rapid absorption. They are used in development of cutaneous vaccines for tetanus and influenza [15].

8.6. Needleless jet injectors

As name suggests a jet injector is a needle free device that can deliver drugs electronically in controlled doses of medication. Use of jet injectors lead to improved patient compliance due to reduced pain to the patient and improved consistency of delivery [49,50]. Jet injectors projects the liquid or solid particles at supersonic speeds through the outer layers of the skin using a reliable energy source for delivering the drug. The mechanism is basically, forcing compressed gas (helium) via a nozzle, such that the resultant drug particles entrained within the jet flow that travels at sufficient velocity for skin penetration [2].

8.7. Heat

It is already known fact that skin morphology changes in response to temperature changes, with more opened skin pores at high temperatures and dilated blood vessels. However, the effect of temperature on the delivery of penetrates greater than 500 Daltons has not been reported [2]. In order to generate the high temperatures needed to ablate the stratum corneum without damaging the underlined epidermis, the thermal exposure should be short, so the temperature gradient across the stratum corneum can be high enough to keep the skin surface

extremely hot but the temperature of the viable epidermis does not experience a significant temperature rise [51].

9. Ideal Characteristics of TDDS [52]

The skin has pH of 4.2 to 5.6, TDDS should have this pH range are used to avoid damage to the skin and maintain biocompatibility.. For the therapeutic action of the drug must have optimum partition coefficient, a low melting point (less than 2000°C), be non-irritating and non-allergic, a molecular weight less than approximately 1000 Daltons

9.1. Types of transdermal drug delivery systems [53]:

(I) Single–layer drug in–adhesive: The adhesive layer of this system also contains the drug. In this type patches the adhesive layer not only serves to adhere the various layer together, along with entire system to the skin but is also responsible for the releasing of the drug. The adhesive layer is surrounded by a temporary liner and a backing.

(II) Multi-layer drug in adhesive: The multi-layer drug in adhesive is similar to the single layer system in that both adhesive layers are also responsible for the releasing of the drug. But it is different however that it adds another layer of drug in–adhesive, usually separated by a membrane. This patch also has a temporary liner–layer and a permanent backing.

(III) Drug reservoir-in-adhesive: Reservoir transdermal system has a separate drug layer. The drug layer is a liquid compartment containing a drug solution or suspension separated by the backing layer. In this type of system the rate of release is zero order.

(IV) Drug Matrix-in-adhesive This matrix system has a drug layer of semisolid matrix containing a drug solution or suspension. The adhesive layer in this patch surrounds the drug layer partially overlaying it.

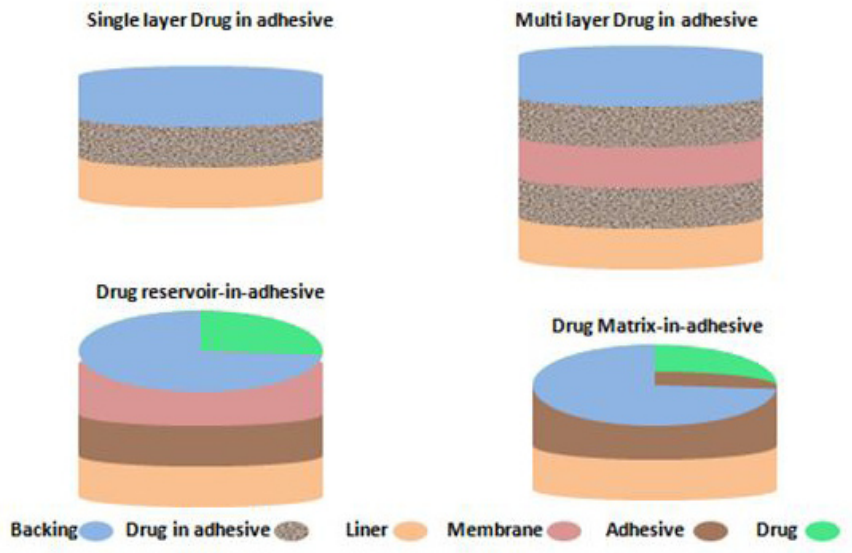

Figure 3: Different types of transdermal patches. [53]

In the fore-mentioned types of transdermal patches (Figure 3), hydrogels can act as drug reservoirs. This assembly of transdermal patch along with skin permeation method can make transdermal drug delivery devices more promising .

10. Hydrogels in Transdermal Drug Delivery

Hydrogels is a fancy word for three-dimensional, cross-linked mesh of water-soluble polymers. Hydrogels can be synthesized from any water-soluble polymer, encompassing a wide range of chemical compositions and bulk physical properties. Hydrogels can be formulated in different physical forms such as slabs, microparticles, nanoparticles, coatings, and films. Hydrogel has consistency between solid and liquid i.e. a gel form with properties of solids such as maintaining its network structure without losing its consistency and at the same time squishy nature which allows it to take the shape of the surface on which it is applied. These formulations have properties of massive water or fluid absorption. This property enables the encapsulation of high amount of drug in mash network of hydrogel. On water or fluid absorption in the spaces among pores, the hydrogels swell, this leads to drug elution. Topical or transdermal application of such biomaterials loaded with drug is how they can be used for transdermal drug delivery. Transdermal drug delivery occurs via diffusion through skin. The concentration gradient between two compartments i.e. hydrogel reservoir and the ventral surface of skin. In such systems hydrogel act as biocompatible drug reservoir.

The unique physical properties of hydrogels have stimulated particular interest in their use in drug delivery applications [54]:

1. Hydrogels are also known to be generally highly biocompatible, as obvious from their use in peritoneum [55] and other sites in *in vivo* systems. This attribute of biocompatibility is due to the high-water content of hydrogels. Furthermore, hydrogel have physicochemical properties mimicking that of native extracellular matrix, both compositionally (particularly in the case of carbohydrate-based hydrogels) and mechanically.

2. Porous nature of hydrogels permits loading of drugs into the gel matrix and subsequent drug release at a rate dependent on the diffusion coefficient of the small molecule or macromolecule through the gel network (**Figure 4**).

3. Their highly porous structure can easily be tuned by controlling the amount of cross-linker and the affinity of the hydrogels for the aqueous environment in which they are swollen.

4. Hydrogels for drug delivery exhibit pharmacokinetic benefits – by allowing the sustained and controlled delivery of drug in the surrounding tissues over an extended period.

5. Biodegradability or dissolution of hydrogels may be tuned via enzymatic, hydrolytic, or

environmental (e.g. p^H, temperature, or electric field) pathways.

6. Hydrogels are deformable due to their sol-gel nature. Hence, they take the shape of the surface to which they are applied. This distinguishing trait of hydrogels is accountable for the muco- or bio-adhesive properties which is profitable in immobilizing them at the site of application.

The term hydrogel was originally introduced by Wichterle and Lim in 1960s and its biological application was put forward. The first paper sighted was by DuPont scientist in 1936 for medical applications, which introduced the spark that was enlightened in 1960 by Wichlerte and Lim who worked on poly (2- hydroxyethylmethacrylate) poly (HEMA).1 It highlighted the properties of this brittle polymer as a highly water swollen, soft and elastic gel. This led to the keen interest in hydrogels as a class of biomaterials and their application as drug delivery systems. Furthermore, because of their high-water content, swollen hydrogels can provide a better feeling for the skin in comparison to conventional ointments and patches. Versatile hydrogel-based devices for transdermal delivery have been proposed so far. The topical application of hydrogels can effectively be used to deliver drugs that can help to alleviate the symptoms of many pathological conditions.

For instance, Nho *et al*. [56] reported a therapeutic hydrogel made of poly (vinyl alcohol) or poly (vinylpyrrolidone) for the treatment of a topic dermatitis.

Peppas and group [104] have reviewed the applications of hydrogels in the pharmaceutical field, hydrogel characterization and analysis of drug release from such devices.

Sun *et al*. [94] have reported the composite membranes comprising of cross linked PHEMA with a nonwoven polyester support. Permeation flux of 4 to 68 mg/cm^2 per h for nitroglycerin can be managed by adjusting the preparation conditions.

Kim *et al*. [95] have reported the preparation of a Carbopol 934w-based formulation containing phosphatidylcholine liposomes (liposome-gel). In their study, the skin absorption behavior of hydrocortisone containing liposome- gel was assessed. Gayet and Fortier have reported bovine serum albumin (BSA) and PEG copolymerized hydrogels [96]. This hydrogel allows the release of hydrophilic and hydrophobic drugs due to their high-water content. Hence this hydrogel has potential application as controlled release devices in the field of wound dressing. Hubbell [97] have reported in-situ photopolymerizable hydrogels made from terminally diacrylated ABA block copolymers of lactic acid oligomers (A) and PEG (B) for barriers and local drug delivery in the control of wound healing.

Currently research in transdermal applications is focusing on electrically assisted delivery, using skin permeation techniques such as iontophoresis and electroporation [98]. For

instance several hydrogel-based formulations are being investigated as medium for transdermal iontophoresis to obtain the enhanced permeation of luteinizing hormone [99] releasing hormone, sodium nonivamide acetate [100], nicotine [101] and enoxacin [102]. On the other hand, a methyl cellulose-based hydrogel was used as a viscous ultrasonic coupling medium for transdermal sonophoresis assisted with an AC current, resulting in an enhanced permeation of insulin and vasopressin across human skin in vitro [103].

10.1. Hydrogel in Transdermal Iontophoretic Delivery

European Patent Application EP 0 524 718 A1 demonstrated hydrogels are suitable for transdermal iontophoretic delivery of drugs [58]. This invention used polyurethane hydrogel matrices as monolithic drug reservoirs.

Transdermal iontophoresis is defined as the transport of ionic drugs through the skin, driven by a very weak electric current as described in previous section. The applied current helps to transfer the ionized drugs through the stratum corneum into the dermis, in which the active ingredient can diffuse into capillaries and then into the systemic circulation.

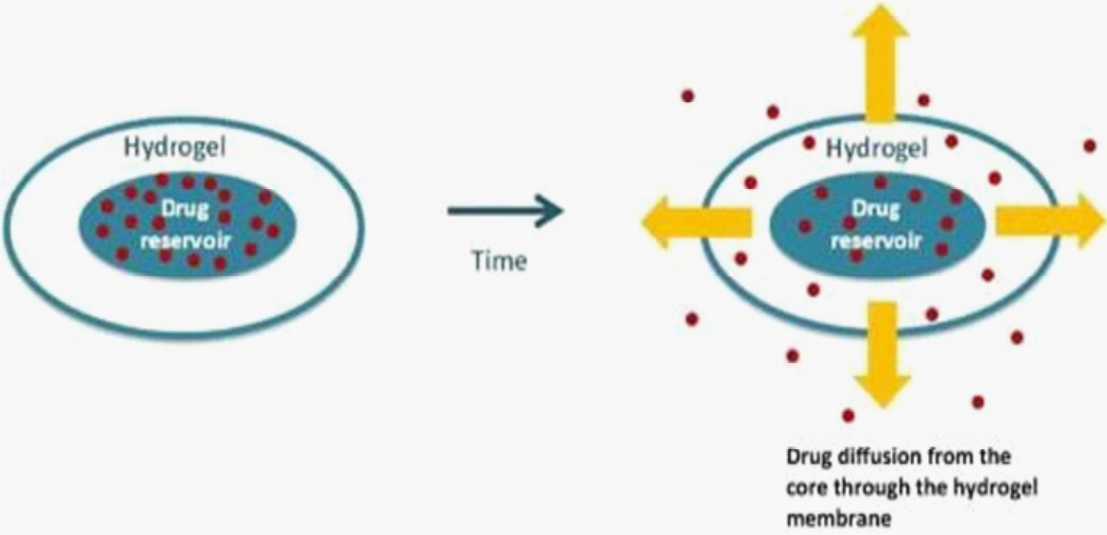

Figure 4: Scheme of drug release through a hydrogel membrane in a reservoir system [57].

Alternatively, hydrogel compositions can be employed as passive transdermal reservoirs. The hydrogels used in the forementioned work showed a high swelling ratio, good flexibility, strength and transparency [58]. Hydrogel-based iontotherapeutic devices as drug reservoir matrices for peptide-based pharmaceuticals have been investigated for transdermal delivery of three model peptides, insulin, calcitonin, and vasopressin [59].

Hydrogels are an ideal candidate for developing the transdermal drug delivery system with dual-functions of moisture and drug delivery. Wang et al. [60] have reported the development of a thermo-sensitive Poloxamer 407/Carboxymethyl cellulose sodium (P407/CMCs) composite hydrogel formulation with twin functions of moisture and drug supply for acute dermatitis treatment. It was found that the presence of CMCs can appreciably improve the

physical properties of P407 hydrogel, which makes it more suitable for tailored drug loading. Transdermal drug delivery behavior revealed that P407/CMCs showed desirable percutaneous performance.

Hydrogels are hydrophilic three-dimensional polymeric networks capable of absorbing a large amount of water or biological fluids [61]. The high moisture content makes hydrogels compatible with most living tissues and thus facilitates widespread application in biomedical and pharmaceutical areas [62,63]. Thermo-sensitive hydrogel exhibits a free-flowing sol form of hydrogel at low temperatures but becomes a gel at body temperature, which facilitates administration and accessibility when applied in drug delivery systems [64,65]. Poloxamer 407 (Pluronic F127) is one of the most typical thermosensitive polymers and has been approved by the FDA. Poloxamer 407 self-assembles into micellar structures and form hydrogels under certain condition. The micellization results from the dehydration of hydrophobic PO blocks and the resultant micelles are spherical with a dehydrated polyPO core and an outer shell of hydrated swollen polyEO chains [66,67]. A high drug loading can therefore be achieved simply by incorporating hydrophilic drugs into the micellar structures. Bioadhesive polymers such as cellulose derivatives are normally added to enhance the bioadhesive property of poloxamer-based hydrogels [68].

High doses cannot be delivered transdermally from a patch of reasonable size, even for molecules whose physicochemical properties are ideal for passive diffusion across the skin's stratum corneum barrier. Therefore, transdermal delivery has traditionally been limited to fairly lipophilic, low molecular weight, high potency drug substances. Since most drugs do not possess these properties, the transdermal delivery market has not expanded beyond around 20 drugs.

Marketed MN-based patches are likely to increase this number of transdermally deliverable drugs in the coming years. However, this increase will only be maximized if high dose molecules can be delivered in therapeutic doses using MN.

12. Hydrogel for Transdermal Delivery Using Miconeedles (MN)

Microneedles are used to pierce the skin to overcome this barrier. Microneedles can be divided into several categories, for instance, solid microneedles, coated microneedles, and hollow microneedles and so on. However, all these types have their weak points related to corresponding mechanisms. In recent years, pioneering scientists have been working on these issues and some possible solutions have been investigated [69]. There are several kinds of MNs [70], namely, solid MNs [71] for skin pretreatment to increase skin permeability, MNs coated with drugs, hollow MNs [72] for drug infusion into the skin, polymeric or polysaccharide MNs [73] that encapsulate drugs and partially or fully dissolve in the skin.

11.1. Solid Microneedles (MNs)

Solid MNs are also called as first generation of MNs. They do not contain drugs themselves and enhance the permeability of skin for drugs, by creating pores into the skin [74]. They are generally made up of silicon or metals [75]. Drawbacks associated with Solid MNs:

- Solid MNs requires a two-step application, which is not convenient for patients.
- Moreover, some of the needles happen to break and are left in the skin, irritation is inevitable, such incidences are not appreciable.
- The fabrication cost is high and the disposition of wastes is also a question.
- Some materials, for example, silicon, require clean room processing and are not FDAapproved biomaterials.

11.2. Hollow Microneedles

- Just like Solid MNs, hollow MNs usually require very specific manufacturing technology and have high production cost, hence massive production is not very feasible [76].
- In case of breakage of hollow MNs in the skin, significant leakage or uncontrolled drug release may occur [77], which may be associated with further complications associated with high dose administration.
- There are also risks that the body tissue blocks the narrow channels which interfares with the drug dosage.

11.3. Polymer Microneedles

Polymer microneedles offer solutions to all above mentioned drawbacks associated with microneedles. The polymer MNs have benefits of ease of fabrication, cost-effectiveness, and the capacity for mass production, as well as controlled drug release with the help of water solubility and degradation properties of polymers [78]. Hydrogel MNs are one kind of polymeric or polysaccharide MNs which are fabricated with polymers or the hydrogel coated on the surfaces of solid MNs. There are three kinds of drug-loading methods (**Figure 5**), some MNs only have drugs in the tips; some in the patches; others have drugs in both.

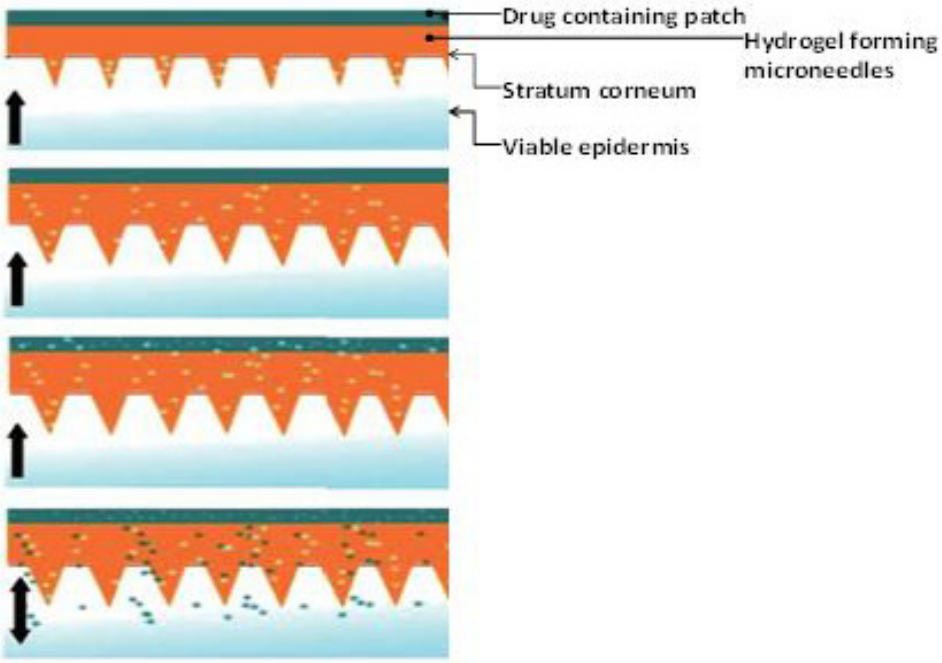

Figure 5: The design of hydrogel MNs [79].

11.4. Advantages of polymer/Hydrogel MNs

- Hydrogel microneedles provide large spaces for drugs to be loaded. The drug loading amount is better than the solid MNs and drug coated MNs as well as the hollow MNs.

- Polymer microneedles also offer the benefits of ease of fabrication, cost-effectiveness, and the capacity for mass production, as well as controlled drug release with the help of water solubility and degradation properties of polymers [79]

- Polymers and hydrogels have excellent biocompatibility, degradability, and nontoxicity [80,81].

- Hydrogel MNs are overall easier and more FDA approvals. The fabrication methods often include the photolithographic process and micro-molding process [78].

According to the function mechanism of hydrogel MNs, they are divided into two categories.

1. Dissolving or degradable MNs

2. Phase transition MNs

11.3.1. Dissolving or degradable MNs:

The dissolution or degradation of the MN matrix, i.e. the polymer or polysaccharide themselves [78,82,83] lead to the drug release. These MNs dissolve or degrade in the skin and release the loaded drugs in a short time leaving no sharp medical waste after use [84-86].

An example of dissolving MNs is reported by Ming-Hung Ling *et al*. [88]. This group

presented a dissolving microneedle patch, made up of starch and gelatin and used insulin as the model drug. An important report by Chin Chen *et al*. [87,89] confirms that drugs loaded in chitosan carriers can be released through swelling and degradation of the chitosan matrix, leading to a clear sustained-release effect. Chitosan with suitable molecular weight is readily biodegradable in *in vivo* systems.

Another way to exploit the biodegradable MNs is to leave the needles in skin to deliver bolus drugs or sustained release of drugs for longer durations by separating the needles from the patches in the skin. Min Kim *et al*. [90] demonstrated one such use of MN, by separation of hydrogel MNs, mediated by hydrogel swelling in response to absorption of fluids on contact with body fluid. In such cases, the tips of biodegradable polymeric MNs are separated because of hydrogel microparticles, which were fabricated between the needle tips and the patches, expand quickly and lose mechanical strength rapidly by swelling and absorbing body fluid. Leonard Y. Chu *et al*. [91] investigated separable arrowhead MNs which upon insertion in the skin, the sharp-tipped polymer arrowheads induced hydrogel part which contained drugs to separate from their metal shafts.

11.3.2. Phase transition MNs

This type of MNs, exhibit phase transition upon in absorption of body fluids by polymer leading to swelling mediated drug release. This kind of MNs leaves few or no residuals after application. These MNs preserve the advantages of other MNs, such as the drug permeating amount, rate improvement, large drug loading amount and relatively easy to be fabricated. They also have the potential to be daily used since few non-drug residuals will be left in the skin, which may increase the patients' compliance. They are so far very promising MN technologies.

Ryan F. Donnelly *et al*. [92,93] developed MNs made of Gantrez AN-139, a copolymer of methyl vinyl ether and maleic anhydride, which could be removed completely and intact from the skin. The needle tips swell in skin to produce continuous, unblockable conduits from patch-type drug reservoirs to the dermal microcirculation, thus allowing prolonged transdermal drug administration. According to their findings, delivery of macromolecules was no longer limited to what can be loaded into the MNs themselves and transdermal delivery drug was controlled by using the crosslink density of the hydrogel system rather than the stratum corneum. The MNs can be fabricated in a wide range of patch sizes and MN geometries by adjusting the molds used.

So far it has become evident that, the hydrogel microneedles are more promising compared with their solid or hollow counterparts. There are also some weaknesses related to the dissolving or biodegradable mechanisms. The most prospective MN type is the hydrogel MN which does not dissolve or degrade in skin but with a controlled or sustained release of drugs.

The application of other methods, for skin permeation, may enhance the drug release rate, but they also increase the costs of MNs while lower the patients' compliance.

12. Conclusion

However, TDDS is very promising drug delivery method with a number of advantages over existing conventional drug delivery methods. But there are some disadvantages associated with TDDS such as high cost, limited drug repertoire that can be administered through skin, allergic response or contact dermatitis due to transdermal patches which can be patient specific.

