CURRENT APPROACHES IN SCIENCE OF LIFE

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(The Licence Number of Publicator: 2014/31220)
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USA: +1 631 685 0 853
E mail: iksadyayinevi@gmail.com
www.iksadyayinevi.com

ISBN: 978-605-74646-3-7
Cover Design: İbrahim KAYA
February / 2021
Ankara / Turkey
Size = 16x24 cm

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PREFACE

I am proud to present the book named "CURRENT APPROACHES IN SCIENCE OF LIFE " to scientists and researchers working in all fields. It is an extraordinary experience to observe the different mechanisms and structures of all living things that we constantly interact with. While examining this book, which deals with different structures in many different living groups, I am sure that you will gain different perspectives on your current knowledge. This book, which consists of 5 chapters prepared by different researchers who are experts in their field, contains very important information on various subjects from humans to plants and insects. In science where multidisciplinary work always leads to more productive results, I hope that there will be many scientists and young people interested in science who will benefit from this book. I would like to thank the staff of ISPEC publishing house for their devoted work, who brought together valuable scientists and scientists for the creation of this book.

With my regards

Dr. Betül AYDIN

CHAPTER 1

STEM CELLS: AN OVERVIEW AND POTENTIAL THERAPEUTIC APPLICATIONS

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INTRODUCTION

Stem Cells - A Short Definition

Stem cells are unspecialized cells that have self-renewal capacity and differentiation ability into a diverse range of specialized cell types of an organism through mitotic cell division. In general, they