In conclusion, the TDD sector continues to grow and develop with rapid expansion in fundamental knowledge feeding industrial development. There is a massive potential in this type of drug delivery systems that needs to be exploited for better. In coming time, it is expected that technological advancements in TDD will lead to enhanced disease diagnosis and control, with concomitant improvement in health-related quality of life for patients worldwide

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Advances in Biochemistry & Applications in Medicine

Chapter 2

Obesity and Endocannabinoids

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1. Introduction

1.1. Obesity and its complications

In the modern-day world, obesity and its associated metabolic disorders like Type 2 Diabetes (T2DM) and other metabolic syndromes have skyrocketed and pose a serious global public health concern as seen in Fig 1. In United States, two thirds of the population are obese [1,2]. The problem of obesity not only exists in prosperous countries but is also present in developing countries like Mexico, China and Thailand [3] and hence serious interventions are required to solve this problem that exists across the world. According to National Institute of Health (NIH), obesity is complex and multifactorial condition. It is also considered as a condition of excess energy stores [4] (NIH). According to the definition of World Health Organization (WHO); in adults, "overweight" is defined as Body Mass Index (BMI) between 25-29.9 while "obesity" is defined by BMI greater than 30kg/m^2 [5]. BMI is defined as persons weight divided by his or her height in meters squared. It is known to correlate with percentage body fat in human subjects [6,7], however sometimes not considered a sufficient parameter [8]. Often waist circumference is also considered as a marker for obesity. There are several ways obesity can affect health. These complications include T2DM [9], Non -Insulin dependent Diabetes Mellitus (NIDDM) [10], hypertension [11], heart disease [12], dyslipidemia [13], osteoarthritis [14], high blood pressure [15], etc. A study [13] showed the distribution of obese individual affected in different diseases. The prevalence of dyslipidemia, hypertension and diabetes are profoundly correlated to obesity. Obesity is caused by the increase in the number and size of fat cells or by the dysfunction of adipose tissue which in turn lead to metabolic disease. Some of the important factors that lead to this pathophysiologic state are modern day sedentary lifestyles, environmental factors, easily available packaged foods, use of cheap soybean oil for food preparation. The metabolic dysfunction leads to altered uptake of nutrients and stor

age of the same. Weight management often reduces the risk of T2DM by increasing insulin sensitivity [16]. It is also known to correct abnormalities in NIDDM [17,18]. However, there are no safe pharmacological therapies for the treatment of obesity so far. The development of effective therapies will be of priorities for the health systems. Among different targets for anti-obesity drugs, endocannabinoids remain at attention. This is because, endocannabinoids (ECs) are known to play a crucial role in the host, as in addition to act as neuromodulator, ECs elicit role in energy homeostasis [19,20], cardiovascular function [21] etc. There are evidences that dysregulated endocannabinoid system play a major regulatory role in energy balance by affecting both central and peripheral nervous systems [20].

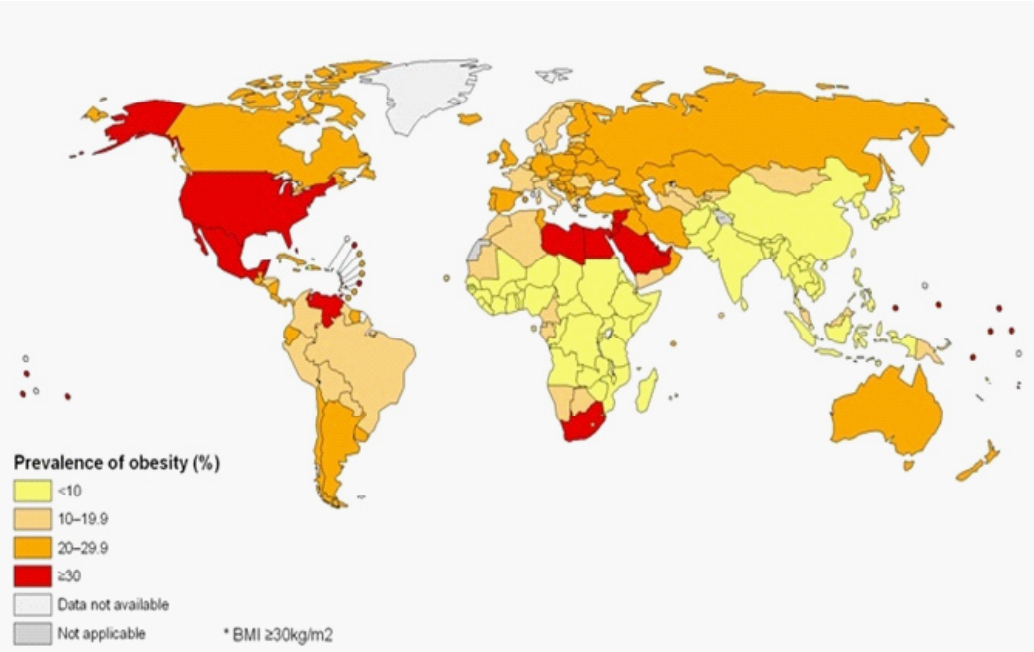

Figure 1: Prevalence of obesity across the world (Source: WHO)

2. The Endocannabinoid system: Overview

2.1 History of cannabinoids

Long back, *Cannabis Sativa*, a herbaceous flowering plant was used as medicine for nausea from arthritic pain, epilepsy etc. *Cannabis Sativa* has originated in Neolithic China. It contains 400 chemicals, 60 of them being cannabinoids [22]. The mechanism of action of cannabis could be known recently because the active compound was isolated, purified and characterized chemically. Cannabinol (CBN) was the first of the cannabinoids to be isolated from red oil extracts of cannabis. Elucidation of its structure was performed in 1930s and it was synthesized in the year 1940. A second cannabinoid, Cannabidiol (CBD) was isolated by Thomas Wood and later its structure was solved by Robert Cahn, Lord Allan Todd [23] and simultaneously by Roger Adams [24]. Although CBD was not the most pharmacologically active compound of cannabis, it led to the discovery of other active compounds present in Cannabis. Later, active compound from marijuana was extracted and named as Δ^9 Tetrahydrocannabinol also known as Δ^9-THC. Both Cannabidiol as well as THC are naturally present

as acids; however, they are decarboxylated when cannabis is heated. Both these compounds are naturally present as (-) enantiomers. Later both (+) and (-) enantiomers were synthesized chemically. The structures of these compounds are elucidated in **Figure 2**.

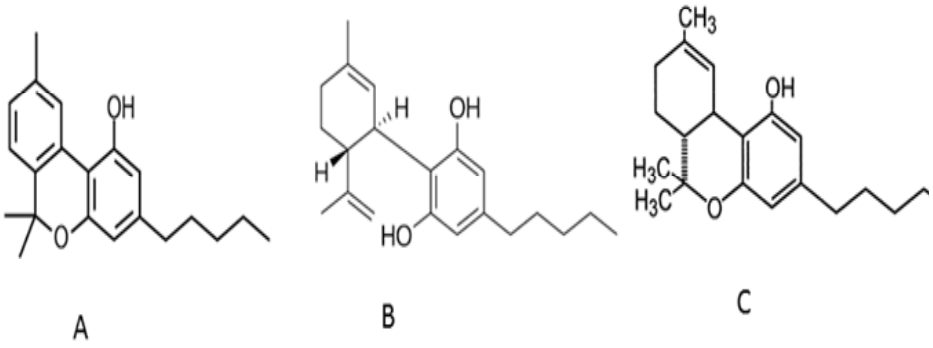

Figure 2: Structures of cannabinoids (A) Cannabinol (B) Cannabidol (C) Δ^9- THC

In mid 1960s and 1970s, several research were performed to understand the pharmacology of cannabinoids. At that time, therapeutic value of these drugs was unknown and recreational value of the drug was widespread. Many experiments were performed in animals and human beings to understand if the psychotropic properties of cannabis could be attributed to Δ^9-THC and the results obtained from those studies were positive in this regard. In one of such studies, it was observed that Δ^9-THC caused "static ataxia" when introduced in dogs. In rodents, cannabis and Δ^9-THC caused immobility index to rise.

Initially the term "Cannabinoids" was used to suggest the C21 compound present in Canabis Sativa. Later the term was used for any compounds that showed pharmacological activity similar to Δ^9 -THC. It was proposed that their membrane fluidity makes them to interfere with the membranes and not to specific receptors. However various groups suggested the binding of the cannabinoids to be stereoselective which kept the search for cannabinoid receptors to be active [25] in the past years. In addition, the binding of THC stereoisomers was studied in different experimental animals and it was concluded that their potencies differed across animal background [26,27]. It took long time to understand the binding site of cannabinoids. Allyn Howlett provided the proof for binding site of cannabinoids as cannabinoid receptors [28]. Her work suggested that the cannabinoids activate G protein coupled receptors which in turn inhibit adenyl cyclase. Studies relating the cellular effects of the synthetic cannabinoids, revealed that they inhibit cAMP production and that they mediate via cell membrane [29].This result is also concluded from numerous studies on the role of cannabinoids in the modulation of cAMP levels in cell cultures, brain homogenates and in *in-vivo*. Initial work indicated that cAMP is altered in biphasic manner in brain. This was because, at lower dose of cannabinoids there was an increase in the level of cAMP while at higher dose there was decrease in the cAMP levels. This study correlated with the initial stimulatory effect of low doses of cannabinoids but depressant effect at the high levels of the same [30]. A second major advance in this field was again made by Allyn Howlett in collaboration with Bill Devane. This was possible

because of the presence of the technique that can detect the binding site of the receptor using radiolabeled ligand and labelled Tritium cannabinoid, CP55940. There was evidence of high affinity binding sites for this in rat brain. Moreover, the ability to displace the labelled by the unlabeled cannabinoids from the binding sites in addition to inhibition of adenyl cyclase was also concluded from their work. It was thus certain that cannabinoids acted via receptor and the receptor was G protein coupled receptor. The cannabinoid receptors were first cloned from rat brain. Later they were cloned from humans, fish, mouse etc. The CB1 receptors are also known to activate mitogen activated protein kinase (MAPK), inhibit voltage activated Ca^{2+} channels and activate K^{+} channels.

2.2 Endocannabinoid System

The endocannabinoid system consists of endocannabinoids, cannabinoid receptors and the enzymes responsible for their degradation [28] (**Figure 3**). This system has been preserved across the species and is selected by the evolution to maximize energy intake and conservation [31,32].

Two more splice variants of CB1 (Cb1b and Cb1a) have been identified in human brain [33,34]. Although these receptors are located in the brain, they are also known to be present in various peripheral tissues like pancreas [35], liver [19] and skeletal muscles [36]. The predominant expression of cannabinoids are the full length CB1 with low expression of CB1a and CB1b in brain [34]. Contrary to this, Cb1b is the major isoform in the liver with 10 fold more expression [34]. The isoforms were different in their N terminal sequence and hence are different in their pharmacological behavior. The isoforms could be detected in hepatocytes and beta cells using modern proteomic approach [37]. Several research also focused in the search of endogenous cannabinoid agonist. Such compound was first isolated from rat brain mem

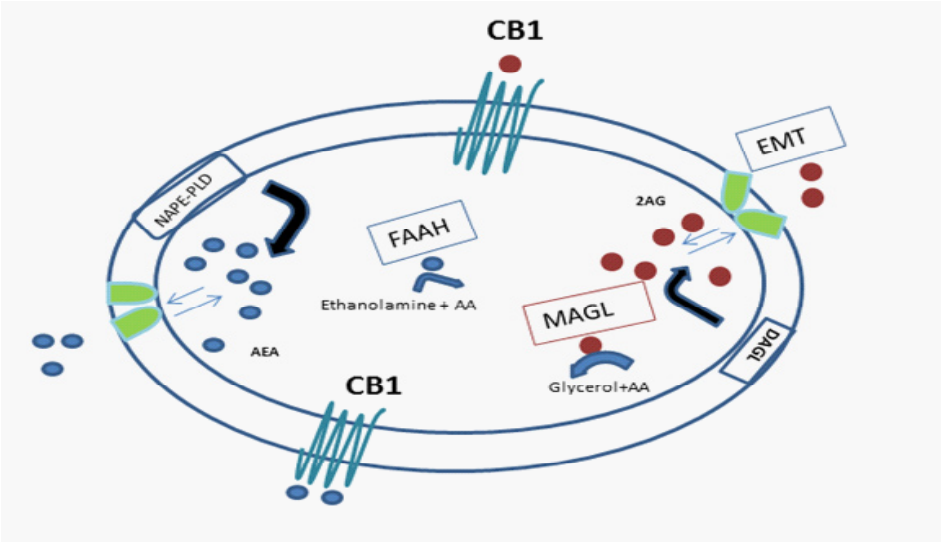

Figure 3: Components of Endocannabinoid system. The biosynthesis of AEA (blue circles) is catalyzed in presence of NAPE-PLD. The biosynthesis of 2AG (orange circles) is catalyzed by MAGL. Endocannabinoids are transported by EMTS on both the direction of cell membrane (EMT stand for Endocannabinoid membrane transporter). FAAH hydrolyze AEA while MAGL hydrolyze 2AG. Adapted from [38].

branes. The molecule was lipophilic and also displaced 3H-HU243. The first endocannabinoid to be discovered was Anadamide (AEA) which was derived from Sanskrit word "Ananda" meaning bliss. Later Anandamide and 2-Arachidonoyl Glycerol (2AG) were identified as the ligands for cannabinoid receptors [39,40] (Fig 4). In the brain, endocannabinoids function to decrease neurotransmitter release at CB1 terminal.Unlike CB1,CB2 is present in the immune cells [41], NK cells. However they are recently also found in brainstem [42] and cerebellar granule cells.

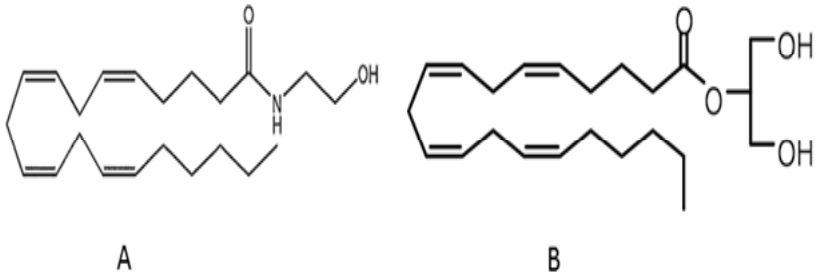

Figure 4: Chemical Structure of Endocannabinoids (A) AEA (B) 2AG

2.3 Endocannabinoids

The term "endocannabinoids" was derived from the name endogenous cannabinoids [43]. They can work as autocrine and paracrine manner on the cannabinoid receptors. The synthesis, transport and degradation of the endocannabinoids are performed on demand. They are not stored in advance for the future use [44]. Thus, the concentration of endocannabinoids varies with respect to energy requirements in the body. It increases during fasting and decreases after refeeding [45]. The synthesis of ECs depends on intracellular Ca^{2+} ions [46,47] and they are derived from arachidonic acid. AEA elicit neuroprotective and immunosuppressive role both by cannabinoid receptor dependent and independent manner. AEA is formed by the hydrolysis of NAPE (N arachidonoyl phosphatidyl ethanolamine) [48] in presence of Phsopholipase D (NAPE-PLD). The hydrolysis of phsopho diester bond of NAPE is brought about in presence of NAPE-PLD which was uncharacterized till recently. It is a member of zinc –metallo hydrolase enzymes. The NAPE precursors of AEA is produced in presence of trans acylase enzyme. This enzyme, catalyzes the transfer of acyl group from sn-1 position of phospholipids to nitrogen of phosphatidylethanolamine. A second pathway is also operative in the brain in which Phospholipase C catalyzes NAPE to phosphorylated AEA which further undergoes de-phosphorylation to form AEA [49]. There are two other pathways for the formation of AEA. The pathways are summarized in Figure 5. 2 AG (**Figure 4**) is mainly formed by the catalytic action of diacylglycerol lipase (DAGL) from arachidonate containing diacylglycerol. Two different isoforms of DAGL are present namely DAGL α and DAGL β. However, another route of synthesis of 2AG is also known. The pathways are summarized in **Figure 6**.

Both AEA and 2AG are removed from the extracellular space by cellular uptake. The

transport of AEA to intracellular space may be facilitated by FAAH like AEA transporter (FLAT), however this finding is controversial. The degrading enzymes for AEA and 2AG are also characterized. They major ones are Fatty acid amide hydrolase (FAAH) and Monoacylglycerol lipase (MAGL) respectively. AEA is also degraded by other enzymes like 12-Lipoxygenase, 15-Lipoxygenase, CYP2B, CYP2D, CYP4F and COX2. AEA and 2AG can also bind to non-cannabinoids such as "Transient receptor potential vanilloid 1" (TRPV1), the activation of which opposes CB1 receptor [51].

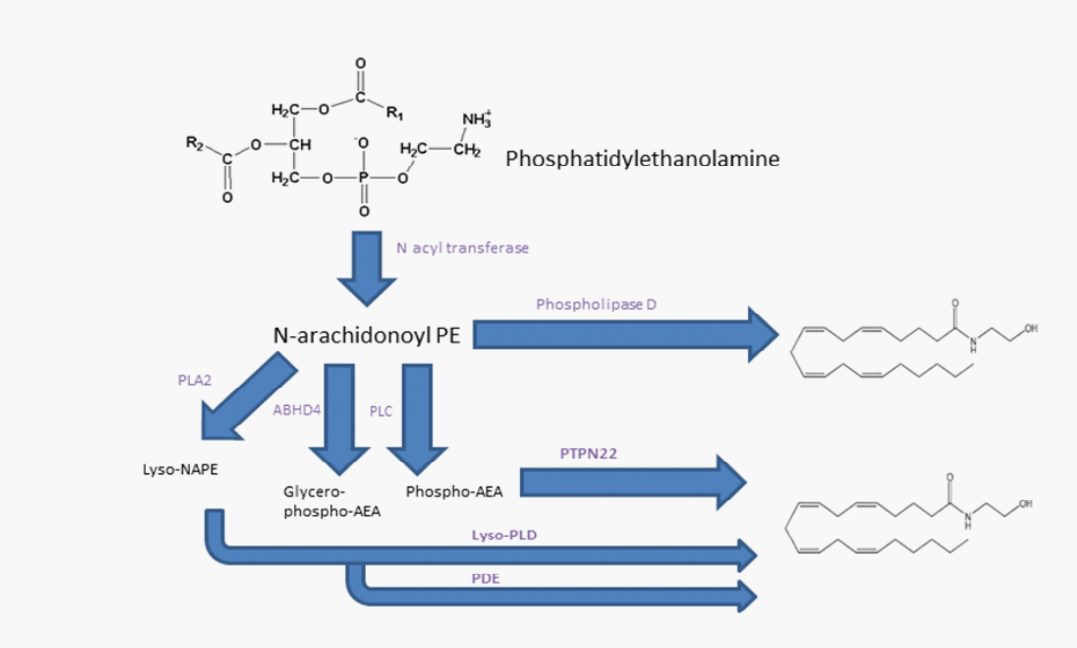

Figure 5: Anandamide biosynthesis pathway. There are four different pathways for the formation of AEA. PlA2 is abbreviated from Phospholipase A2 while PDE is abbreviated from Phosphodiesterase. This is adapted from [50]

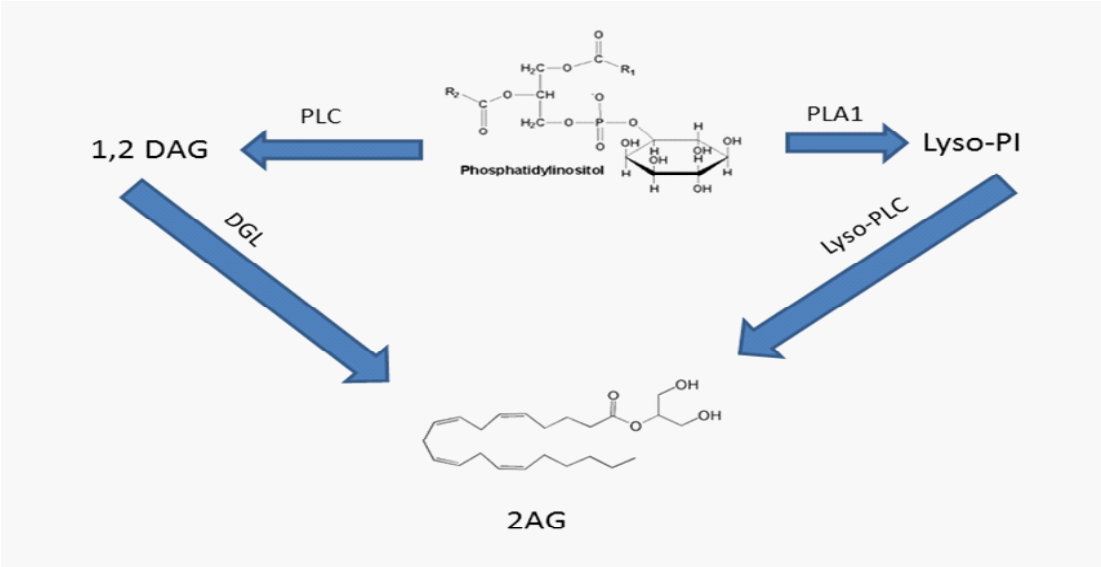

Figure 6: Biosynthetic pathway of 2AG. The abbreviations are used as follows. PLC- Phospholipase C, DGL –Diacylglycerol Lipase, PLA1- Phospholipase A1, Lyso-PI-Lysophospholipid, Lyso-PLC- Lisophospholipase C. This is adapted from [52].

In addition to AEA and 2AG, many EC like molecules have been discovered, but their functions are not completely known yet. For example anti-inflammatory lipid, lipoxin A4 could be the endogenous allosteric enhancer of CB1 [53,54]. More studies are required in this direction.

Within CNS, the endocannabinoids work in the retrograde manner to the cannabinoid receptor in the presynaptic neurons leading to suppression of release of neurotransmitter [55]. Other than CB1, the next prevalent cannabinoid receptor that exists is CB2. Although there exists 44% homology between CB1 and CB2 [56] the ligands for CB1 and CB2 are similar. This could be because of 68% identity in the binding domain of CB1 and CB2. However, the affinity of the endocannabinoids towards the cannabinoid receptors are not similar. While AEA exhibits higher affinity towards CB1R compared to CB2R, 2AG has similar affinity towards both the receptors. One of the challenges in the study of endocannabinoids is their measurement. This is because of rapid degradation and isomerization of 2AG. The sampling treatments are crucial for the assay of endocannabinoids because their half - life is in the order of minutes. Thus, for the measurement of endocannabinoids in blood, blood collection should be performed in ice, centrifuged immediately and kept in -80^{0}C until the analysis.

2.4. Role of age and sex in the modulation of ECs

Endocannabinoid level exhibit variations in sex and age. A study in 2006, suggested AEA level to be different in men and in women, but no difference was exhibited in the level of 2AG, however it was correlated to visceral fat. Moreover only 2AG was correlated to age [57]. In a different study no difference was observed in AEA levels in different sex while 2AG was shown to be different [58]. In addition, 2AG was also shown to be correlated with age only in women. Also, AEA correlated with BMI, waist circumference and fasting insulin. 2AG exhibited correlation with triglycerides irrespective of sex. The discrepancy of the results in two studies could be due to the variations, selection of the cohort. While in the first study obese individuals were recruited, in the second one, normal individuals are studied. Although no concrete conclusions could be drawn from these studies, it pointed out that age, as well as sex might have potential role to play in ECs levels which could be due to role of gonadal hormones in the modulation of ECS [59]. However more investigations are required in this front.

3. Endocannabinoids and Eating Disorder

Cannabis can induce weight gain and is known to occur by the stimulation of central nervous system(CNS). The first report of increase in appetite was reported in AD 300. Smoking cannabis in patients with the history of HIV was shown to modulate leptin and ghrelin but not insulin levels demonstrating their effect on hormones [60]. They were also prescribed as appetite enhancing medicine in patients with AIDS and cancer [61,18].

Endocannabinoids mediate eating disorder by the activation of the cannabinoid receptors, CB1 which are present in central [57] as well as in peripheral nervous systems [62]. Elevated levels of AEA and 2AG have been reported in different studies in obesity. Administration of AEA in ventromedial hypothalamus or systemically, induces hyperphagia. Like AEA, 2AG also evoke increase in feeding behavior when injected systemically or to lateral

hypothalamus. It is known that both endogenous and exogenous administration of cannabinoids in rats increases feeding [63,64]. The key area in brain responsible for the motivation of feeding is hypothalamus. CB1 receptors present in hypothalamus are responsible for food intake behavior. In one of the studies, it has been shown that administration of endocannabinoids in nucleus accumbens (NAc) increases intake of sucrose solution in rats [65]. In addition, administration of Δ^9 tetrahydrocannabidol increases hedonic taste response after sucrose administration by releasing dopamine in NAc. Administration of one of the selective CB1 antagonist, Rimonabant reduces food intake suggesting a role of CB1 in the energy intake because dopamine release was prevented in NAc [66]. Thus, intake of palatable food is associated with increase in dopamine in the brain. This mechanism has been attributed to the activation of dopaminergic neurons in ventral tegmental area in the brain. This is achieved by the activation of CB1 receptors by endocannabinoids in glutamatergic neurons which in turn inhibit GABA-ergic neurons that project from NAc to ventral tegmental area thus disinhibiting dopaminergic neurons in ventral tegmental area [67]. Besides, modulation of ECS leads to change in level of neuropeptide hormones that are responsible for the signaling of appetite. Administration of Rimonabant has reduced expression of neuropeptide Y and increased in the expression of other anorexigenic peptides such as CART and αMSH levels in hypothalamus. Thus, increase in the food consumption is brought by increasing the motivation associated with the food intake. Several experiments by different groups were performed on role of CB1 in food intake. It can be further noted that the mice with CB1 receptor knocked out ate less than their wild type littermates. While food intake in the CB1 knock out (CB1KO) mice is independent of Rimonabant dose, it reduced food intake in the wild type mice. Apart from cannabinoid receptors, ECS are also known to exhibit their properties by other G protein coupled receptors like GPR55, GPR18, CB2 etc. The role of CB2 in this context has also been studied by two different groups. It has been shown that mice deficient in CB2 exhibited hyperphagia and administration of CB2 selective agonists increased food intake [68]. Moreover, selective over expression of CB2 in brain led to decrease in feeding that is induced by fasting and hence lean phenotype [69,70]. These receptors have opposing effects to CB1 in the chemically induced liver damage. It has been demonstrated that CB1 antagonist and CB2 agonist protect against liver injury [71]. The role of CB1 and CB2 could be opposing in nature and more experiments are required to understand the role of each receptors in context of energy metabolism. Both the cannabinoid receptors (CB1 and CB2) are present in pancreas although there remain discrepancies about the exact location of CB1 and CB2. While CB1 receptors are mostly present in α and β cells, CB2 is present mostly in δ cells. High fat diet increase in the concentration of AEA and 2AG in the whole pancreas, however loss of islets in diabetic mice did not alter the endocannabinoids level in pancreas. *In vitro* experiments conclude that stimulation of CB1R enhance secretion of insulin and glucagon but stimulation of CB2R lowers glucose dependent insulin secretion [72,73]. In a different study it was demonstarted that Rimonabant was useful to prevent the islet loss as well as weight of pancreas in obese Zucker rats along with proper

renal function and hence led to decreased mortality [74]. Under hyperglycemia, AEA and 2AG are dysregulated in pancreas. RIN-m5F β cells, known as a model of pancreatic islets β cells when kept in low glucose medium show low ECs. In addition, it did not show glucose induced rise in ECs when co-stimulated with insulin [75,73]. Thus, pancreatic ECs also play a role in energy metabolism.

Along with food intake, ECs also modulate smell and taste that guide the organism towards the food [76]. In this context, it is important to note that malfunctioning of olfaction is known to occur in obesity in various organisms. Hunger is known to stimulate 2AG in olfactory epithelium. In mice, olfactory neuronal circuits are modulated by ECS [77]. Also, CB1R in the mouse taste cells co-localize with sweet receptor component, T1r3 and the neural response to the sweet taste is enhanced by endocannabinoid signaling. Taste related signal is processed in the hindbrain specially in parabrachial nucleus and nucleus of solitary tract which also receive information from the gastrointestenial tract, and thus affect the meal size [78,79]. Endocannabinoids in parabrachial nucleus thus enhance the intake of palatable food by acting through CB1. An interesting fact regarding olfaction and fat ingestion is that, the oral cavity leads to the production of endocannabinoids in the gastrointestinal tract by efferent vagal signaling which in turn also leads to further fat intake [80,31]. Endocannabinoids in the gut are known to modulate food intake and hunger signals and are known to vary under fasting and satiety [81]. Their concentration rises during fasting and fall after feeding. CB1R expression in gut is modulated by cholecystokinin, a hormone secreted by gut to induce satiating effect [82]. Some studies suggest that orosensory properties of AEA and 2AG lead to increase in fat intake when present in high concentration while their reduction lead to meal termination. In addition, food intake is also modulated by CB1 signaling in olfactory bulb. Besides, in recent times, a link between ECS and cephalic phase response has been established. The role of ECS in cephalic phase response is well established in sham feeding model. In this model it has been established that gut derived endocannabinoids are responsible for fat intake based on its orosensory properties [80,83]. Gut endocannabinoids have been identified to participate in positive feedback mechanism of fat ingestion. It has been suggested by other studies that oral exposure to fat cause the release of dopamine in ventral striatum [84] which is a hub for evaluating rewarding sensory stimuli [85]. This also prove the cephalic phase response to the fat rich foods. Blockade of CB1 in gut just before sham feeding lowers food intake [80]. CB1 in the gut cells also induces ghrelin which in turn can increase fat taste perception [86]. In addition to this, there exists literature that gastrointestinal tone is controlled by gut microbiota. However the connection between microbiota and ECS level is yet to be studied. Apart from digestion, gut also functions to convey satiety function.

Besides, CB1 receptor is also found in the fundus of stomach, although cellular localizations are not particularly known. A small dose of Rimonabant was able to reduce the effect of

ghrelin [87], the production of which takes place in gastric endocrine (X-) cells [87,88]. Thus, stomach has also a role to play in energy metabolism.

Another molecule known to regulate glucose homeostasis is adiponectin [89]. In obese animals it exhibits improvement in hyperglycemia, insulin resistance [90] etc. It has been suggested by various studies that Rimonabant exhibit its effect on adiponectin. *In- vitro* studies have suggested that Rimonabant increases adiponectin, while activation of CB1 inhibits adiponectin concentration [91]. This is further supported by *in-vivo* experiments in Zucker rats. Further in CB1KO mice, Rimonabant had no effect in adiponectin mRNA levels in adipose tissue. This suggests that Rimonabant affects adiponectin in a CB1 dependent manner.

Although endocannabinoids are well studied for their role as neuromodulators very less is known about their biological functions. In different studies, the concentration of endocannabinoids is shown to be positively correlated to BMI, waist circumference etc. However, whether that results from the spillover from the tissues or if its bears a biological relevance to obesity is yet to be understood [92].

4. Dietary Long Chain PUFA Disrupts ECS Tone in Centrally and Peripherally and Results in Obesity

In the modern time, diet induced obesity (DIO) is prevalent in western countries and is a good model to study obesity in humans. Some of the constituents in the high fat diet is known to modulate ECS system and hence play a role in obesity. High brain concentration of AEA was observed in piglets fed with arachidonic acid (20:4n-6) [93]. It has been proposed in an epidemiological study that an increase in the prevalence in obesity was due to increase in arachidonic acid pool due to intake in linoleic acid which in turn lead to increase in 2AG concentration. A study showed that obesity is directly linked to consumption of soybean oil which has high content of linoleic acid [94]. Moreover, increase in dietary linoleic acid from 1% to 8% cause increase in weight gain, arachidonic acid phospholipid (ARA-PL); AEA and 2AG. Also, ARA-PL pool is decreased by increase in Eicosapentanoic acid (EPA) and Docosahexanoic acid (DHA) which also reduced the obesogenic effect of linoleic acid in rodents by reducing the stimulation of endocannabinoid system. In addition to this, in a separate study, it has been shown that a low fat diet could be made obesogenic by increasing the concentration of linoleic acid [95]. Thus, excess in the EC activity is associated with obesity. In a separate study, by a different group, DIO was shown to lower CB1 density in the extrahypothalamic regions such as hippocampus, nucleus accumbens etc. It was hypothesized that increase in endocannabinoids has resulted in lowering density of CB1 [96] in these regions.

CB1KO mice were resistant to DIO and have decreased weight gain [19]. The mice with this phenotype also have reduced feed efficiency [97] and have reduced leptin, triglycerides, insulin and increased adiponectin, suggesting improved lipid metabolism and hormone

sensitivities [98,99]. Mice with selective CB1 knock out in forebrain and sympathetic nervous systems are also resistant to DIO because they display thermogenesis, lipid oxidation and decrease in energy absorption [100]. Besides, virally mediated CB1R mRNA knockout in mice led to decrease in body weight gain and increase in energy expenditure [101]. It is also been noted that deletion of CB1 receptor from Sim-1 expressing neurons protect mice against DIO by increasing the expression of thermogenic genes in white adipose tissue [102,45].

The peripheral EC system is also stimulated in obesity in humans. In a study, DIO was associated with increase in the size of adipocytes. It is important to note that adipocytes from humans as well as rodents are known to express all the components of ECS including CB1 and CB2 [57]. Also human adipocytes metabolize and bind to 2AG and AEA [103,104]. Lipogenesis is mediated by AEA and stimulation of CB1 in adipocytes. AEA is also known to activate peroxisome proliferator activated receptor γ (PPARγ) inducing differentiation of adipocytes [105]. It has been shown that activation of CB1R in obese mice lead to decrease in mitochondrial biogenesis in white adipose tissue [106]. The study also demonstrate the positive effect of Rimonabant in lipolysis and hence reduction in fat mass [107,69]. In addition, administration of Rimonabant in wild type mice has led to induction of certain genes which are involved in β oxidation and mitochondrial biogenesis. The study suggests that blocking CB1R, improves mitochondrial oxidative capacity and hydrolysis of triglycerides in adipocyte. Several other studies also indicate that obesity induced inflammation in adipose tissues. It has been shown by a research group that inhibiting CB1R attenuate LPS induced pro-inflammatory cytokines like Interleukin-6(IL-6), Tumor necrosis factor-α, (TNFα), in human adipocytes [108]. Similar result is obtained by a different group which showed decrease in circulatory cytokines in obese Zucker rats by Rimonabant [109]. CB1 protein expression in adipocytes was seen to increase while exercise training reduced the effects of DIO in subcutaneous and visceral adipose tissues. The authors found that high fat feeding decreased while exercise increased the protein expression of PPARδ which in turn inhibited CB1 expressions. This study suggested a new regulatory pathways towards expression of CB1 [110]. Along with CB1, CB2 also play an important role in inflammation in adipocytes. *In vivo* administration of selective agonist of CB2; JWH-133 led to increase in inflammatory genes in mice under both normal and high fat diet. As an extension, CB2R KO mice were resistant to inflammation. Also administration of CB2R antagonists; AM630 in ob/ob mice led to reduced adipose tissue inflammation [111]. It must be noted that the role of CB2R in adipocytes are quite contrary with respect to other tissues, where stimulation of this receptor in other tissues led to attenuate inflammation [112,69].

High fat diet is also associated with increased expression of CB1 in liver. There is an elevation of AEA in liver after high fat diet along with decrease in activity of FAAH [13]. Both increase in synthesis as well decrease in degradation resulted in activation of ECs. In rodents, stimulation of CB1R in the hepatocytes results in expression of lipogenic transcription fac

tor SREBP-1c [113], Acetyl coenzyme carboxylase-1(ACC1), and Fatty acid synthase (FAS) which in turn leads to de novo lipogenesis [19]. Lipogenesis pathway can be activated by AEA which could result in DIO. Treatment of isolated primary hepatocytes with 2AG cause increase in gluconeogenic gene expression as well as hepatic glucose. This effect was attenuated by application of Rimonabant thus explaining the role of CB1 in hepatic gluconeogenesis [114]. Mice deficient in FAAH (AEA degrading enzyme) have elevated fasting glucose although having elevated fasting plasma insulin levels [115]. This result suggests the unfavorable effect of ECs activation in host under high fat diet.

Both AEA and 2AG levels are increased in diabetic patients [116]. Some studies have elucidated whether ECS is correlated to visceral fats as it is regarded as hallmark of obesity. Studies show that there is negative correlation between plasma 2AG levels and insulin sensitivity independent of body mass. In humans, plasma 2AG concentrations is correlated to visceral fat but there is no difference in 2AG level between the lean and the obese subjects [57,117]. However, in both these studies it was shown that 2AG level was correlated to decrease in insulin sensitivity, increase in free fatty acids, triglycerides, cholesterol etc. CB1 receptor is also known to be dysregulated in white adipose tissue (WAT) in humans in DIO. However, the nature of dysregulation is controversial as one group report CB1 to increase in WAT in obese condition, other suggested the opposite. FAAH is known to be lowered in subcutaneous WAT in obese individuals [57]. Also in visceral fat mass, FAAH expression was negatively correlated to circulating 2AG [57,73]. An interesting data from morbid obese subjects indicate increased level of 2AG in visceral fat (and not subcutaneous) compared to controls. The enzyme levels associated with the formation and degradation of 2AG was not seen to be different in the adipose tissue. The authors predicted that elevated fatty acids in the diet to be the reason for increased bioavailability of 2AG [118].

It has also been argued in literature that cannabinoid receptors and the related enzymes undergo site specific perturbation. In a study, while the enzymes involved in the endocannabinoid pathway were shown to be decreased in gluteal subcutaneous adipose tissue in the obese individuals, the same individuals exhibited elevated abdominal fat [119]. This results shed light on the role of peripheral endocannabinoids in obesity. An important aspect to note in this regard is that, although ECs are correlated to weight gain, a decrease in weight gain by exercise and diet does not lead to decrease in EC concentration. In two independent studies it was shown that loss in weight in the obese individuals did not result in decrease in circulating endocannabinoids, although the metabolic parameters were improved [20,120]. This study was followed by a different study in which there was intervention in the physical activity and healthy eating which led to decrease in plasma AEA and 2AG in men after one year. The decrease in 2AG levels was correlated to decrease in visceral fats, decrease in triglycerides and increase in HDL cholesterol levels [121]. The different results obtained from different studies

could be because of different diet being used in different studies to reach the weight loss goal and also different time frame being used. In addition to liver, fats and plasma, perturbation of endocannabinoids also exists in other biofluids and tissues. In a study it was shown that intervention in the lifestyle leads to change in salivary content of AEA while no change in 2AG [122]. Apart from liver, pancreas, CB1 is also present in skeletal muscle [123]. Impaired glucose utilization by skeletal muscle led to insulin resistance. It is known that ob/ob mice exhibited insulin resistance along with hyperglycemia and hyperinsulemia however treatment with Rimonabant leads to proper glucose uptake in isolated soleus muscle preparation [124]. An *in-vitro* model of skeletal muscle, E6 cells are known to modulate glucose uptake by ECS at the level of PI3 Kinase leading to change in the activity of downstream PI3Kinse; like Protein kinase B, Pyruvate dehydrogenase, Protein kinase C, although protein level expression of glucose transporter like Glut1 and Glut4 were not affected by ECs [125,73]. *In-vitro* studies have also suggested that CB1 antagonist AM251elevated the level of AMP activated protein kinase (AMPKα1) in myotubes of both lean and obese individuals which in turn lead to fatty acid oxidation [123]. CB2R deficient obese mice, exhibited elevated insulin mediated glucose uptake in skeletal muscle relative to wild type mice indicating that CB2R also has role to play in insulin sensitivity in skeletal muscle [126].

5. Leptin and Endocannabinoids

One of the key regulators of endocannabinoids in the hypothalamus is serum leptin. Leptin is known to reduce endocannabinoids in brain. In obese mice the dysregulation of leptin signaling give rise to higher endocannabinoid level in hypothalamus and is known to interfere with ECS signaling [127]. It prevents the ECS synthesis by lowering the levels of calcium ions. While in the normal rats, injection of leptin reduce endocannabinoids in the brain, EC levels increase in db/db, ob/ob as well as fa/fa mice [128]. All these three categories of mice have leptin deficiency or defective leptin receptor signaling. These results were specific to endocannabinoids in hypothalamus [127]. In addition to this, endocannabinoids in the uterus of ob/ob mice were elevated and could be reversed by using leptin treatment. Leptin administration was effective to restore all the enzyme activity of the endocannabinoid pathways; both the synthesis as well as degrading process related to endocannabinoids [129].

Leptin requires hypothalamic CB1 to exert its anorexic effects. It has been noted that partial deletion of CB1 from the hypothalamus has resulted in stopping the ability of leptin to reduce food intake [101]. In addition, leptin interacts with glucocorticoids for the regulation of endocannabinoids in paraventricular nucleus (PVN). Glucocorticoid can lead to repression of synaptic excitation in PVN through endocannabinoids. Leptin can cause a decrease in glucocorticoid mediated ECS synthesis and hence the excitation in PVN neuron [130]. Increase in EC in hypothalamus not only interfere with leptin signaling but also lead to insulin resistance in periphery [131]. In some other studies it has been noted that when CB1 is deleted from ste

roidogenic factor-1 expressing neurons, of ventromedial hypothalamus, it increases the sensitivity of leptin to function as anorexigenic agent during consumption of normal chow, however leptin resistance is caused during the consumption of high fat diet [132].

In addition, Ghrelin is also known to be associated with the endocannabinoid level in the brain. Endocannabinoid is known to mediate the orexigenic effect of ghrelin when the hormone is administered to PVN [133]. Ghrelin require CB1 machinery to be active which in turn recruit AMP activated protein kinase, and which is required for ghrelin to function in hypothalamus. Ghrelin and 2AG are both elevated in human plasma when food for pleasure is ingested. This indicates that endocannabinoids and ghrelin are closely associated in reward related actions [134].

ECS have been studied in various animal models. It has been observed that the mice developed obesity due to mutation in leptin or leptin receptor gene. Dysregulation of ECS and leptin deficiency is also referred to be confounders of obesity.

6. CB1 Blockade Improves Obesity

One of the many causes of obesity is the presence of dysregulated and overactive ECS system. Several cannabinoid antagonists were developed. Both plant derived as well as synthetic compounds are known to suppress the food intake. Chronic treatments lead to improvement in weight loss in genetic and DIO.

Synthetic compound like Rimonabant also referred as SR141716A acts as an inverse agonist to CB1 has shown potential therapeutic use in obesity both in animals as well as in humans [135,136]. It has been noted that weight reducing effect of Rimonabant in mice can be enhanced by blocking μ type opoid G protein coupled receptor or by co-treatment with the gut hormones like oxyntomodulin or YY3-36 [137,138].

Peripheral administration of Rimonabant decrease the synthesis of Stearoyl Coenzyme A Desaturase 1(SCD1) in DIO induced obese mice. This suggests that CB1 blockade reduce synthesis of monounsaturated fats in WAT, independent of food intake however central administration gives same result as pair fed group [139]. In addition, other groups have reported that Rimonabant results in enhanced expression of Acetyltransferase, Palmitoyltransferase2 which are involved in fatty acid oxidation [107]. Due to psychiatric side effects Rimonabant was withdrawn from the market [140]. However, it has been shown that co-administration of melanin concentrating hormone receptor (MCHR) antagonist can augment the effect of CB1R, while normalizing the behavior changes [141].

The neutral CB1 antagonists are AM4113 and VCHSR1. VCHSR1 has lower affinity to CB1 and decreases milk ingestion in mice [142] and AM4113 reduce food intake in mice

under high fat, high carbohydrate and lab chow [143]. A lot of research was dedicated to find the antagonist of peripheral CB1in past years as it was shown to be an efficient way to suppress appetite, increase energy expenditure and reduce lipogenesis in both liver and adipose tissues. Several factors are studied and evaluated. JD 2144 as well as JD-5006 have been shown to be very effective to reduce weight and improve metabolic parameters in obese mice. AM6545 shows promising results as it reduces food intake in mice under high fat and high carbohydrate diet, however fails for normal chow diet. In addition, URB447, CB1 antagonist and CB2 agonist is known to decrease weight gain and also reduced brain penetration [144]. Recently LH-21 which also has low brain penetrability was used as potential drug in rodents. It is reported to have reduced high fat diet induced weight gain in obese rats modulating the lipogenic pathway [145]. One of the other approaches was to reduce the formation of 2AG. The mice lacking DAGLα were lean. Thus inhibiting DAGL-α using O-7460 was actually shown to be effective in reducing high fat diet intake in mice [146]. In addition to this, nonsteroidal anti-inflammatory drugs (NSAIDS) is shown to alter cannabinoid receptor induced response [147]. This is because these drugs can inhibit cyclooxygenase2 (COX2) which is also the degrading enzymes for AEA and 2AG. Another class of compounds known as "allosteric modulators" are developed which are known to decrease activity of CB1 in presence of their ligands [31]. Some of the examples being homopressin, pepcans and pregnenolone. Hemopressin [148] is known to modulate circuits in mediobasal hypothalamus and not the reward related areas. Pregnenolone is neurosteroid which restricts weight gain and adiposity in DIO. Another approach of reducing ECs would be to reduce the ω-6 pool which is the precursors of ECs. This could be achieved by increasing the ω-3 fatty acids pool which in turn could be achieved by introducing of docosahexaenoic acid and eicosapentanoic acid in the diet. This technique is known to reduce fat in adipose tissue, heart in Zucker rats [149]. More research is ongoing worldwide in search for a suitable compound to reduce obesity, with no side effects. Since different isoforms are present for CB1, with varied pharmacological properties, researchers should take into account the localizations of different isoforms in tissues when designing for drugs.

7. References

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Advances in Biochemistry & Applications in Medicine

Chapter 3

Childhood Obesity, an Emerging Threat to Global Public Health: A Neutraceutical Approach to Contain

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Abstract

Global health reports indicate alarming levels of childhood obesity (CHOB) in recent decades across the world. Childhood obesity (CHOB) has a significant impact on both physical and psychological health and is a known precursor to metabolic disorders in adulthood. Apart from genetic aspects, changed lifestyle preferences, environmental factors, food habits, cultural aspects and declining physical activity are the prime causes of rising prevalence of obesity world over. In most populous and developing countries like China and India increased purchasing capacity of the middle classes, increased publicity and mushrooming of fast food centres, supermarkets, attraction of the children towards fried-roasted foods, increased conveyance facility and information technology has direct impact on food habits and contributed to growing over weight-obesity. Both obese children and adults are at increased risk for several health complications including hypertension, dyslipidemia, type 2 diabetes, CVDs, arthritis and infertility. Additional health complications associated with overweight-children include sleep apnea, asthma and liver diseases. Since it is difficult for children to have bariatric surgery or to be on synthetic drugs for a long time, a natural product based nutraceutical approach may find fit to deal with childhood obesity. This review discusses and updates various causative aspects and consequences of childhood obesity and necessary interventional options with emphasis on phytochemicals to contain CHOB.

Keywords: Childhood obesity; Causative factors; Neutraceuticals

1. Introduction

1.1. Epidemiology of childhood obesity

In recent decades prevalence and severity of childhood obesity has reached epidemic proportions, [1]. In 2016, it was estimated that globally 200 million children under 5 years were overweight, with more than 75% of overweight or obese children living in low and middle income countries [2,3]. While the incidence of obesity is not new to the developed countries, the main drivers of the escalating trends of childhood obesity in the developing countries are cheap foods with high content of sugar and fat, sedentary lifestyles, rapid nutritional transitions, increasing affluence, socioeconomic transitions, urbanization, mechanization and rural-to-urban migration [4]. For instance, the socio economic transitions in most populous countries like China and India have profound impact on world statistics of obesity and overweight [5]. In developing countries, the requirement for physical labour has considerably reduced due to mechanization, availability of advanced technologies, various implements, instruments and sophistication of life styles. Growing and rampant rural to Urban migration and consequent changes in lifestyles and food habits is an important cause for high incidence of obesity and other metabolic disorders.

Since 1986, several studies in preschool children show increasing obesity in most countries in Latin America and the Caribbean, along with the Middle East and North Africa, which is comparable with prevalence rates of childhood obesity seen in the United States [6]. Similar trends have also been observed in India, Mexico, Nigeria, and Tunisia over the past two decades [7]. Increase in the prevalence of overweight among older children and adolescents has been seen as well; from 6.4 to 7.7% between 1991 and 1997 in China, and from 16 to 24% between 2002 to 2007 in New Delhi, India [8,9].

1.2. Obesity parameters: Body mass index (BMI) as a measure of overweight and obesity

Overweight is defined as a body mass index (BMI) in the 25 to 29 kg/m^2 range, where as obesity is a BMI in excess of 30 kg/m^2. Overweight and obesity result from an energy surplus over time that is stored in the body as fat. The definition of childhood overweight and obesity based on body mass index (BMI) is complicated but it is made clear by recent studies [10]. Due to differences in maturation and growth, the measurement of overweight and obesity in children and adolescents is very difficult. There are two periods when adiposity increases they are about the age of 5 to 7 years and early puberty. So, BMI in childhood changes substantially with age. The international cut-offs defined the BMI values at the age of 18 years but BMI reference of the WHO is based on growth standard and growth reference. Using LMS (Skewness L, Medain M, Variation S) curves coefficients are related to the international child BMI cut-offs of thinness, overweight and obesity and they make it simple to compare them with

other methods like BMI cut-offs [11].

2. Contributing factors

There are regional differences in the prevalence of childhood obesity that have occurred overtime [12] and in many countries childhood obesity depends on lifestyle behaviours such as physical activity and dietary intake, but childhood obesity may not depend on same lifestyle behaviour across the world [13,14]. Complex interaction of multiple behavioural, biological and environmental factors which adversely impact long time energy balance and this energy balance leads to obesity in children. The major contributing factors are showed in **Figure 1**.

2.1. Genetic background

Obesity is developed by complex interactions between environment, behaviour and genetic predisposition. Of late, dietary and lifestyle changes are said to be major contributing factors to develop obesity, but previous studies reported the genetic basis for development of obesity [15-18]. There is growing evidence that genetic factors are cornerstone in the development of obesity [19]. Specific gene expression pattern of obesity may help to understand the pathogenic mechanisms of obesity and associated metabolic diseases [19].

2.2. Epigenetic aspects: Environmental factors

Influence of environment encompasses several aspects like social, cultural, economic and political factors [20]. The obesogenic environments are divided in to two stages- the micro and macro settings/sectors [21] and there are four types of environments like physical environment (what is available), economic environment (what are the costs), policy environment (what are the rules) and socio-cultural environment (what are the beliefs and attitudes) [20]. In recent decades, sea changes in environmental aspects have been greatly contributing to the tremendous raise of obese children rather than genetic aspects. Physical activity and television viewing may have independent effects on adiposity and cardiovascular risk factors [22]. Increased purchasing power, increased availability of readymade foods and decreased necessity for walking because of increased conveyance facilities are also their due role in this regard.

2.3. The impact of TV and advertising

A special focus is given in the present paper on TV and advertising. Within the environmental context, the impact of TV viewing and advertising on children, their seating behaviour and health seems to have a potential association with their overweight or obesity problems. Television is suspected to be linked to a reduction in physical activity whilst advertising seems to promote an over consumption of sugar rich and high fat foods [23]. Most of the studies showed [24] a positive correlation between times spent watching television and nutritional status of the subjects involved.

Two main aspects have been considered by researchers' about the TV effects on children's obesity: (1) reduced energy expenditure linked to screen time [25] and (2) augmented energy intake driven by advertising and snacking in front of the TV (**Figure 2**). The first issue seems to be related to long hours of TV watching, influencing positive energy balance through displacement of physical activity. It has been suggested that youth may decrease their physical activity when sedentary behaviours are increased.

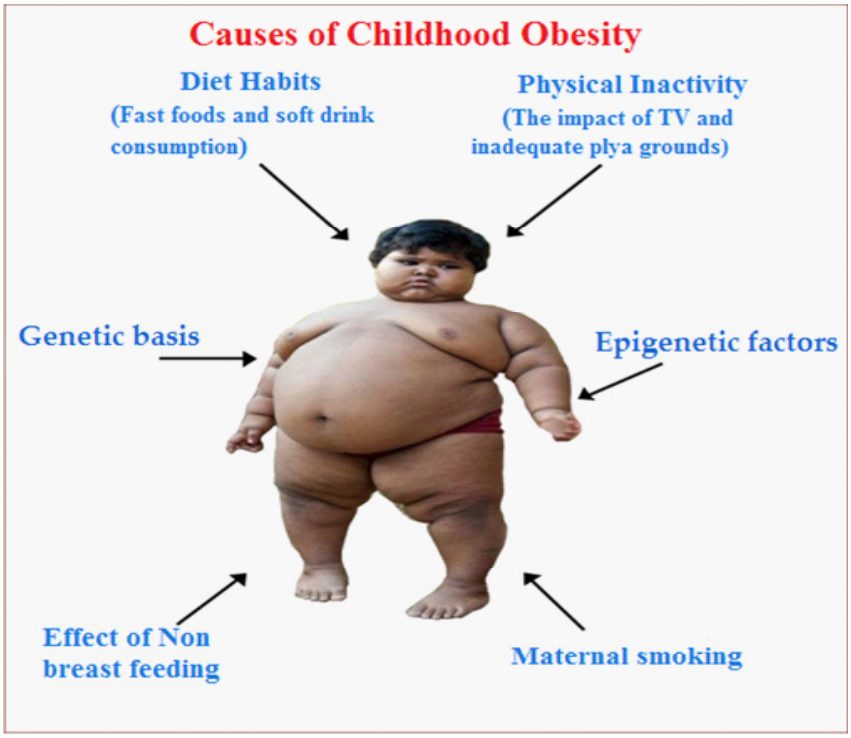

Figure 1: Causes of Childhood obesity

Figure 2: Children eating high calorie foods and drinks

2.4. Fast foods and soft drinks consumption

Fast food consumption is another leading suspect in the childhood obesity epidemic. Fast foods typically include all of the things that nutritionists warn against: 'saturated and trans-fats', high glycemic index, high energy density, and large portion sizes. This alarming trend should be of particular concern to health authorities and public communities. Usually,

food industries release attractive “messages” and “advertisements” to which children are easily attracted and thus increased consumption of high-fat, high-sugar foods (HFSS) can profoundly impact a child’s eating habits and weight status [14]. It is therefore undeniable fact that the food industry has successfully created a highly obesogenic environment. Consumption of meat foods like chicken and fish that are grown using growth boosters is also reported to have obesogenic effect. As children are an important asset of any nation, this disorder adversely affects the future generations as it results in metabolic syndrome and causes higher mortality rate in adulthood [26].

Further studies reveal that the soaring rates childhood obesity has been due to innate propensity of children to respond to external food advertising cues [27]. Some research supports the hypothesis, that there are no differences between obese and leaner individuals in their liking for sweetness in food products and subsequent food intake in relation to brand exposure [28]. It is equally important to note that the amount of television-viewing among children denotes the possibility of better fast food brand recognition which can be a threat to children’s health [9,29]. However, conflicting evidence revealed that obese children did not consume more than non-obese individuals when meals were branded with famous food logos [28]. Employing strategic branding on healthy foods may be a novel and effective way of helping children develop healthy eating habits and lifestyles from an early age [30].

2.5. Effect of smoking during pregnancy

Many previous studies support the ‘fetal origins of adult diseases’ hypothesis for maternal smoking: these studies showed a positive association between maternal smoking during pregnancy and overweight in children aged between 5 and 16 years. One study found that maternal smoking during pregnancy was mainly related to childhood overweight in the upper percentiles [31]. The number of cigarettes smoked during pregnancy also appeared to be associated with childhood overweight. Symeet et al. [32] used magnetic resonance imaging-based (MRI) studies to measure adiposity and reported that maternal smoking during pregnancy was not associated with fat distribution in early puberty but higher subcutaneous and intra-abdominal fat mass was noticed in late puberty [32,33]. Other studies on maternal smoking showed that over weight increased with age suggesting a lasting effect that may increase even further in puberty, adolescence and adulthood, with major health implications [34].

2.6. Effect of breast feeding

Breast feeding has been associated with a decreased risk of obesity, along with other health benefits for the child and mother. According to the WHO recommendations, infants should be exclusively breast fed for the first six months, and breast feeding should be supplemented with additional foods for the first two years or beyond. Breast milk is considered the ideal food for infants, as it provides adequate energy and nutrients to meet the infants’ needs.

In addition, as breast milk is safe and contains antibodies, breast feeding could reduce the risk of neo natal infection, gastro-intestinal infection, and pneumonia during infancy [35,36].

In recent decades, there is a growing work culture among women of developing countries of Asia and African continents to work in industrial and service sectors. So, due to economic compulsions, work timings and other reasons the mothers are forced to skip breast feeding to their babies. Babies fed on tinned milk/formula milk are more prone to develop health complications including obesity ailments.

Prolonged breast feeding is directly related to a decreasing risk of obesity in children [37]. More particularly, children being breastfed for ≥7 years are significantly less likely to be obese in later childhood [38]. Moreover, breast feeding has long-term benefits through-out a child's life time. Usually, children who were breast fed have lower rates of overweight/obesity, type-2 diabetes, hypertension and are known score higher on intelligence tests than persons who were formula-fed [39]. Breast feeding has been identified as a protective factor for childhood obesity in many studies [40,41].

2.7. Changed school environments: without adequate playgrounds

More particularly in developing countries like India, mushrooming of private schools and colleges with inadequate play grounds could be widely noticed. As a result, children are not adequately exposed to playing games and sports regularly and hence do not get required physical activity. In school going children, increased snaking frequency, consumption of junk foods, soft drinks, milk products and ice creams that contains excessive sugar, fat and preservatives, coupled with steady decline in physical activity have been major contributing factors for rising rates of obesity [42]. Surprising observation is that some school managements allot more time periods for teaching and learning and give less time for physical activity because they are more concerned about students scoring higher makes from their school. Among apparently healthy school going children the higher prevalence of obesity is unnoticed and in obese children the systolic and diastolic hypertension was higher [42].

3. Consequences of childhood obesity

Many of the outcomes associated with obesity which were previously thought of as diseases of adults are now affecting children as well. Outcomes related to childhood obesity include hypertension, diabetes, dyslipidemia, CVDs, non-alcoholic steatohepatitis, obstructive sleep apnea, and orthopedic problems in addition to social and psychological problems (**Figure -3**). Hence, obesity is popularly described as "New World Syndrome".

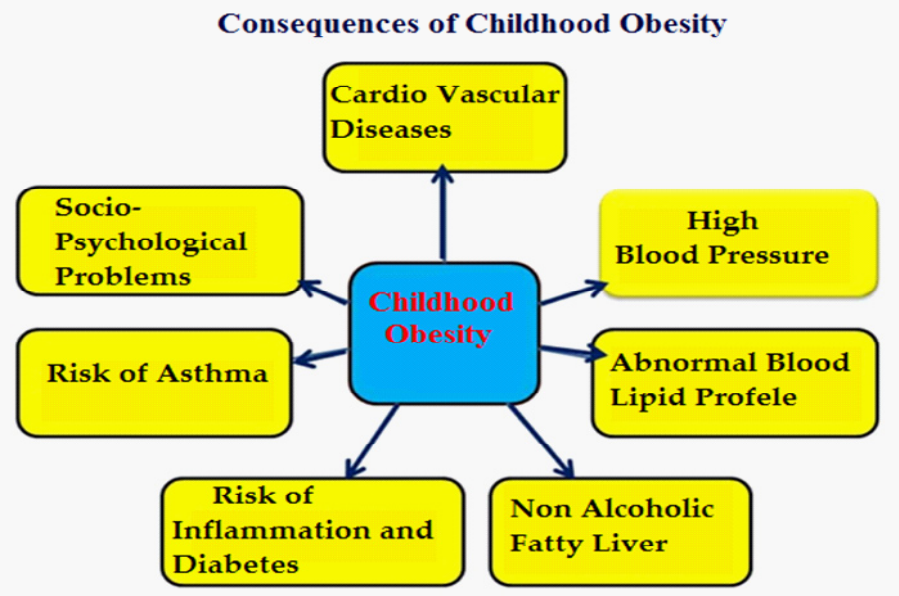

Figure 3: Consequences of childhood obesity

3.1. Cardiovascular diseases (CVDs)

The World Health Organization (WHO) reported in 2008 that 17.3 million deaths worldwide were due to CVDs [43]. A major contributor to CVDs is atherosclerosis which is a dynamic process that can begin in childhood and develop or regress, depending on the presence or absence of a range of risk factors including obesity, inflammation, hyperglycemia, hypertension and hyperlipidemia [44,45]. The International Obesity Task force estimates that approximately 40-50 million school aged children are obese [2].

Abdominal obesity in children is associated with low grade inflammation, a significant contributor to the development of atherosclerosis [44]. Both BMI and WC correlate with intra-abdominal fat in primary school aged children [47] and are used as clinical measures to identify CVD risk [48]. Obese children are also at increased risk of hypertension and dyslipidemia as they age [49,50]. Overweight boys with high dietary intakes of fat and carbohydrate in particular had significantly more CVD risk factors than girls.

3.2. Blood pressure alterations

The prevalence rates of hypertension and obesity are increasing worldwide in children [51]. Increased blood pressure leads to damage of capillaries and tissues in brain, heart, kidneys etc. Administration of antihypertensive drugs like diuretics, ACE inhibition, adrenegenic receptor antagonist, renin inhibitors and vasodilators on children for longer period is not suggestive as they cause side effects. One report quoted that, the blood pressure lowering effect of docosa hexanoic acid (DHA), observed in adults, could be mediated by ATP release from the endothelium, which increases vasodilation by stimulating the release of nitric oxide, and by the decrease in nor-adrenaline levels [52].

A systematic review stated that breast feeding has a small protective effect against high

systolic blood pressure, although residual confounders had to be eliminated [53]. One of the plausible mechanisms that have been suggested to explain this protective effect is represented by the presence of long chain poly unsaturated fatty acids (LCPUFAs), including DHA, which are important structural components of the vascular endothelium [53].

3.3. Abnormal blood lipid profile

The alterations of blood lipid profile associated with metabolic syndrome are usually characterized by increased triglycerides (TG), very-low-density lipoproteins (VLDLs), low density lipoproteins (LDL) and reduced High density lipoproteins (HDL) [54,55]. Visceral obesity and insulin resistance could be key factors in the promotion of atherogenic dyslipidemia by increasing the synthesis of TG-rich VLDLs in the liver [56].

Long term administration of statins or other anti hyperlipidemic drugs have side effects on children and adults. A study performed on 32 obese children showed that plasma phospholipids, DHA content was negatively associated with VLDL-triglyceride, a major factor involved in the development of metabolic syndrome [57].

3.4. Non-alcoholic fatty liver disease (NAFLD)

In children of industrialized countries, Non-alcoholic fatty liver disease NAFLD is the most common chronic liver disease reaching prevalence up to 80% in obese or overweight children [58]. NAFLD includes different diseases ranging from “simple” liver steatosis, with pathological accumulation of fat, non-alcoholic steatohepatitis (NASH), with different degree of inflammation and fibrosis to end-stage liver disease with cirrhosis and hepato-cellular carcinoma [59].

Only one study showed that in obese children with single-nucleotide polymorphism (SNP), 276G>T at adiponectin gene, the increased liver echogenicity could be associated with higher levels of n-6 PUFA in plasma phospholipids (Presented at 44th ESPGHAN Annual Meeting, Sorrento) [60]. However, some trials evaluated the effect of extracts supplementation on paediatric NAFLD ([61,62]. A reduced liver hyper-echogenicity was observed in children with NAFLD after DHA supplementation for 12 and 24 months [61].

Breast feeding might be protective against NASH and liver fibrosis, suggesting a long-lasting effect of breast milk [63]). The authors speculated that DHA supplied by breast milk, could be protective, acting as a Peroxisome proliferator-activated receptor (PPAR)-agonist, a transcription factor involved in protection against fibrosis [60,64].

3.5. Risk of diabetes mellitus

Childhood obesity is a condition where children below the age of 14 years have high

BMI. Many of them are prone to diabetes when they become adults. Day by day we witness that the effect of junk food is clutching the children's future into neuroleptic malignant syndrome (NMS). It seems that children of very low age are suffering from either type-1 or type-2 diabetes. Type-1 diabetes is generally found in children and was initially termed as juvenile diabetes. About 10% of children worldwide is suffering from type-1 diabetes only. The diabetic figures of Asia and African subcontinents are shocking. The increased longing for consumption of polished rice and wheat foods, dairy products, ice creams, stored-preserved products, fast foods and other junk foods by school children has been the major cause of raise in diabetes. Added to this, decreased preference for sports and games at schools and home resulted in decreased physical activity [65].

With changed typical physical work and dieting activity, the BMI of the persons is changing to considerable extent in every decade and it has been noticed in Americans mostly [66]. Eating disorders are the main reasons of obesity, as obese person when eat more become more fat and then the tendency to lose the fat will get decreased. Moreover, the fat person feels sleepy all the time and lazy too which does not allows him/her to work and they remain in the same lying/siting posture for long time. Not only children alone, but with the changing times eating habits are also changing due to which obesity has been considered as the centre of metabolic syndrome as it causes different chronic disorders which some-times may leads to death also [67].

3.6. Risk of newly diagnosed asthma

An association between a greater BMI and increased risk of asthma in both children and adults has been repeatedly shown in prospective, population-based studies [68]. Previous studies demonstrated significant associations between overweight/obesity with asthma and eczema [69].

The age of the weight gain may play a role, as pronounced weight gain in early life was identified as a risk factor to develop asthma before 10 years of age [70]. Two types of asthma in obese subjects can be distinguished by the age of onset and clinical presentation. Early onset of asthma in obese cases before the age of 12 years might occur in boys and girls and is characterized by severely decreased pulmonary function, significant airway hyper-responsiveness, and poor asthma control. These patients are atopic; serum immunoglobulin E (IgE) is increased, airway inflammation is eosinophilic, and fraction of exhaled nitric oxide (FeNO) is high [71]. In contrast, obese late-onset asthmatics become symptomatic after the age of 12 and are predominantly females without atopic characteristics. Compared to early-onset asthmatics, they have little airway obstruction with less airway hyper-responsiveness and better asthma control.

3.7. Risk of psychosocial problems

Overweight in children and adolescents may be associated with a range of psychological and social problems that have a considerable deleterious impact on the behavioural development and quality of life in them. By the ages of about 5 years, obese boys have more conduct problems, hyperactivity, inattention problems and peer relation problems than normal weight children. Interestingly, obese girls of these respective ages present only with more peer relation problems in comparison to normal weight girls, indicating obese boys to be at a greater risk for the development of emotional and behavioural problems already at an early age [72]. The school and social performance of obese children, their academic and extracurricular activities and their overall quality of life are often less favourable compared to their normal weight peers. This is a multi-faceted problem, related to greater school absenteeism, less nutritious diet and physical activity, more emotional and behavioural problems, less favourable neuropsychological functioning and overall psychosocial stress.

By contrast, negative body esteem is usually correlated with the severity of obesity. Normal weight children tend to show negative attitudes towards their overweight peers. Obese youth are usually considered less popular than normal-weight teens, and overweight during childhood and adolescence may be associated with low esteem, considerable societal victimization and peer teasing. Moreover, obese children themselves perceive negatively many of their own characteristics and attach them to their overweight [73].

3.8. Risk of metabolic syndrome

The worldwide epidemic of childhood obesity is responsible for the occurrence in paediatric disorders once mainly found in adults, such as the Metabolic Syndrome (MS). The prevalence of childhood obesity has been increasing in the last four decades with an estimated sixty millions of children being overweight worldwide by 2020 [74]. MS in children is commonly defined as the co-occurrence of three or more of the following features: severe obesity (high BMI), dyslipidemia (increase of triglycerides and decrease of HDL), hypertension and alterations of glucose metabolism such as impaired glucose tolerance (IGT) and type 2 diabetes [75]. This causes a large accumulation of lipids into the liver, which results in hepatic steatosis and higher triglycerides production. From a molecular point of view the link between lipid accumulation and insulin resistance seems to be represented mostly by diacylglycerol (DAG) [76]. In children aging between 5-8, only 16% can be regarded as metabolically healthy, while about 36% fulfilled the criteria for metabolic syndrome [77]. The metabolically healthy children are characterized by lower waist circumference, less visceral fat content, increased peripheral insulin sensitivity, less pronounced inflammatory status, lower melondialdehyde concentration etc.

4. Interventional Strategies

4.1. Awareness campaigns

Both sedentary life style and high energy diet contribute to increased global obesity rates for the past three decades [78,79]. Along with obesity, the associated ailments like diabetes and heart disease [80] are cause of public health concern in both children and adults. Hence, education campaigns and health promotion programs are recommended by the World Health Organization as prevention strategies of obesity [81,3]. While implementation of WHO recommended prevention strategies of obesity in multicultural countries, variation in cultural, religious, demographic and ethnic backgrounds needs to be considered [82-86]. For instance, a study showed that, the assimilated Iranian migrants were more active to accept and endorse the Australian culture of physical activity. Otherwise, this contributes to the development of obesity in migrant populations [87]. At nursery and school level the teacher/trainers need to bring greater awareness about the junk foods and emphasize on physical activity. The non-governmental organisations (NGOs) and other institutes need to play active role in this regard as the epidemic of childhood obesity affects the future of their countries. Information about new lifestyle and environmental interventions is necessary for implementation.

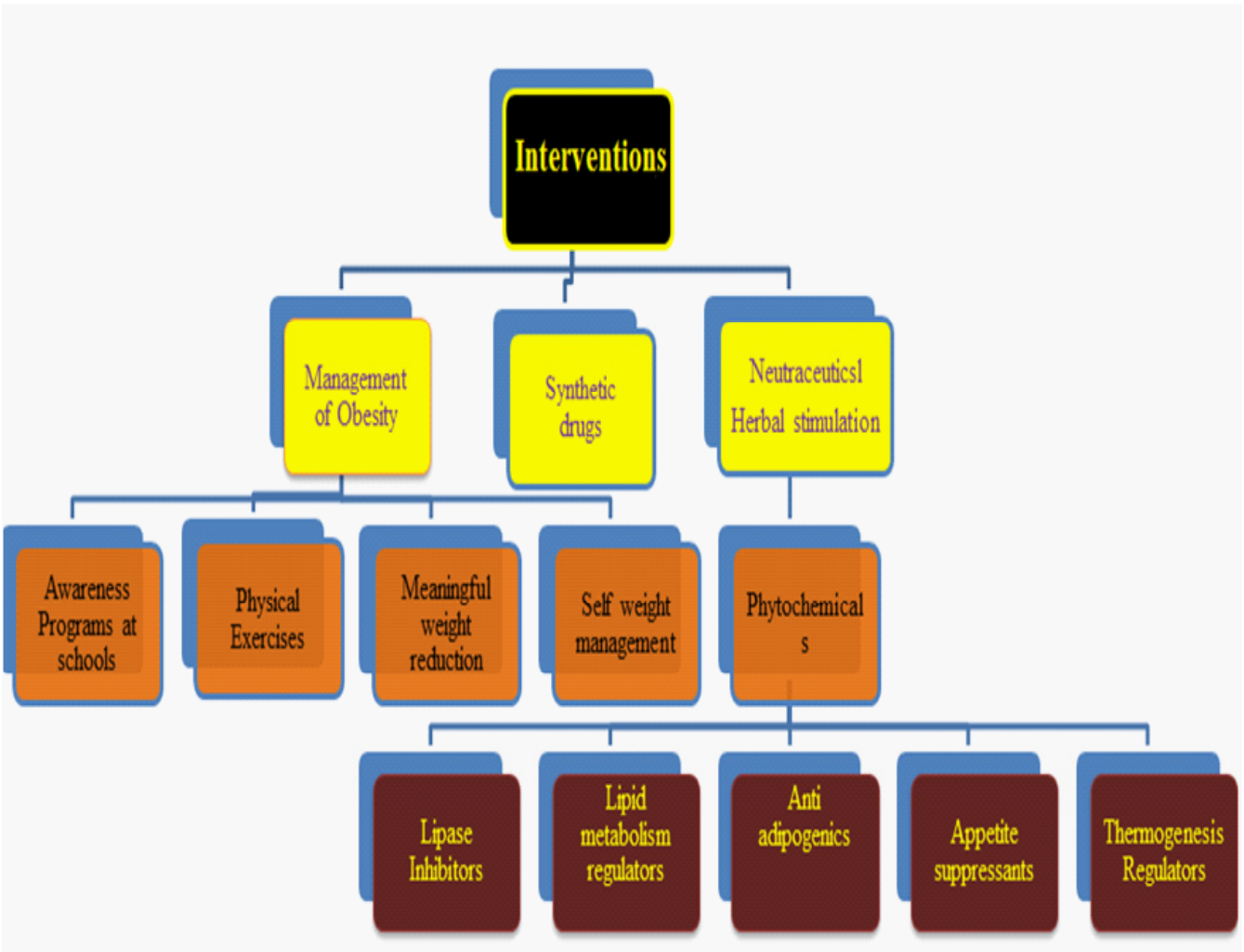

Figure 4: Interventional strategies

4.2. Management of childhood obesity

Management of weight in the child includes strategies to both reduce overweight/obesity and promote sustained change. Most weight loss programs are based on promoting behavioral change in the child and parent. As in the adult, caloric intake must be less than energy expended for weight loss to occur. Therefore, the key to weight loss in the children is making changes in both diet and exercise. Behavioural interventions included meeting with individual case managers, group and individual counselling sessions, self-management training, individualized adherence strategies, and clinical support help to manage obesity. Although many family physicians are pessimistic about their ability to influence patients to make necessary lifestyle changes in order to achieve weight loss, research suggests that patients are more likely to attempt weight loss when their primary care physicians recommend it [88]. In another study, patients who lost weight credited their physicians with having helped them by explaining the health risks of obesity, making physical activity recommendations, and providing referrals to weight-loss groups or programs.

It is also essential to keep in mind that while pharmaceutical agents can help patients achieve clinically meaningful weight loss, the medications must generally be continued to maintain the reduction [89]. Multivitamins contain fat-soluble vitamins to offset potential losses from fecal fat excretion [90]. Physical exercise and activity are particularly important for maintaining weight loss over the long term (and for preserving lean body mass during dieting) [89].

4.3. Synthetic drugs

Although some drugs/formulations are available in the market, only a few drugs are approved by FDA and European commission, and some of them are withdrawn due to their side effects. So it not advisable for children to take such drugs for a long time. In view of the difficulties in implementing dietary restriction and physical exercise regularly and in place of pharmaceutical approach, nutracetical approach gained moment to treat childhood obesity.

4.4. Nutraceuticals

Nutraceuticals are products derived from food sources with extra health benefits in addition to the basic nutritional value found in foods. In this context, an effective nutraceutical is that which can increase energy expenditure and/or decrease caloric intake is desirable for body weight reduction. Several phytochemicals have been reported for their anti lipidemic and anti obesity activities (**Figure 4**) [45]. Herbal stimulants, such as caffeine, ephedrine, chitosan, ma huang-guarana, and green tea are effective in facilitating body weight loss [91]. However, their use is controversial due to their ability to cause side-effects. Green tea extract and 5-hydroxytryptophan may promote weight loss, while the former increases the energy expenditure,

the latter decreases appetite. [91,92].

Active ingredients of fruits, vegetables, and other edible plants, comprising of flavonoids, saponins, tannins, glucosinolates, phenols, phytates, phyto-estrogens are capable of efficiently combating metabolic syndrome. Anti-inflammatory properties contribute to counteract the obesogenic state [93]. Dietary phytochemicals (**Figure 4**) act on various targets associated with obesity aliments [94]. Some probable mechanisms of action of these plant derived products are reduction in adipose tissue mass by inhibition of precursor cell proliferation, enhancing apoptosis of fat cells and hindering the absorption of triglyceride by reducing formation of pancreatic lipase and amylase. Some work as appetite suppressants, interfere in lipid metabolism, decrease intake of energy and enhance energy expenditure [45,95,96].

For example asparagus is also effective in weight control. It works by flushing out toxins and other wastes from the body [97]. Tomatoes, the low calorie vegetables are consumed raw as well as cooked. Lycopene, the active constituent in tomato is famous for its anti-oxidant and anti-carcinogenic properties. Tomatoes are good for health as they also help to lose weight [98].

Oats, a popular breakfast item, is rich in antioxidants and other minerals. Fibre in oats brings down the cholesterol level [99]. Blueberries, rich in anthocyanins are responsible for breaking down fats and sugars and thus curtail extra fat from our body [100]. The most popular beverage in the world next to water is green tea, obtained from the leaves of Camellia sinensis, helps in reducing weight. Other fruits and vegetable like papaya, kera, grapes, lemen, curry leaves, carrot spices like piper, cumin seeds, cinnamon etc., possesses anti-obesity effects [101,45].

5. Conclusion

Recent medical and health reports have shown childhood obesity as an emerging threat to the global health in 21st century and termed it “New World Syndrome”.

The present study focused on various causative factors for raising prevalence of obesity world over. One of the major contributing factors for alarming world statistics on childhood obesity is increased purchasing power of millions of middle class people in most populous countries like China and India which has direct bearing on changed food habits and life styles. Other reasons include decreased preference for physical activity in schools and avoiding of breast feeding by mothers for various reasons. Since children are vulnerable and cannot be put on pharmacological intervention for a long time, nutraceutical approach has been highlighted in this study. Several fruits, vegetables and spices like cucumber, tomato, curry leaves, bitter guard, moringa, piper, cumins seeds, cinnamom etc., have significant beneficial effects against obesity ailments. Finally the parents at home and teachers at school need to educate children

and emphasize to avoid junk foods, encourage physical activity and include anti-obesity nutraceuticals in regular diet because today's healthy children are tomorrows wealthy citizens.

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Chapter 4

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Anti-advanced glycation end product therapies in diabetic vascular complications

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Abstract

Advanced glycation end products (AGEs) are formed by non-enzymatic reaction between reducing sugars and proteins, lipids or nucleic acids. Interaction of AGE with its receptor; receptor for advanced glycation end product (RAGE) elicit various signal transduction pathway leading to vascular complications in diabetes mellitus (DM). Therefore, inhibition of AGE may be a useful strategy to ameliorate pathogenesis of several diseases including diabetic vascular complications. Several AGE inhibitors have been identified till date, which differ from each other in their mechanisms of action, although all have the same outcome, and lead to reduction in AGE formation or accumulation. Therefore, anti-AGEs drugs are also being intensively studied in the recent time. Therapies that target multiple pathways may indeed be more successful than those that target one pathway alone. It remains to be determined whether a combination of hemodynamic and metabolic pathways is more effective than any individual therapy in preventing diabetes-associated injury. Therapies against the AGE-mediated effect can act through diverse pathways, like inhibiting the production of Amadori products, decreasing AGE-RAGE interaction, detoxifying dicarbonyl intermediates and interrupting biochemical pathways that impact on AGE levels. However, food and drug administration does not approve any agents for AGE modification to date, though some such medications are in clinical and preclinical testing.

In this chapter, various agents which are known as inhibitors of formation of AGE and AGE breakers reported till date are being discussed. Also, exploring the existing drugs in AGE inhibition, which are developed for other therapeutic interventions have been demonstrated to be potent inhibitors of glycation and AGE formation.

Keywords: advanced glycation end product; diabetes mellitus; receptor for advanced glycation end product; anti-AGE therapy

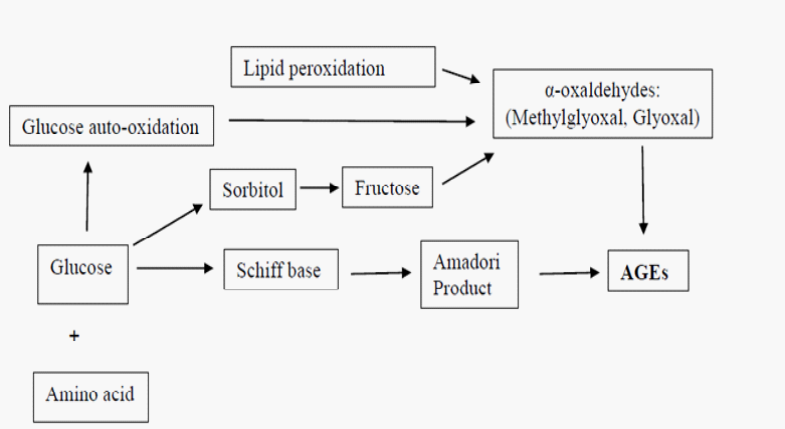
1. Advanced Glycation End Product (AGE)

Hyperglycemia facilitates formation of advanced glycation end product (AGE). AGEs are heterogeneous compounds that are formed mainly via the Maillard reaction. The formation of AGE has been first identified in 1992 by Maillard and is known as the Maillard or "Browning" reaction. The Maillard reaction occurs when reducing sugar reacts in a non-enzymatic way with amino group of proteins, lipids or DNA [1]. The Maillard reaction has been considered for years in the food industry because its products add a desirable colour and taste to foods. Association of AGE with certain pathological conditions such as diabetes mellitus(DM), cardiovascular disease, Alzheimer's disease and also aging process has drawn increasing attention towards the role of AGEs in these diseases [2, 3].

1.1. Formation of advanced glycation end product (AGE)

The formation of AGE through the Maillard reaction occurs in three phases as shown in **Figure 1**. First, glucose attached to a free amino acid (mainly lysine and arginine) of a protein, in a non-enzymatic way to form a Schiff base which has a carbon to nitrogen double bond where the nitrogen is not attached to hydrogen. The initiation of this step depends on glucose concentration and takes place within hours. If concentration of glucose decreases, this reaction is reversible. During the second phase, the Schiff base undergoes chemical rearrangement over a period of days and form Amadori products. The Amadori products are more stable compound but the reaction is still reversible. They, undergo complicated chemical rearrangement (oxidations, reductions, and hydrations) and form cross-linked proteins. This process takes place in weeks or months. These are very stable and accumulate in the cells and interfere with protein function [4].

Other pathways can also form AGE alongwith the Maillard reaction. For instance, the autoxidation of glucose and the peroxidation of lipids into dicarbonyl derivatives such as α-oxaldehydes (glyoxal, methylglyoxal) and 3-deoxyglucosone by an increase in oxidative stress can lead to formation of AGE [5]. Another pathway for the formation of AGE is through polyol pathway, where glucose is converted to sorbitol by the enzyme aldose reductase and then to fructose by action of sorbitol dehydrogenase. Fructose metabolites such as fructose 3-phosphate converted into α-oxaldehydes to form AGE [6,7].



(Adapted from: Ojigbo., 2014 [8])
Figure 1: Formation of AGE

1.2. AGE-mediated pathogenesis

Advanced glycation end product (AGE) and its interaction with RAGE mediated-intracellular consequences has been reported in several diseases including diabetic vascular complications. High levels of blood AGE and enhanced expression of RAGE is associated with activation of various downstream signaling cascades such as activation of MAP kinases and JAK/STAT pathway. These pathways lead to the activation of nuclear factor-kappa B (NF-κB) which induces various target genes such as pro-inflammatory genes, cytokines (e.g.TGF-β1, CTGF) and other adhesion molecules (e.g. VCAM-1). In addition, AGE-RAGE interaction also enhanced production of reactive oxygen species (ROS) via activation of nicotinamide adenine dihydrogen phosphate (NADPH) oxidase.This enhanced oxidative stress and inflammation implicated in the development and induction of vascular complications in diabetes mellitus (DM) [9,10]. Besides a receptor-mediated action, AGEs are also responsible for alteration in protein function and theirstructure which lead to impaired cell function [11].

2. Inhibitor of Formation of AGE and AGE-Cleaving Agents

Various agents as inhibitor of formation of AGE or AGE breaker have been reported in several studies [12,13]. The following are the agents which known for their anti-AGE properties.

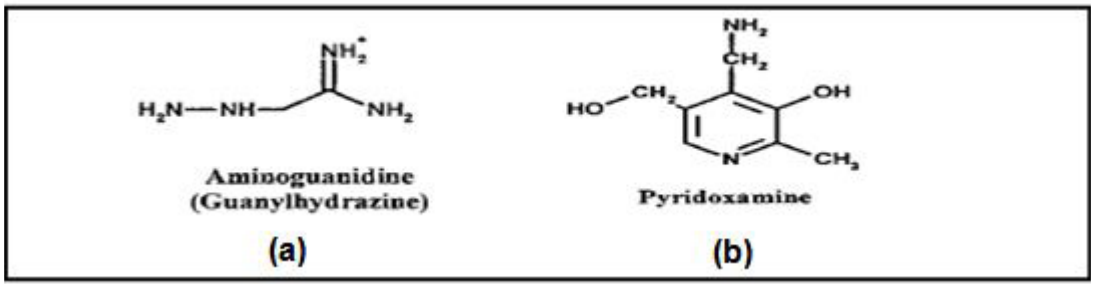
2.1. Aminoguanidine

Aminoguanidine (AG) [**Figure 2 (a)**], nucleophilic hydrazine compound, is known as pharmacological inhibitor of AGE [14]. It was the first drug designed to inhibit the glycation process by inhibiting the conversion of early stage products into AGE. It prevents the formation of advanced glycation end product by reacting with Amadori-derived fragmentation products such as 3-deoxyglucosone, methylglyoxal, and glyoxal and also by trapping of reactive carbonyl intermediates in the Maillard reaction [15]. Inhibitory effect of AG for vascular complications has been observed in experimental DM and has beneficial for diabetes related

vascular complications [16].

2.2. Pyridoxamine

Pyridoxamine [**Figure 2 (b)**], is natural form of vitamin B_6. It also inhibits the formation of AGE. Pyridoxamine has multiple mechanisms of action such as blocking of oxidation of the Amadori intermediate, trapping of reactive carbonyl and dicarbonyl compounds derived from the Amadori compound, chelation of metal ion catalysts of oxidation and scavenging of reactive oxygen species (ROS) [17, 18]. It delays the development of diabetic nephropathy in animal models of both Type 1 and Type 2 diabetic nephropathy [19, 20].

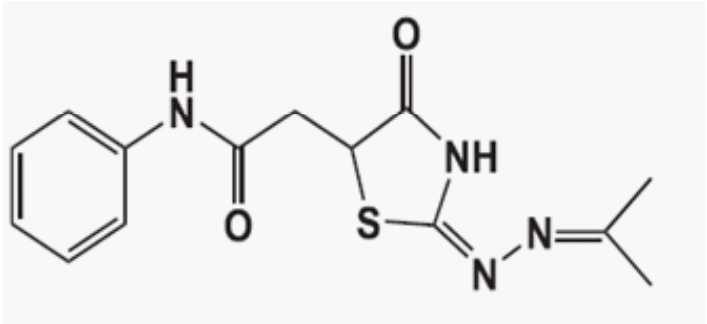


(Adapted from: Booth et al., 1997 [21])

Figure 2: Chemical structure of (a) Aminoguanidine and (b) Pyridoxamine

2.3. OPB-9195

OPB 9195 [(±)-2-isopropylidenhydrazono-4-oxo-thiazolidin-5-ylacetalinide][**Figure 3**], is a synthetic thiazolidine derivative. It decreases AGE production as cross-link breaker and inhibits cross-linking of AGE [22, 23]. It has shown inhibitory actions on glycoxidation and lipoxidation reactions and decrease the formation of AGE and dicarbonyl intermediates [24].



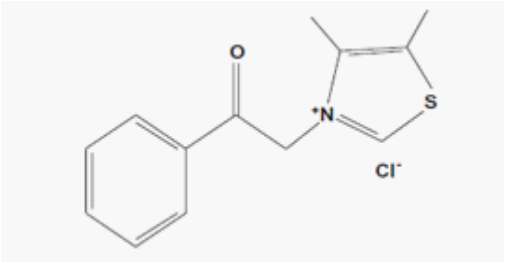
(Adapted from: Nagai et al., 2012 [25])

Figure 3. Chemical structure of OPB-9195

2.4. Alagebrium (ALT-711)

Alagebrium (ALT-711) [**Figure 4**], is another potential cross-link breaker. ALT-711, a small easily synthesized compound (3-phenacyl- 4, 5-dimethylthiazolium chloride) was developed for heart failure and systolic hypertension [26]. Its treatment has been found to significantly decrease plaque area or complexity within the thoracic and abdominal aortas and inhibited the accumulation of AGE-modified collagens in the aortas in animal model [27]. It

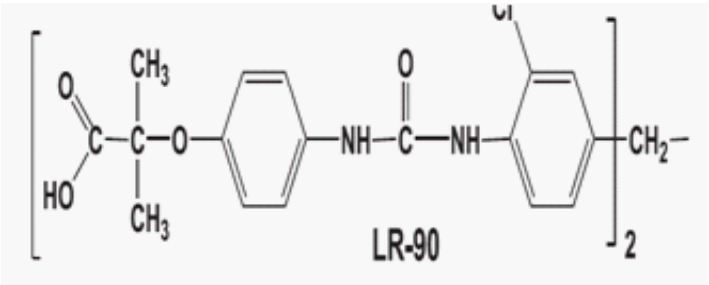
also decreased AGEs and collagen accumulation in the diabetic kidneys, inhibited glomerulosclerosis and tubulointerstitial injury in streptozotocin-induced diabetic rats [28].



(Adapted from: Dhar et al., 2012 [29])
Figure 4: Chemical structure of Alagebrium

2.5. LR-90

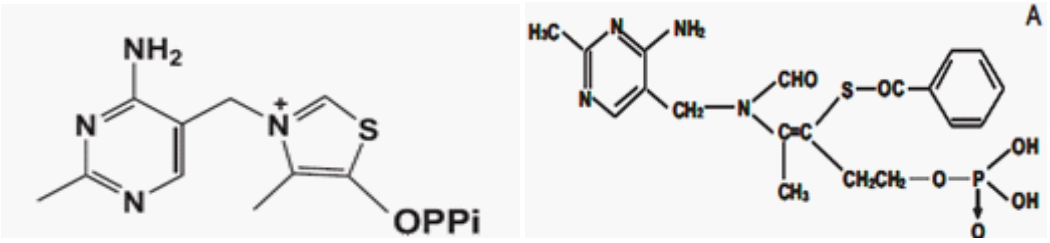
LR-90; 4-4'-(2 chlorophenylureido phenoxyisobutyric acid) [**Figure 5**], is an aromatic compound. LRs were named after their developers as Lalezari-Rahbar (LR) compounds [30]. It inhibits AGE production by chelating transition metals that catalyze the production of AGE. In experimental diabetic models, it has been shown to reduce the formation of AGE, oxidative stress and prevent the progression of nephropathy [31].



(Adapted from: Nagai et al., 2012 [25])
Figure 5: Chemical structure of LR-90

2.6. Thiamine and Benfotiamine

Thiamine [**Figure 6 (a)**] is vitamin B_1 and benfotiamine [**Figure 6 (b)**] which is derivative of vitamin B_1 show AGE-lowering properties. These are also known to decrease the formation of reducing sugars and intermediates from the polyol pathway [32]. Both thiamine and benfotiamine have beneficial role in experimental models of diabetic nephropathy [33]. Furthermore, administration of benfotiamine to type 2 diabetes mellitus (T2DM) patients, who consumed a high AGE content diet, reduced the circulating AGE levels [34].

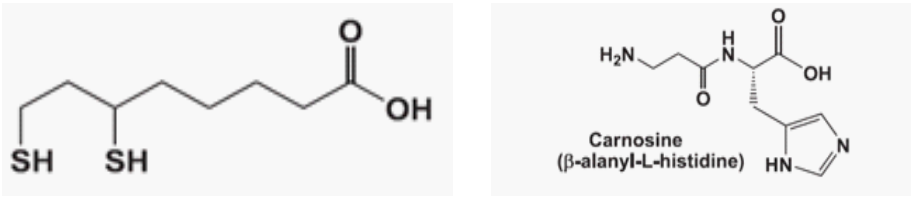


(a) **(b)**

(Adapted from: Nagai et al., 2012 [25]) (Adapted from: Yadav et al., 2009 [35])
Figure 6: Chemical structure of (a) Thiamine pyrophosphate and (b) Benfotiamine

2.7. Lipoic acid and carnosine

Lipoic acid [**Figure 7 (a)**] and carnosine [**Figure 7 (b)**] act as an antiglycating agent and reduce the rate of formation of AGEs. These compounds have shown their anti-AGE role through carbonyl-trapping activity as well as potent chelating activity [36].

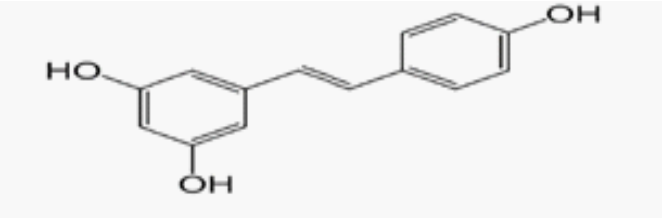


(a) **(b)**

Adapted from Nagai et al; 2012 [25]
Figure 7: Chemical structure of (a) Lipoic acid and (b) Carnosine

3. Resveratrol

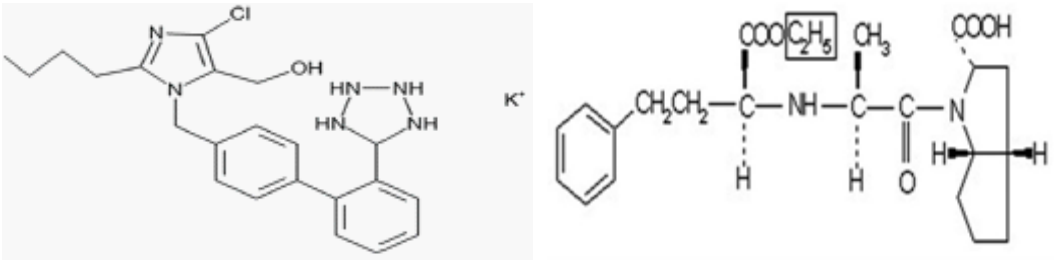
Resveratrol (RSV; 3, 4, 5-trihydroxy-trans-stilbene) [**Figure 8**], is a stilbenoid, a type of natural phenol, found in plants and red wines. It is a member of a group of plant compound called polyphenols [37]. RSV has gained considerable attention because of its beneficial effects as anti-oxidant, anti-inflammatory, anti-atherosclerotic, and anti-cancer properties [38, 39]. However, RSV have also ability to inhibit the formation of AGE and several studies have shown its anti-AGE role in pathogenesis of diseases [26, 40, 41].



Adapted from: Kim et al., 2014 [42]
Figure 8: Chemical structure of Resveratrol

4. Antihypertensive Drugs

Recently, it has been shown that antihypertensive drugs such as losartan, olmesartan, and hydralazine, seem to inhibit formation of AGE [43-45]. Ramipril [**Figure 9 (a)**] and losartan [**Figure 9 (b)**] are widely used anti-hypertensive drugs in the treatment of diabetic nephropathy [46, 47]. These drugs have shown that in addition to their hemodynamic role, they have added additional benefit of reducing AGE formation and accumulation [48, 49]. The mechanisms of action of these drugs with regard to decrease AGE by trapping reactive carbonyls, hydroxyl and also via chelation of metal ions have been reported [44]. Ramipril and valsartan have reduced AGE accumulation in kidneys of STZ-induced diabetic rats [48, 50]. The AGE inhibiting property of ARB and ACEI has opened more possibilities for newer therapeutic interventions.



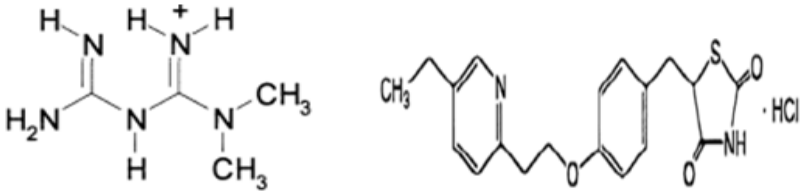
(a) **(b)**

(Adapted from: Diego et al., 2010 [51]) (Adapted from: Das et al., 2015 [52])
Figure 9: Chemical structure of (a) Ramipril and (b) Losartan

5. Hypoglycemic Drugs

By minimizing hyperglycemia, oral hypoglycemic agents can decrease the formation of AGE, but some have other AGE preventive mechanisms as well. Metformin and pioglitazone are anti-hypoglycemic drugs used routinely in the treatment of diabetes.

Metformin (1, 1-dimethylbiguanide)[**Figure 10 (a)**], is an orally effective synthetic anti-hyperglycemic drug, is structurally similar to aminoguinidine [53]. The mechanism of action of metformin with regard to inhibition of AGE formation is trapping of reactive carbonyl molecules through presence of its guanidine moiety [54]. It inhibits glycation at multiple steps with maximum effect observed in post Amadori stages [55].



(a) **(b)**

(Adapted from: Khouri et al., 2004 [56]) (Adapted from: Prakash et al., 2013 [57])
Figure 10: Chemical structure of (a) Metformin and (b) Pioglitazone

Pioglitazone (5-(4-(2-5 Ethylpyridin-2-yl) ethoxy) benzyl) thiazolidine-2, 4-dione hydrochloride)[**Figure 10 (b)**], is an oral anti-diabetic drug used in the treatment of type 2 diabetes mellitus (T2DM) or adult onset diabetes. It is known as oral and well-tolerated drug for diabetes, proved to have a role in anti-AGE treatment because of their peroxisome proliferator-activated receptor (PPAR) γ-agonist activity, which determine an increase in soluble RAGE (sRAGE) expression, which is inversely associated with atherosclerosis. The reduction of endothelial RAGE expression by Thiazolidinediones (TZD) such as rosiglitazone and pioglitazone have been reported by Marx et al. (2004) [58]. It anti-AGE action is similar to metformin in trapping dicarbonyl compounds. It also has metal-chelation property [55].

6. Soluble AGE-Binding Peptides

Soluble RAGE, which is isoform of full length RAGE bind to RAGE ligand such as

AGE, thus preventing RAGE activation and prevent cellular dysfunction [59].

7. Anti-RAGE agents

Recently, several molecules such as low-molecular weight heparin and neutralizing anti-RAGE antibodies which inhibit RAGE, which is receptor for AGE, have been identified-beneficial towards the inhibition of AGE-mediated consequences. They block the RAGE and inhibit the AGE-RAGE interaction [60-62].

8. Glycemic Control

Hyperglycemic environment has been associated with enhanced formation of AGE, making obvious that the good glycemic control can reduce the total body AGE pool. Decrease in AGE levels improved the glycemic control in diabetic rats has been reported by Odetti et al. 1996 [63].

9. Dietary AGEs Restriction

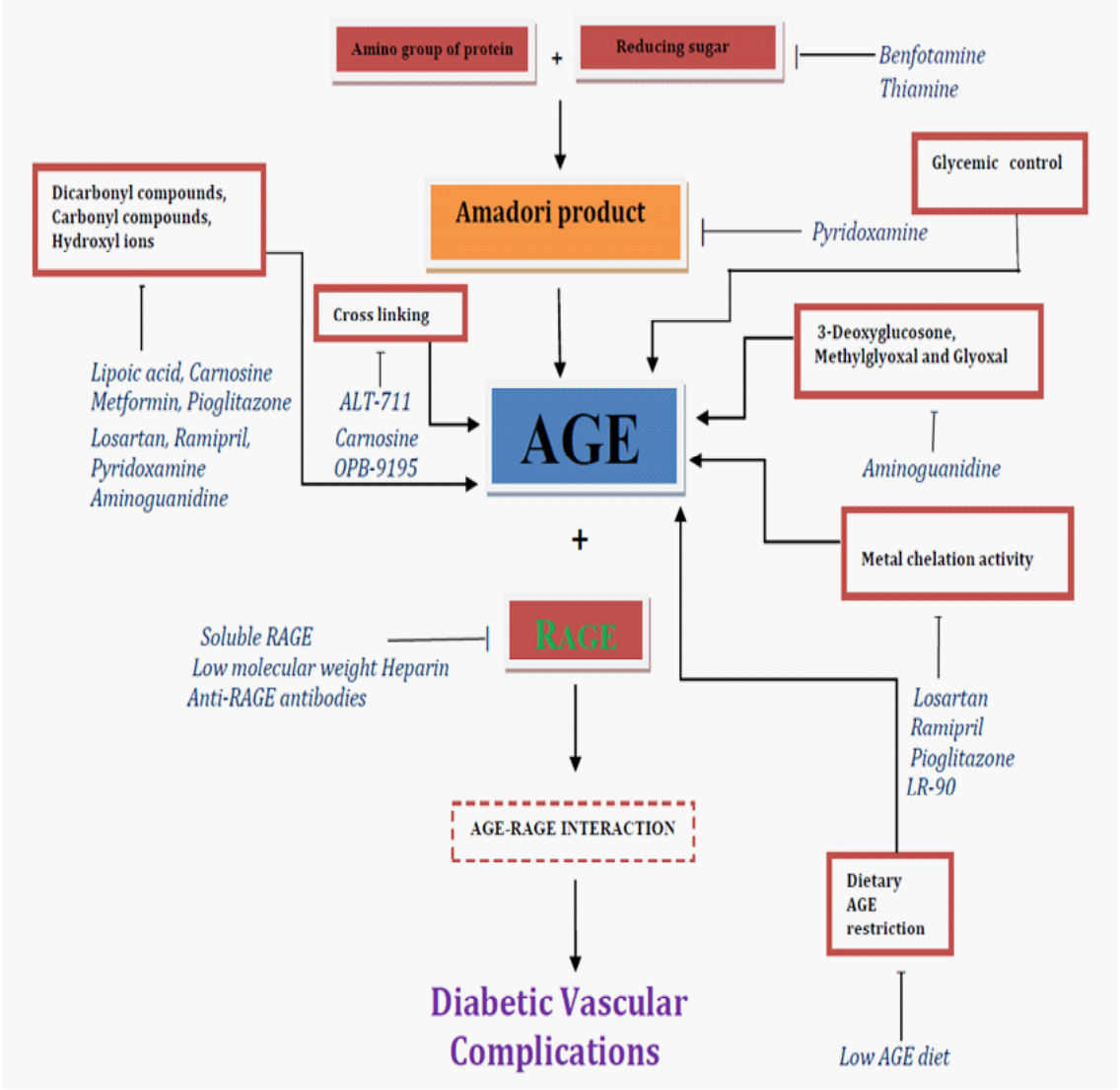
Dietary AGE intake is a significant determinant of circulating and tissue AGE levels [64, 65]. Studies have shown that a low-AGE diet results in decreased serum AGE levels, inflammatory markers levels such as C-reactive protein, total body AGE pool and AGE- related pathology [66-68].

10. Antioxidants

In several studies although antioxidants have been proposed as anti-AGE agents however, further studies are needed with the purpose of establish the effectiveness of antioxidant treatment in reduction of AGE levels [69-73].

11. Conclusion

It is well established that AGEs are involved in the pathogenesis of various diseases, however, the mechanism involved is yet to be fully elucidated. Several efforts have been made in the past decade towards development of drugs, which can inhibit AGEs formation and accumulation without any significant breakthrough. Anti-AGE strategies acting synergistically with conventional approaches may be an important therapeutic option for amelioration of AGE-mediated consequences. Finding newer anti-AGE therapeutics with lesser toxicity level is extremely essential for arresting vascular complications in T2DM.



**Inhibitory action of compounds (|—)*

Figure 11. Inhibitory action of anti-AGE compounds

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Chapter 5

Multidrug Resistance Proteins: A Family of ATP Dependent Transporters and their Role in Cancer

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1. Introduction

Multidrug resistance (MDR) or chemo-resistance is a serious phenomenon utilized by cancer cells that hinder the success of cancer chemotherapy. Owing to the chemo-resistance to antineoplastic drugs, either by acquired or intrinsic mechanisms, the 5-year survival rates remain dismal despite the significant advances in the field of chemotherapy [1]. This was first demonstrated in 1973, where it was found that Ehrlich ascites cells lowered the intracellular daunorubicin concentration by active outward transport [2]. Later it was discovered that the large glycoprotein, now known as multidrug resistance proteins (MRP), in the plasma membrane of MDR cells is responsible for the active outward transport of antineoplastic drugs [3, 4]. The identification of drugs and conjugates efflux pumps of MRP family was started with the discovery of MRP1 in 1992 [5].

Multidrug resistance proteins are the subfamily of the transmembrane transporters superfamily ATP- binding cassette (ABC) [6,7]. It is the largest family of transmembrane proteins which use the energy of ATP hydrolysis to drive a wide range of organic and anionic conjugates such as sulfate, glutathione, glucuronide conjugates and leukotriene C_4 across the

cell membranes [7]. Based on the alignment and phylogenetic analysis with a number of methods, the ABC superfamily can be categorized into seven major subfamilies [6]. The multidrug resistance proteins or ATP binding cassette subfamily C (ABCC) is one of the seven major subfamilies.

MDR uses various mechanisms for the transport of drugs which can be classified as target dependent and drug dependent [8]. Target dependent multidrug resistance mechanism mainly uses factors which cause deletion, mutation and translocation to the target of drugs [9]. Drug dependent MDR is caused by the overexpression of detoxifying enzymes and efflux drug transporters which results into increased efflux of drugs from cell [10]. The aim of this chapter is to discuss the general properties such as structural and functional and to highlight the role of MRPs in cancers cells.

2. General characters of MRPs

The MRP subfamily contains nine members of drug transporters. All the members of the subfamily may have multiple names as several laboratories characterized the MRP family as displayed in **Table 1**. Based on the presence or absence of extra N-terminal membrane spanning domain (MSD), the MRPs are of two types. MRP1, MRP2, MRP3, MRP6 and MRP7 falls into one category which contains an extra N-terminal MSD as presented in **Figure 1** whereas rest of the MRPs contains only two MSDs i.e. MSD1 and MSD2 (**Figure 2**).

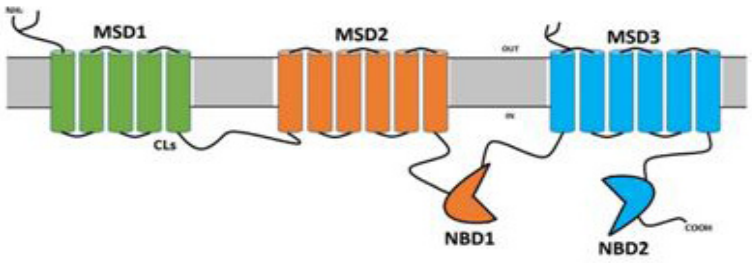

Figure 1: Domain organization of MRP1, MRP2, MRP3, MRP6 and MRP7 with extra N-terminal membrane spanning domain and 17 transmembrane α-helices (MSD–Membrane Spanning Domain; NBD–Nucleotide Binding Domain; CLs – Cytoplasmic Loops)

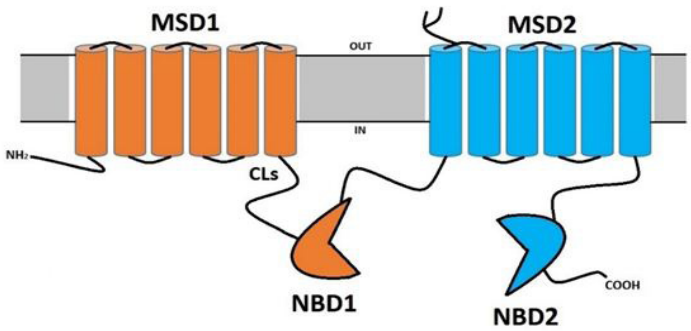

Figure 2: Domain organization of MRP4, MRP5, MRP8 and MRP9 without extra N-terminal membrane spanning domain and 12 transmembrane α-helices (MSD–Membrane Spanning Domain; NBD–Nucleotide Binding Domain; CLs – Cytoplasmic Loops)

Along with all MSDs, the MRPs also have two cytoplasmic nucleotide binding domains (NBDs) and the 17 transmembrane α-helices in case of three MSDs whereas 12 transmembrane α-helices in case of two MSDs [7,11,12]. The binding and the hydrolysis of ATP at NBDs is required for the passage of substances across membrane.

The amino acid sequence lengths of MRP subfamily range between 1325 amino acids for MRP4 to 1545 amino acids for MRP2 (**Table 1**). As compared to MRP1, the amino acid percent identity of MRP3 shares 58 % which is closest member to MRP1 along with MRP2. While the MRP4 and MRP5 shares below 40 % identity which appear to lack the extra N-terminal MSD [13,14]. Furthermore, several studies have revealed that the extra N-terminal MSD is not essential for the transport of drugs across the membrane [14].

Table 1: The human multidrug resistance protein family and some general characters

S. No.	Name	Synonyms/ Symbols	Chromosomal Localization	Amino acids	Amino acid identity	Protein Accession number	References
1.	MRP1	ABCC1, GS-X	16p13.11	1531	100	NP_004987	[5, 15]
2.	MRP2	ABCC2, cMRP, DJS	10q24.2	1545	50	NP_000383	[16, 17]
3.	MRP3	ABCC3, cMOAT2, EST90757, MLP2, MOAT-D	17q21.33	1527	58	NP_003777	[18, 19]
4.	MRP4	ABCC4, EST170205, MOAT-B, MOATB	13q32.1	1325	41	NP_005836	[19, 20]
5.	MRP5	ABCC5, EST277145, MOAT-C, SMRP	3q27.1	1437	38	NP_005679	[18, 19]
6.	MRP6	ABCC6, EST349056, MLP1, URG7	16p13.11	1503	46	NP_001162	[21, 22]
7.	MRP7	ABCC10, EST182763, SIMRP7	6p21.1	1492	35	NP_258261	[19]
8.	MRP8	ABCC11	16q12.1	1382	33	NP_149163	[7, 23]
9.	MRP9	ABCC12	16q12.1	1356	36	NP_150229	[7, 23]

3. Overview of the MRP Family

The localization and distribution of multidrug resistance proteins vary in different human tissues as their expression pattern is cell and tissue type specific such as kidney, lung, skeletal and cardiac muscles specific. To understand the function of the MRPs efflux pump, it

is required to see the domain-specific localization of MRPs in various cell types. Along with the localization and distribution of MRPs, it is also needed to know the substrates of the members of MRP family. The amphiphilic organic anions of molecular mass between 0.3 to 1.0 kDa are the substrates of the MRP subfamily members [11,12]. **Table 2** summarizes the location of members of MRPs and their substrates.

3.1. MRP1

The MRP1 or ABCC1 is localized mainly in the cells of blood-tissue barriers which is shown by the immunofluorescence and immunohistochemical analysis [24]. It is highly detectable in several human cells and tissues such as macrophages, kidney, lung, placenta, testis, umbical cord, skeletal muscles, cardiac muscles and gestational tissue [12,25]. During pregnancy, MRP1 expression level changes have been associated with pre-term birth, growth restriction, and pre-eclampsia [26]. There is lack of detectable amount of MRP1 in normal hepatocytes but in proliferating hepatocyte-derived cells MRP1 appears to be upregulated [12, 27]. The cells that do not express MRP2, MRP1 plays an important function in detoxification from those cells [12].

The first physiological substrate of MRP1 to be identified was the cysteinyl leukotriene LTC4. This finding was discovered during the search for the efflux pump that cause the release of LTC4 from mastocytoma cells [28]. Later by the studies in Abcc1-/- mice it was confirmed that LTC4 is a physiologically relevant substrate [29]. MRP1 can identify a wide range of substrates by making a single bipartite substrate-binding site. The substrate binding site of MRP1 can be categorized into two parts – one with the positively charged region that directs the GSH moiety and other with the hydrophobic area that incorporates the lipid tail [30]. Glutathione containing LTC4, which is high affinity MRP1 substrate, discovery preceded the finding of several glucuronosyl and S-glutathionyl substrates for MRP1 as displayed in Table 2. Another MRP1 substrate, oxidized glutathione (GSSG) with comparatively low affinity suggests the role of MRP1 against oxidative stress [12,31]. GHS plays various role in MRP1-mediated transport such as it act as co-substrate together with the other compounds like Vinca alkaloids. Moreover, it plays a role as transport enhancer without being co-transported itself in case of glucuronidated and sulfated conjugates [32].

3.2. MRP2

The second member of MRP subfamily, MRP2, was first localized in the canalicular membrane of the human and rat hepatocytes [33] and afterward in the apical membrane of rat and human kidney proximal tubules [34,35], placenta [36], small intestine [37], colon [38], gall bladder [39] and bronchi segments [38]. In various human cells and tissues such as blood-brain barrier, pancreas and skin the expression of MRP2 protein either remain below detection limit or remain absent. The apical localization of MRP2 remain in line with its function in the

efflux of many endogenous substance and phase II conjugation product of drugs into extracellular fluids including urine, bile and intestinal fluid.

Table 2: Tissue distribution and substrates of human multidrug resistance proteins

S. No.	Members of MRP subfamily	Location of MRPs in human body	Substrates of MRP transporters
1.	MRP1	Macrophages, kidney, lung, testis, placenta, umbical cord, skeletal and cardiac muscles	Leukotriene C4, Leukotriene D4, Glutathione disulphide, GSH, S-Glutathionyl prostaglandin A2, Glutathionyl melphalan, Estrone 3-sulphate, Bisglucuronosyl bilirubin, folate, cobalamin-OH
2.	MRP2	Liver, kidney, small intestine, colon, gall bladder, placenta, segment of bronchi	Leukotriene C4, Mono- and Bisglucouronosyl bilirubin, 17β-glucuronosyl estradiol, cholecystokinin peptide, Estrone 3-sulfate
3.	MRP3	Gut, Liver, kidney, adrenals, colon, spleen and pancreas	Leukotriene C4, Mono- and Bisglucouronosyl bilirubin, 17β-glucuronosyl estradiol, Cholyglycine, Dehydroepiandrosterone 3-sulfate
4.	MRP4	Prostate, testis, ovary, lung, muscle, gall bladder and pancreas	Leukotriene C4, B4, Prostaglandin E2, F2α, Thromboxane B2, , 17β-glucuronosyl estradiol, cGMP, cAMP, Cholyltaurine (+GSH), cholate (+GSH), Folate, Urate, ADP
5.	MRP5	Urethra, heart, placenta, blood brain barrier	Methotrexate, cGMP, cAMP, Folate, 2'-Deoxyuridine 5'-monophosphate, 9-(2-Phosphonomethoxyethyl)adenine (PMEA)
6.	MRP6	Kidney and Liver	Leukotriene C4, S-Glutathionyl N-ethylmaleimide
7.	MRP7-9	Cerebral cortex and Secretory cells	17b-Glucuronosyl estradiol, Leukotriene C4, Dehydroepiandrosterone 3-sulfate, cGMP, cAMP, Folate, Cholylglycine

MRP2 and MRP1 substrates are quite similar, however the kinetic properties are different i.e. the Km values of MRP1 for 17β-glucuronosyl estradiol and LTC4 are five and tenfold lower, respectively, than those for MRP2 [40]. Similarly, MRP2 have higher affinity for the mono- and bisglucuronosyl bilirubin as compared to the MRP1 [41]. Additionally, as compared to MRP1, MRP2 have low affinity for the transport of GSH and GSSG [42]. The amino acid identity of both MRPs i.e. MRP1 and MRP2, is only 50% (**Table 1**). Thus the similar substrate specificity was initially unexpected, however advancement in the research leads to the finding of the similar structural determinants for substrate binding of both proteins which are responsible for the similar substrates [12].

3.3. MRP3

ATP-dependent efflux transporter mainly MRP3 localized to basolateral (sinusoidal) hepatocyte membrane which transport compounds from hepatocytes to sinusoidal blood [43]. It was initially demonstrated in the human and rat hepatocytes [44,45] and now localized in several human cells and tissues including cholangiocytes, pancreas, kidney, enterocytes, spleen, gall bladder and adrenal cortex [12,43]. The level of MRP3 in human liver may fluctuate upto

80 fold among people. In hereditary MRP2 deficiency and different types of cholestatic liver disease, the MRP3 level increases which leads to elevated serum concentration of bilirubin glucuronosides [46].

MRP3 transports a broad range of xenobiotics and endogenous organic anions, mostly conjugated as presented in **Table 2**. Mono- and bisglucuronosyl bilirubin efflux across the basolateral membrane of hepatocytes into sinusoidal blood is MRP3-mediated transport [47]. It also transports methotrexate in addition to LTC4 and S-(2, 4-dinitrophenyl) glutathione. Human MRP3 transports bile acids (e.g., cholylglycine, cholyltaurine, and sulfatolithocholyltaurine) with low affinity in case of bile acid cholylglycine or below detectability in case of bile acid cholyltaurine [48]. However, the MRP3 of rat transports bile acids with high affinity [49]. This indicates that the MRP3 substrates are species specific, particularly for bile acids.

3.4. MRP4

The MRP4 protein is expressed in a variety of polarized cells and localized in the apical and basolateral membrane domain [43]. Initially it was localized in the glandular epithelial cells of the prostate gland in basolateral membrane [50]. Additionally MRP4 protein express in platelets [51], erythrocytes, astrocytes, adrenal glands and in many cultured cell lines used for the transfection studies such as V79, HEK293, HL60 and HeLa [12,52]. Moreover, MRP4 localization is detected in human and rat hepatocytes, choroid plexus epithelial cells and in polarized MDCKII cells [53-56].

The substrates first identified for MRP4 protein were the nucleosides monophosphate analogs used as antiretroviral drugs, mainly the 9-(2-phosphonylmethoxyethyl)adenine (PMEA) [57]. In delta granules of human platelets, MRP4 mediates the ADP transport which results into accumulation of ADP in delta granules [58]. In addition, the cGMP, cAMP and LTC4 are the important physiological substrate for the MRP4. Other MRP4 protein substrate includes eicosanoids such as prostaglandins E1, E2 and F2α and leukotrienes C4 and B4 [52].

3.5. MRP5

Localization of MRP5 has been detected in basolateral membrane of polarized epithelial cells. However in brain capillary endothelial cells, MRP5 aka ABCC5 is detected in apical membrane [59]. Relatively higher level of MRP5 protein has been demonstrated in smooth muscle cells, astrocytes and in various tissues of human genitourinary system [59]. Furthermore MRP5 protein expresses in epithelial cells of urethra [60], endothelial cells of heart [61] and in fetal vessels of placenta [62].

MRP5 protein substrates includes anionic dye fluorescein diacetate, a number of nucleosides monophosphate analogs, the cyclic nucleotides cGMP and cAMP and some GSH

S-conjugates [63,64]. MRP5 mediated transport is inhibited by various phosphodiesterase inhibitors some of which are structurally similar to cGMAP. MRP5 along with the MRP4 may contribute to regulation of cAMP and cGMP tissue level. Moreover, the affinity of MRP5 to cAMP and cGMP seems to vary depending upon the cellular system [11].

3.6. MRP6

MRP6 protein is highly expressed in the basolateral membrane of human and rodent hepatocytes and epithelial cells of proximal tubule of kidney [65]. Recently it has been identified that MRP6 acts as basolateral efflux pump for nucleotides mainly ATP which after hydrolysis by ecto-enzymes leads to extracellular pyrophosphate [43].

MRP6 protein of inside-out vesicles transports the glutathione S-conjugates LTC4 and NEM-SG and BQ-123 with low affinities [66]. MRP6 mutation leads to a serious genetic disorder, Pseudoxanthoma elasticum (PXE), with ectopic mineralization affecting eye, skin and cardiovascular system. This is hypothesized that PXE is a consequence of hepatic accumulation of MRP6 substrate(s) as it seems that MRP6 remain absent in the affected organs whereas a high expression is seen in hepatocytes [67].

3.7. MRP7-9

On the basis of mRNA analysis it is assumed that the MRP7-9 are expressed widely in various human cell types and tissues [12]. MRP8 was detected in axonal membrane of neurons in human cerebral cortex and in the HepG2 apical membrane [68]. Additionally, MRP8 protein express in the luminal membrane and large vacuoles of secretory cells such as apocrine sweat glands [12,69,70].

A number of substrates are mediated by the MRP7 protein including 17β-glucuronosyl estradiol and LTC4 [12]. It also confers the low level of resistance to Vinca alkaloids and paclitaxel [71]. MRP8 mediates the transport of a number of physiological substrates including dehydroepiandrosterone 3-sulfate, LTC4, cholylglycine, cyclic nucleotides, 17β-glucuronosyl estradiol and folate. Recently a new substrate, Tenofovir disoproxil fumarate, of MRP8 is identified which is a nucleotide reverse transcriptase inhibitor [72]. The substrate of MRP9 has not been detected so far [12].

4. Role of MRPs in Cancer

MRPs are the proteins which are responsible for the resistance of cancer cells to a broad variety of mechanistically and structurally anticancer drugs. The chemotherapeutic failure is the result of either intrinsic resistance or acquired resistance of cancers cell which leads to the malignant tumor progression [73]. The major mechanism of multidrug resistance can be categorized into various groups such as inhibition of apoptosis pathways, metabolic modifica

tion, activation of DNA repair, decreased drug influx, altered drug targets, and detoxification, increased drug efflux mainly via MRP subfamily transporters and via higher expression levels of these efflux transporters [8,74]. These efflux transporters or efflux pumps reduce the concentration of several intracellular exo- and endotoxins via the above-mentioned mechanisms.

Recently, an overexpression of members of MRP subfamily, particularly MRP1 and MRP8, was reported in the aggressive breast carcinoma subtypes [75]. Similarly overexpression of MRP1, MRP2 and MRP3 was observed in lung cancer patients [76]. The expression level of these pumps varies based on the lung cancer subtypes i.e. In non-small cell lung cancer (NSCLC) cell lines, higher expression of MRP1, MRP2, and MRP3 was found than small cell lung cancer (SCLC) cell lines, with the highest level of MRP3 [76]. Additionally, the decrease in drug sensitivity towards etoposide, cisplatin, vincristine and doxorubicin in lung cancer patients is owing to overexpression of MRP1 and MRP3. Likewise in colorectal carcinoma, the levels of MRP1 and MRP2 are found to be higher [77]. As compared to the patients who respond to chemotherapy, there is higher expression of MRP6 and MRP8 in non-responders. The overexpression of MRPs in drug resistance of specific types of cancer are summarized in **Table 3**.

RT-PCR and northern blotting exhibited the MRP1 overexpression in prostate cancer lines resistant to doxorubicin. Similarly, overexpression has also been detected by immunohistochemistry in pancreatic carcinoma cell lines and in renal cell carcinoma patients [78–80]. Recently, MRP7 and docetaxel-treatment failure were confirmed by *ex vivo* study where MRP7 was greatly expressed in ER and Her2 breast cancers and to reverse MDR in chemotherapy, inhibition of MRP7 was suggested [81]. Therefore, in patients with high expression level of MRPs, to inhibit drug efflux function by developing modulators is a feasible approach.

Table 3: MRPs overexpressed in drug resistance of specific types of cancer

S. No.	Specific type of Cancer	MRPs Overexpressed	Reference
1.	Breast cancer	MRP1, MRP8	[75]
2.	Non-small cell lung cancer	MRP1, MRP2, MRP3	[76]
3.	Small cell lung cancer	MRP3, MRP5	[76,82]
4.	Colorectal cancer	MRP1, MRP2	[77]
5.	Prostate cancer	MRP1, MRP4	[78,83]
6.	Pancreatic cancer	MRP1	[79]
7.	Renal cancer	MRP1	[80]

4.1. Current strategies for MRP modulators

In order to reverse the MRP-mediated MDR, several attempts have been performed. Recently a few approaches are being used to develop MRPs modulators to reverse MDR in chemotherapy such as off-target small molecular inhibitors as modulators and miRNA based

therapy [73].

MicroRNA (miRNA) is small RNA molecules of approximately 20-25 nucleotides in length which bind directly to 3'UTR of targeted mRNAs. Various miRNA based modulators are being used such as miR-326, miR-297, miR-534 and miR-134. The overexpression of MRP1 mRNA and its protein was observed in the breast cancer cell line MCF-7/VP. On MCF-7/VP and MCF-7 cells, 17 of miRNAs were distinctly expressed by utilizing a microarray consisting of human mature miRNA probes. All the expressed miRNA showed increased expression but miR-429, miR-92-2, miR-7, miR-187 and miR-326 exhibited decreased expression [84]. Quantitative RT-PCR result revealed that the decreased expression of miR-326 was 3.3 fold less as compared to MCF-7 and the expression was inversely correlated with the MRP1 mRNA. It was observed that expression of MRP1 were lowered in miR-326 miRIDIAN mimic-transfected MCF-7/ VP cells [84]. From this finding, it was suggested that by blocking the MRP1, the miR-326 could strengthen the cytotoxic effect of doxorubicin on MCF-7/VP cells.

A number of small off-target molecular inhibitors are used as modulators of MRPs i.e. Ibrutinib as modulator of MRP1, Masitinib, Lapatinib, Imatinib, Erlotinib, Nilotinib and Tandutinib as Modulators of MRP7. MRP1 modulator ibrutinib can potentially block the efflux of doxorubicin in HL60/Adr cells which leads to increased intracellular doxorubicin accumulation [85]. In recent years, it has been reported that Masitinib, Lapatinib, Imatinib, Erlotinib, Nilotinib and Tandutinib could reverse MDR in transfected HEK/MRP7 cells [73,86,87].

5. Summary

This chapter summarizes about the multidrug resistance proteins which are a subfamily of ATP dependent transporters, ABC family. The MRP family is the transmembrane proteins which use the energy of ATP hydrolysis to drive a wide range of organic and anionic conjugates such as sulfate, glutathione, glucuronide conjugates and leukotriene C4 across the cell membranes. The MRP subfamily contains nine members of drug transporters i.e. MRP1-9. All the members of the subfamily may have multiple names as several laboratories characterized the MRP family. The localization and distribution of multidrug resistance proteins vary in different human tissues as their expression pattern is cell and tissue type specific such as kidney, lung, skeletal and cardiac muscles specific. The amphiphilic organic anions of molecular mass between 0.3 to 1.0 kDa are the substrates of the MRP subfamily members. The major mechanism of multidrug resistance can be categorized into various groups such as inhibition of apoptosis pathways, metabolic modification, activation of DNA repair, decreased drug influx, altered drug targets, and detoxification, increased drug efflux mainly via MRP subfamily transporters and via higher expression levels of these efflux transporters. The chemotherapeutic failure is the result of either intrinsic resistance or acquired resistance of cancers cell

which leads to the malignant tumor progression. Recently a few approaches are being used to develop MRPs modulators to reverse MDR in chemotherapy such as off-target small molecular inhibitors as modulators and miRNA based therapy. These recent strategies to engage the MRP transporters to enhance the cancer treatment reflect the creativity of cancer researchers and hopefulness that at least this basis of MDR can be defeated.

6. References

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Advances in Biochemistry & Applications in Medicine

Chapter 6

Sirtuins: Its Role in Metabolic Homeostasis

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1. Sirtuins: Its Role in Metabolic Homeostasis

Sirtuin proteins are evolutionarily conserved enzymes that function in critical cellular processes such as DNA repair, transcription and stress resistance. The name Sirtuin (Sir2) is derived from the yeast gene 'silent information regulator' which is responsible for controlled expression of the silent mating type loci and also required for telomere hyper cluster formation in quiescent yeast cells [1]. It was not until fifteen years later the significance of sir2 proteins was identified as beta-nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, which deacetylate lysine at a specific site and accounts for silencing, recombination suppression, apoptosis, mitochondrial biogenesis, lipid metabolism, and extension of life span *in-vivo* [2,3]. The sirtuins were originally classified as histone deacetylases but many non-histone targets are described recently.

1.1. Biochemistry of sirtuins

Sirtuins possess a conserved catalytic core (~275 amino acids) that is flanked by N- and C- terminal extensions. These N- and C- terminal extensions play a key role in ensuring proper cellular localization, regulating the interaction with other proteins and targets for post-translational modifications that affect the functions of sirtuins [4]. The larger sirtuin domain consists of a Rossmann-fold, that is characteristic for NAD^+-binding unit, and the smaller domain: a

zinc-binding motif and a α-helical region which shows the highest diversity among family members [5].

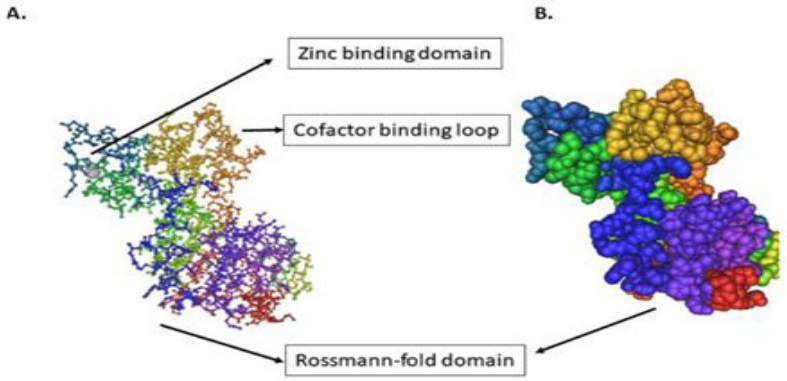


Figure 1: structure of NAD-dependent protien Deacetylase Sirtuin-1 (PDB ID 41G9): A. Ball and stick model B. space fill model

Protein acetylation is regulated by protein acetyltransferases and deacetylases. Sirtuins are a family of NAD^+ - dependent protein deacetylases (class III) which are widely distributed in almost all phyla of life. Sirtuins differ from other classes of deacetylases in that they are absolutely dependent on NAD^+, deacetylates lysine residue and releases nicotinamide (NAM), 2' O-acetyl-adenosine diphosphate-ribose (AADPR) and a deacetylated substrate. As sirtuins are dependent upon the presence of NAD^+, sirtuin activity is directly linked to the metabolic state of the cell [6]. In the first step of the reaction, ADP-ribose is covalently attached to the acetyl moiety of the substrate, accompanied by the release of free NAM. Hydrolysis of the acetyl-lysine bond then occurs, liberating the AADPR. NAM acts as an inhibitor of the reaction and thus provides negative feedback inhibition of the sirtuins *in vivo* [7]. The byproduct O-acetyl ADP-ribose (AADPR) which is released in the deacetylation reaction has an essential role in modification of proteins [8,9]. Macrodomain, a conserved globular protein domain of 130-190 amino acids recognizes the terminal ADP-ribose of poly ADP ribose (PAR). Only 11 human macrodomain-containing proteins have been identified so far which are capable of binding to AADPR. Macrodomains are involved in DNA repair, transcriptional regulation, signalling events, control of NAD^+ metabolism, chromatin remodelling and developmental processes [9].

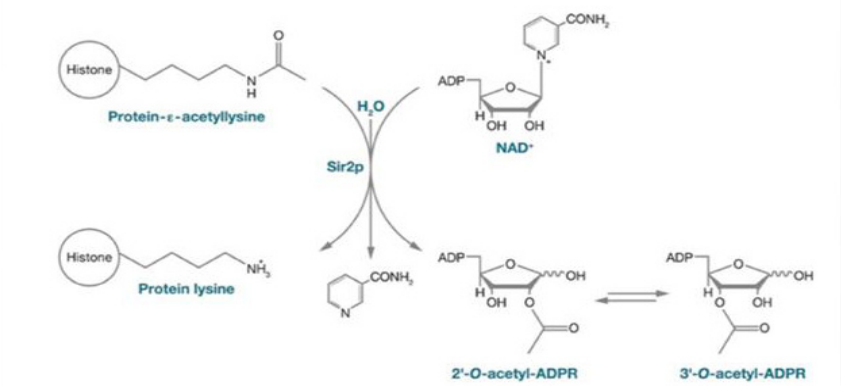


Figure 2: the sir2 reaction. Deacetylation of protien acetyllysine catalysed by sir2p. Acetyl-group transfer to the ADP-ribose (ADPR) motely of NAD* occurs via sir2p chemistry to form 2'-o-acetly-ADPR. (Adopted from sauve et al. 2006

Sirtuins are absolutely dependent on NAD^+ the abundance of free NAD^+ and its biosynthetic and breakdown products in cells are relevant to the enzymatic activity of sirtuins [10]. The root substrates for different NAD^+ biosynthetic pathways include the amino acid tryptophan (Trp), nicotinic acid, NAM and nicotinamide riboside (NR). The intermediate nicotinamide mononucleotide (NMN) can also directly stimulate NAD^+ synthesis [11]. Nicotinamide phosphoribosyltransferase (Nampt) catalysis is a rate-limiting step which transfer of phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate to nicotinamide forming NMN and pyrophosphate. The cellular levels of Nampt which synthesize NAD^+ vary during different pathophysiological conditions in metabolism like inflammation and cancer [12]. Increased expression of Nampt in response to various stresses elevates cellular NAD^+ levels, which in turn regulate catalytic activity. For example, intracellular NAD levels regulate tumour necrosis factor protein synthesis in a sirtuin-dependent manner [13].

1.2. Modulators of sirtuins

The sirtuins promote longevity in diverse species and mediate many of the beneficial effects of calorie restriction (CR), such as reduced incidence of cancer, cardiovascular disease and diabetes. Sirtuins attracted considerable interest as a therapeutic target for the development of drug targets [7]. Many inhibitors and SIRT1-activating compounds (STACs) have been discovered for sirtuins but at the molecular level, the mechanism by which sirtuins is activated remains elusive [14].

Nicotinamide which is a byproduct of the deacetylation process by sirtuins acts as an inhibitor. The synthetic molecule iso-nicotinamide (iNAM) can act as a pan-sirtuin activator within the limits of pharmacological concentrations. Several classes of plant-derived metabolites such as flavones, stilbenes, chalcones and anthocyanides activate SIRT1 *in vitro*. Resveratrol (3,5,4' – trihydroxystilbene), is the most potent of the natural activators of the sirtuins. Unfortunately, resveratrol as a drug is not likely to succeed due to insolubility, it's poor bioavailability and rapid half-life [15]. The first synthetic STACs were derivatives of an imidazothiazole scaffold (e.g. SRT460, SRT1720 and SRT2183) and chemically distinct from the polyphenol backbone of resveratrol. More potent second-generation STACs based on benzimidazole and urea-based scaffolds were subsequently described [7,16].

The sirtuin inhibitors include sirtinol (reduces inflammation in capillary endothelial cells of the skin), cambinol (competitive inhibitor that competes with acetylated polypeptides), suramin (urea derivative competes for binding with both NAD^+ and the acetylated lysine of the substrate), EX-527 (SIRT1 inhibitor, increase the acetylation of P53 protein at K382 following the induction of DNA damage in human mammary epithelial cell and some tumor cell lines), oxyindole (SIRT2 inhibitor, inhibits α-tubulin deacetylation in mammary cell lines [5].

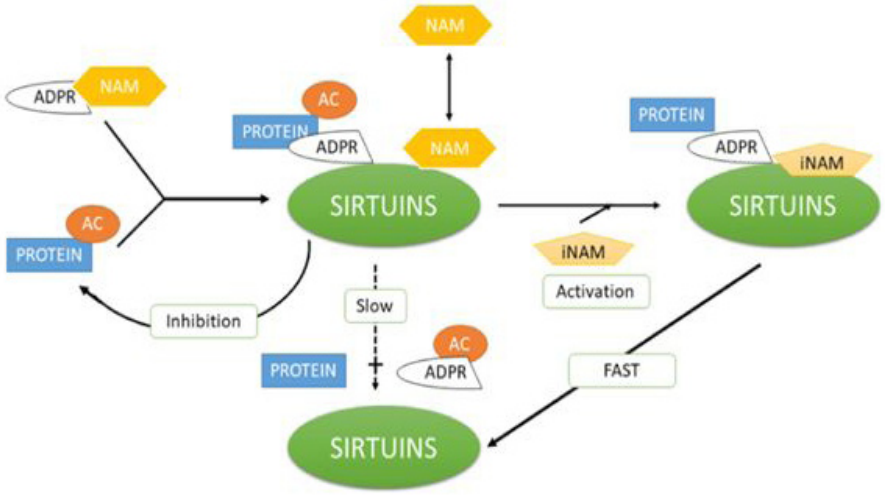


Figure 3: Activation mechanisam of sirtuins by isonicotinamide (INAM). In the presence of nicotinamide the deacetylation reaction is slow because of depletion of the imidiate by nicotinamide reactivity. isonicotinamidecan bind the nicotinamide pocket and prevent reaction reversal. The INAM-bound complex can complete deacetylation more effeciently, leading to activation of sirtuin catalytic activity in cell. (Adapted from "The Biochemistry of sirtuins" by sauve et al. 2006)

1.3. Localization of mammalian sirtuins

The seven mammalian sirtuins are nuclear encoded and ubiquitously expressed in human tissues and occupy three different subcellular localization nucleus, cytoplasm and mitochondria. SIRT 1, -2, -6, -7 are found in nucleus. SIRT1 and 2 are found in the cytoplasm as well. SIRT3, 4 and 5 are found in the mitochondria [5,17]. Sirt1 localization is predominantly nuclear but can be translocated to the cytoplasm in a cell-specific and cell-autonomous manner, in response to various physiological stimuli and disease states. The subcellular localization, enzymatic activity and the diverse functions of sirtuin isoforms are shown in **Table-1**. The various sirtuins have very diverse substrates which can be broken down into three major overlapping classes: transcriptional, apoptosis regulating and metabolic regulating. The substrates for sirtuins in *Homo sapiens* include Histones (H1, H3, H4), P53, FOXO3a, MyoD PGC-1α and other transcription factors which are deacetylated with transcription activation. In most cases, these transcription factors control genes related to growth, cell cycle and apoptosis control [10].

Table 1: Mammalian isoforms of Sirtuins, its enzyme activity and function

Sirtuin	Subcellular localization	Enzymatic activity	Function	Reference
SIRT1	Nucleus Cytoplasm	Deacetylase	• Formation of facultative and constitutive chromatin • Mitochondrial biogenesis • Fatty acid oxidation • Repress adipocyte differentiation • Regulation of cholesterol and bile acid homeostasis • Stimulate gluconeogenesis • Inhibit apoptosis	[18–26]

SIRT2	Nucleus Cytoplasm	Deacetylase Demyristoylase	• Cell cycle regulation through mitosis • Promotion of lipolysis in adipocytes • Inhibit adipocyte differentiation • Tumour suppression/promotion • Neurodegeneration	[27–33]
SIRT3	Mitochondria	Deacetylase Decrotonylase ADP-ribosyltrans-ferase	• Regulation of mitochondrial activity • Protection against oxidative stress • Enhance adaptive thermogenesis • Tumor suppression • Accelerate acetyl CoA conversion • Facilitate TCA cycle and mitochondrial energy production • Enhance fatty acid oxidation	[34–43]
SIRT4	Mitochondria	ADP-ribosyltrans-ferase Lipoamidase	• Glucose metabolism • Aminoacid catabolism • Tumor suppression • Repress aminoacid stimulated insulin	[44–48]
SIRT5	Mitochondria	Deacetylase Demalonylase Desuccinylase Deglutarylase	• Enhance urea cycle • Fatty acid metabolism • Amino acid metabolism	[49–52]
SIRT6	Nucleus	Deacetylase Deacylase ADP-ribosyltrans-ferase	• Genomic stability / DNA repair and prevent against ageing related disorders • Promote apoptosis • Reduce glycolysis and increase mitochondrial respiration • Reduce inflammatory response	[53–57]
SIRT7	Nucleus	Deacetylase	• Ribosome biogenesis • Tumor promotion	[58–61]

2. Sirtuins Mediate Effects of Calorie Restriction

There are many studies showing that sirtuins mediate the effects of calorie restriction (CR) in mammals. Restricting calorie intake, a reduction of calories by 20-40% is known as calorie restriction which can increase lifespan in lower organisms [62]. The two hallmark of CR is metabolic reprogramming (towards oxidative metabolism so as to gain most energy during the restricted diet) and resistance to stress (particularly oxidative stress) [63]. Calorie restriction induces the expression levels of SIRT1 and SIRT5 similarly, loss of function mutation of specific sirtuins ablates specific outputs of CR. However, high-fat diet leads to the loss of SIRT1 in mice [64]. The transgenic overexpression of SIRT1 or STACs mitigates disease syndrome like CR, these include diabetes, neurodegenerative diseases, liver steatosis, bone loss and inflammation [63]. Different groups of neurons in the hypothalamus control mammalian physiology & energy homeostasis, including feeding behaviour, nutrients inputs, energy expenditure, physical activity, body temperature and central circadian control.

Sirt1 is absolutely dependent on NAD+ and function as nutrient/redox sensor, and its expression is regulated by changes in nutritional status and is involved in a wide range of

metabolic processes. Nutrient sensors have the ability to sense and respond to fluctuations in environmental nutrient levels which characterize a fundamental requisite for life [65]. There are diverse nutrient sensor pathways detecting intracellular and extracellular levels of sugars, amino acids, lipids and other metabolites that incorporate and correspond at the organismal level through hormonal signals. During the period of food-richness, nutrient-sensing pathways engage in anabolism and energy storage. Conversely, shortage of food triggers homeostatic mechanisms including mobilization of internal stores through autophagy. The expression levels of SIRT1 increases upon CR in rodent and human tissues. Levels of NAD rise under CR-like conditions, which in turn induces expression of SIRT1 [66]. Thus, sirtuins act as a metabolic sensor by detecting fluctuations in the NAD^+/NADH ratio in cells. When the amount of nutrients decrease, especially in the case of glucose, levels of NAD^+ increase and sirtuins are then elevated [67]. Calorie restriction increases SIRT1 deacetylase activity in skeletal muscle in parallel with enhanced insulin-stimulated phosphoinositide 3 kinases (PI3K) signalling and glucose uptake.

3. Sirt1 in Control of Metabolic Homeostasis

The brain plays a critical role in the regulation of systemic energy homeostasis. The hypothalamus is directly sensitive to nutritional changes [68]. Among several key brain areas involved in the regulation of energy balance, the hypothalamus is the primary structure that interprets adiposity and nutrient-related inputs [69]. Among the most prominent regulators within the hypothalamus, neurons in the circumventricular organ called the arculate nucleus (ARC) of the hypothalamus located in the mediobasal hypothalamus, anteriorly juxtaposing the median eminence (ME) is of critical importance for the regulation of energy balance. Leptin is a hormone released from adipose tissue binds to leptin receptors (LEPR) on agouti-related protein (AGRP)- producing neurons and pro-opiomelanocortin (POMC)- producing neurons in the ARC of the hypothalamus [70,71]. During fasting circulating leptin levels decline rapidly. The fall in leptin stimulates the expression of AGRP and neuropeptide Y (NPY) and suppresses POMC and cocaine- and amphetamine-regulated transcript (CART), thereby increasing food intake and decreasing energy expenditure [72,73]. Recent studies have shown that SIRT1 may play a role in central energy regulatory area. For instance, it has been shown that both CR and fasting enhance SIRT1 expression and activity in the hypothalamus [69].

At the molecular level, FoxO1 is a metabolic sensor that integrates both leptin and insulin signalling. FoxO1 is the master regulator of energy metabolism across species. FoxO1 is one of the four FoxO isoforms of transcriptional factors and is highly expressed in insulin-responsive tissues including pancreas, liver, skeletal muscle and adipose tissue [74,75]. In all these tissues FoxO1 orchestrates the transcriptional cascades regulating glucose metabolism. During fasting, FoxO1 promotes adaptation by inducing gluconeogenesis in the liver and a transition from carbohydrate oxidation to lipid oxidation in the muscle. In the pancreas, FoxO1

inactivation is required for β-cell proliferation. Insulin suppresses FoxO1 activity through activation of PI3K / AKT signalling pathway. Activated AKT (also known as protein kinase B) phosphorylates FoxO1 at three highly conserved phosphorylation sites resulting in its nuclear exclusion and thus inhibition of transcription [75].

The hypothalamic mTOR (mammalian target of rapamycin) plays a role in cellular energetics by inducing numerous anabolic protein processes and lipid synthesis and it signals suppression of food intake. AMP-activated protein kinase (AMPK) is a serine/threonine kinase which is also a nutrient /energy sensor whose activity is coupled to the energy status of the cells. AMPK is activated by increased AMP/ATP ratio that occurs during fasting [76]. AMPK is involved in the regulation of numerous biochemical pathways to turn off anabolism including fatty acid degradation. AMPK is an important regulator of energy homeostasis and is stimulated by a decrease in cellular energy status, nutrient and oxygen deprivation and increased energy expenditure. Activation of AMPK results in increased expression of Nampt and supply of NAD^+ to support SIRT1 activity [77,78]. AMPK might phosphorylate SIRT1, disrupting the interaction with its negative regulator DBC1 (Deleted in Breast Cancer1, also known as KIAA1967).

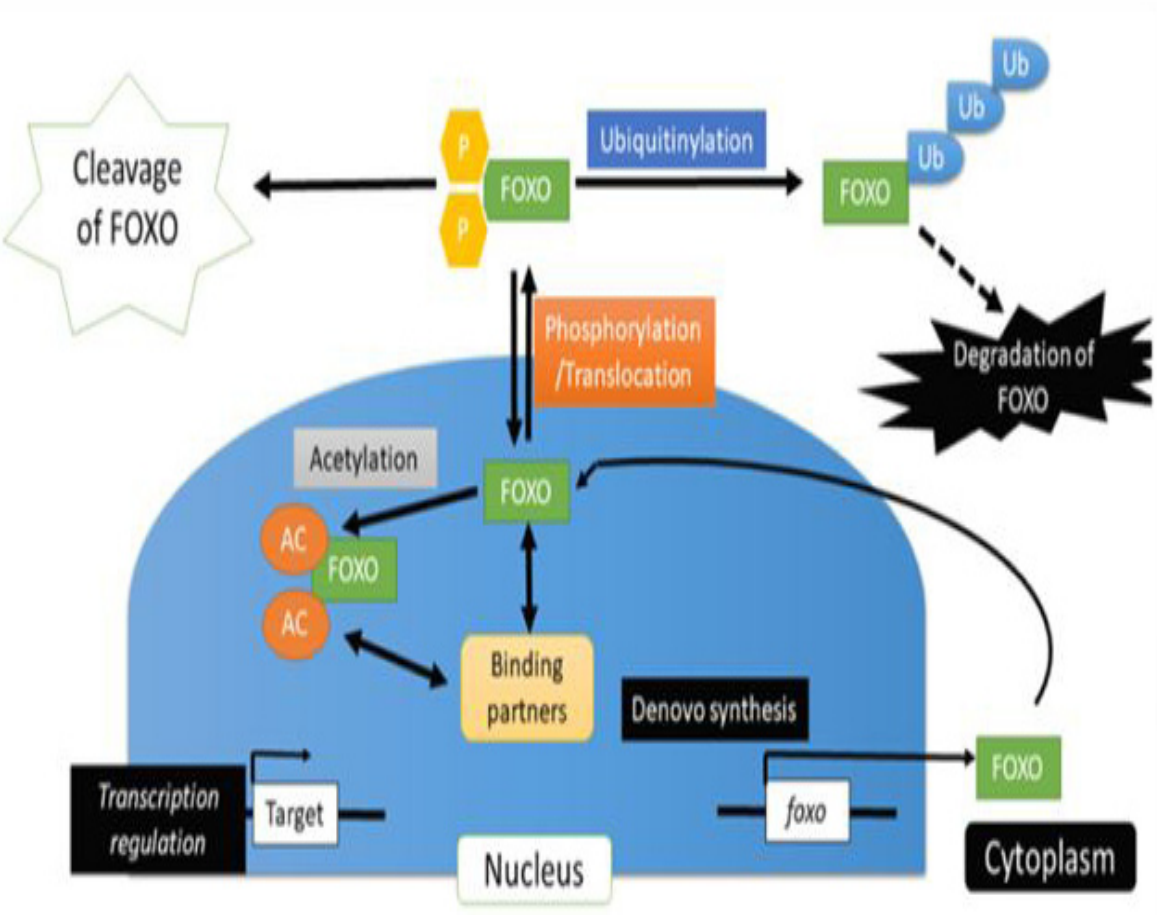


Figure 4: Mechanism regulating FOXO transcription factors. Acetylation of FOXO transcription factor is retained in the nucelus, wheresas, phosphorylation excludes the FOXO from nucleus to cytoplasm. ubiquitin dependent degradation is an irreversible step. Ub, ubiquitin; p, phosphate group; Ac, acetyl group.

3.1. Lipid metabolism

In mammals, most energy is stored as fat in adipose tissues. White adipose tissue (WAT) is the main "storage site" of excess energy, primarily in the form of triglycerides. During fasting, WAT releases fatty acids that are used as fuel by other organs [21,79]. In addition, a functionally and morphologically distinct adipocyte with dense mitochondria was found in Brown adipose tissue (BAT). Brown adipose tissue dissipates energy as heat (non-shivering thermogenesis). Brown adipocytes uncouple mitochondrial electron transport from ATP synthesis to a greater extent permeating the inner mitochondrial membrane to allow inter-membrane proton to leak back into the mitochondrial matrix, primarily through uncoupling protein-1 (UPC1) [80,81]. One of the critical regulators of fat storage in WAT is the nuclear peroxisome proliferating-activated receptor- γ (PPAR γ), whose activity promotes adipocyte differentiation and lipid anabolism. One of the suggested mechanism for loss of SIRT1 in obese animals is suggested by the finding that a high-fat diet in mice triggers cleavage of SIRT1 in WAT by caspase1 of the inflammasome [63]. The gain of function of NAD-dependent deacetylase SIRT1 or loss of function of its endogenous inhibitor DBC1 promotes "browning" of WAT by deacetylating PPAR- γ on K268 and K293 [82].

During fasting SIRT1 associated with PPAR γ and promoted the binding of the nuclear receptor corepressor1 (NCoR1). SIRT1 directly deacetylates PPAR γ which allows the recruitment of PRDM 16 to drive browning of white fat. Brown adipocytes are characterized by the expression of mitochondrial uncoupling protein 1 (UCP1), which allows dissipation of energy as heat for thermogenesis. The binding of PGC-1α to PPARγ promotes brown adipocyte-like features in white adipocytes through an upregulation of brown-adipocyte specific genes, such as UCP1, and a down-regulation of white-adipocyte specific genes. SIRT1 activation might prevent excessive accumulation of fat in adipocytes by boosting fat consumption and enhancing thermogenic function.

SIRT1 inhibits lipogenic gene expression by acting as a negative regulator of the Sterol Regulatory Element Binding Protein (SREBP)-1c. SREBP-1c is a transcription factor that promotes the expression of lipogenic and cholesterogenic genes in order to facilitate fat storage [83]. The deacetylation of SREBP-1c by SIRT1 renders the protein susceptible to ubiquitin-mediated degradation [84]. Hence, SIRT1 activation leads to decreased SREBP-1c protein levels. This results in decreased occupancy of SREBP-1c on the promoter of lipogenic genes and a concomitant reduction in their expression levels. Deacetylation of SREBP1 by SIRT1 results in targeting of SREBP1 for proteasomal degradation, which inhibits the expression of lipogenic and cholesterol synthesis genes [85]. SIRT1 also promotes reverse cholesterol transport by deacetylating and activating the LXRα nuclear receptor, and promotes the cholesterol catabolic pathway by deacetylating and activating BAR, bile acid receptor; LXRα, oxysterols receptor; SREBP-1, sterol regulatory element binding protein 1 [86,87]

Indeed, SIRT1 has been shown to modulate cholesterol metabolism *in vivo* through positive regulation of the Farnesoid X receptor (FXR) and the Liver X receptors (LRX), LXRα and LXRβ. In FXR, SIRT1 can directly deacetylate Lys 157 and Lys 217. Down-regulation of hepatic SIRT1 increases FXR acetylation, which inhibits its heterodimerization with the Retinoid X receptor (RXR) α and therefore, its transcriptional activity [88,89]. Hence, SIRT1 deletion in the liver is sufficient to downregulate FXR-related transcriptional programs and lead to the formation of cholesterol gallstones [23,86]. In LXR, ligand binding promotes the interaction with SIRT1 and subsequent deacetylation on Lys 432 (LXR α) and on Lys 433 (LXR β), promoting their activation.

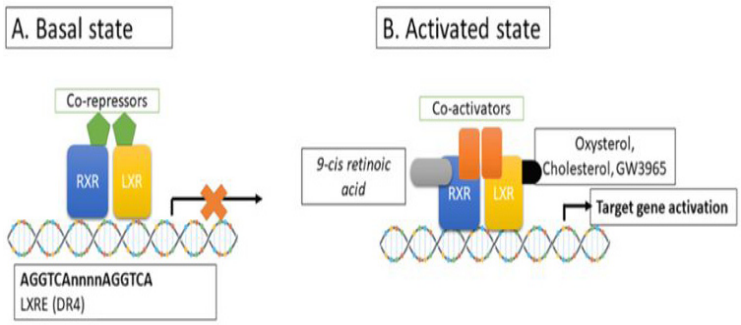


Figure 5: the liver X receptors (LXRs) and retinoid X receptors (RXRs) from heterodimer and blind to direct repeat 4 (DR4) response element in regulatory regions of their target genes. A. In basal state co-repressors are bound to heterodimer, which inhibits transcription of target genes. B. when RXR and LXR are activated by biniding of retinoic acid and oxysterols respectively the co-activators are recruited and initiate transcription of target gene.

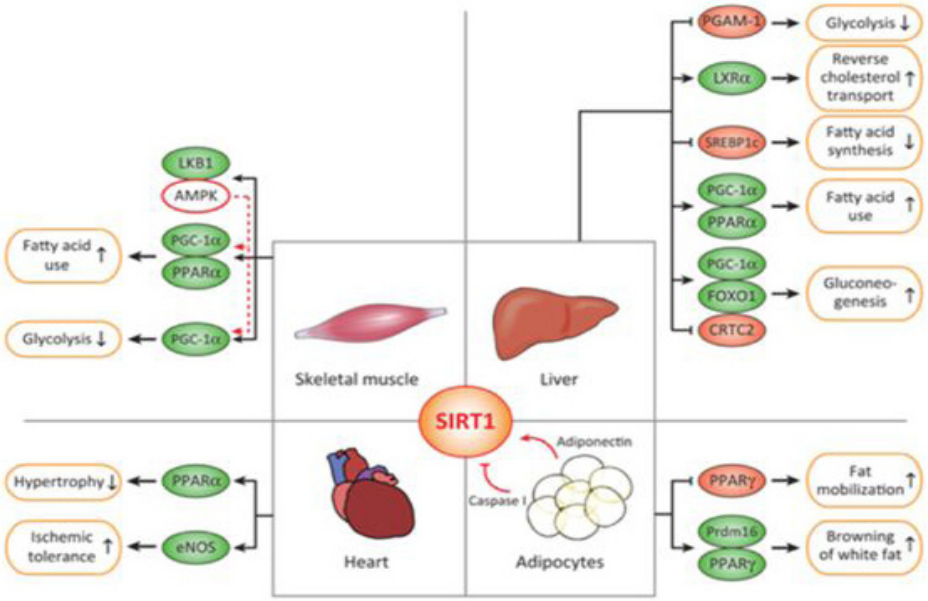
Liver X receptor (LXR) forms an obligate heterodimer with retinoid X receptor (RXR) that binds to a DR4 (direct repeat spaced by four nucleotides) LXRE (LXR response element) in the regulatory regions of target genes, thereby repressing gene expression [86,90]. Following ligand binding to LXR or RXR, the heterodimer changes conformation, this leads to the release of corepressors and the recruitment of coactivators. This results in the transcription of target genes. Similarly, farnesoid X receptor (FXR) forms a heterodimer with RXR and binds to the FXR response element (FXRE), which is typically an inverse repeat spaced by one nucleotide (IR1), in its target genes to induce gene expression. Knockdown of SIRT1 in the liver also leads to decreased expression of CYP7A1, a bonafide LXR target. LXRs are also a potent inducer of lipid anabolism by increasing SREBP-1c activity. However, SIRT1 can deacetylate SREBP-1c resulting in proteasomal degradation [83]. The liver X receptor (LXR) controls both sterol regulatory element–binding protein (SREBP)-2 and SREBP-1c. SREBP-2 regulates the genes involved in cholesterol synthesis, while SREBP-1c stimulates the production of genes involved with the lipogenic enzymes. Inhibition of LXR would result in a decrease in both cholesterol and triglyceride synthesis [90].

Therefore, SIRT1 activation might promote the beneficial effects of LXR activity on cholesterol homeostasis while preventing the detrimental effects on lipid anabolism by deacetylating SREBP-1c. Altogether, SIRT1 overexpression improves cholesterol metabolism and prevents hepatic steatosis, while SIRT1 deletion in the liver favours lipid accumulation.

3.2. Hepatic glucose metabolism

SIRT1 participate in the energy regulation in metabolically active tissues such as liver and muscle. Gluconeogenesis during starvation can be triggered by the hormone glucagon, which induces dephosphorylation and nuclear translocation of the transducer of regulated CREB activity2 (TORC2) [91]. TORC2 activates DNA transcription factor Cre binding protein (CREB), which then induces the expression of the transcription coactivator PGC-1α. PGC-1α complexes with transcription factor PPARα, FOXO1, glucocorticoid receptor (GR) and hepatocyte nuclear factor 4α (HFN4 α) [92]. These, in turn, induce the transcription of key gluconeogenic genes encoding the rate-limiting enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [93].

In the fed state, PCG1α is phosphorylated by insulin-stimulated Akt kinase activity thereby reducing the transcription of gluconeogenic genes. At low levels of cellular ATP, in liver, AMPK inhibits gluconeogenesis by phosphorylating TORC2. AMPK activation is a key pharmacological target for developing drugs for type-II diabetes which work to suppress hepatic glucose output such as metformin [92,94]. Besides phosphorylation, PGC1 α is also regulated by acetylation and deacetylation. PGC1α is activated by SIRT1-mediated deacetylation during prolonged fasting which results in fatty acid oxidation and improved glucose homeostasis. This regulation of PGC1α activity is not dependent on glucagon or glucocorticoids but on the levels of metabolic intermediates like pyruvate and NAD. SIRT1 also affect gluconeogenic activity through PGC1α in an indirect manner. In the liver, STAT3 is known to suppress the expression of PGC1α and gluconeogenic activity gene expression. Regulation of gluconeogenesis by STAT3 could be linked to nutritional status via SIRT1, which can directly deacetylate and attenuate the anti-gluconeogenic transcriptional activity of STAT3 [25,92,95]. SIRT1 also deacetylates and activates transcriptional factor Foxo1, resulting in increased gluconeogenesis [96].



work in part through chromatin modification and epigenetic control of gene expression [69]. The autonomous and self-sustainable nature of circadian timing is largely dependent on the molecular circadian clockwork. Cells modify their biochemical activities depending upon the food intake and energy expenditure. This can be achieved by fine tuning the central biochemical pathway by various metabolic targets- mainly rate-limiting enzymes. The expression of these metabolic targets is modulated by the chromatin conformation and the accessibility of transcription factors that encode these enzymes. In turn, these chromatin conformations are controlled by histone acetylation, the levels of which are controlled by the concerted enzymatic activity of HATs and Histone deacetylases [102]. The circadian rhythms are directly dictated by the food availability, which is an external cue that entrains peripheral clocks. Further, several stimuli, including insulin, glucose, the glucocorticoid hormone analogue dexamethasone, forskolin and phorbol ester can trigger circadian expression *in vitro* by activating signalling cascades [103].

The molecular mechanism underlying the mammalian circadian clock consists of a transcriptional-translation feedback loop involving CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like1), which recognize E-box elements. CLOCK, a transcription factor is crucial for circadian function, has intrinsic histone acetyl transferase (HAT) activity [102]. The CLOCK/BMAL1 heterodimer activates the transcription of the period (Per1, 2 and 3) and cryptochrome (Cry1 and 2) leading to the subsequent repression of CLOCK/BMAL1 activity. Indeed, the cellular DNA-binding activity of CLOCK/BMAL1 is strongly influenced by the ratio of reduced to oxidized NAD cofactors. SIRT1 is a component of CLOCK/BMAL1 transcription complexes and affects the expression of clock genes [104]. SIRT1 interacts with CLOCK-BMAL1 to control the amplitude and extent of circadian clock-controlled gene expression through deacetylation of PER2 and BMAL1. Circadian regulation of SIRT1 activity is due to circadian oscillations of the cellular NAD^+ levels. It has been found that Nampt is a direct transcriptional target of CLOCK-BMAL1. Therefore, the feedback loop in the circadian clock that involves CLOCK-BMAL1, Nampt, NAD^+ and SIRT1 provide an important connection between physiological rhythm and cellular metabolism.

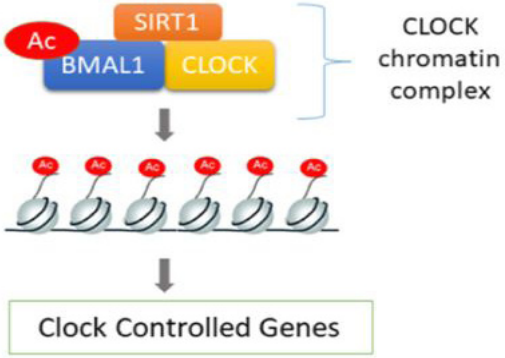


Figure 7: CLOCK is associated in a nuclear complex with BMAL1 and SIRT1, an HDAC that is directly regulated by cellular metabolism. This comples is recruited to circadian gene promoters in a cyclic manner and is thought to be responsible for the circadian acetylation of histone H3 at K9 and K14. (Adopted from "Mammalian circadian clock and metabolism-the epigenetic link" Bellet & Sassone-Corsi 2010)

6. Summary

Sirtuins are a conserved protein with NAD^+- dependent deacetylase activity and their functions are intrinsically linked to cellular metabolism. Sirtuins in lower organisms prolong the life and regulate the ageing process against metabolic stresses. The versatile functions of seven sirtuins are sustained by the distribution within the cellular compartment and tissues allowing cells to sense nutrient levels. SIRT1 plays a critical role in maintaining metabolic homeostasis with systemic effects via the hypothalamus and helps to deliver the benefits of calorie restriction. Sirtuins have numerous targets in many tissues such as liver, muscle, adipose tissue etc. which perceive the nutrient levels and respond through deacetylation of histones, key transcription factors and metabolic enzymes. SIRT1 networks with CLOCK-BMAL1 to regulate the physiological rhythm and cellular metabolism.

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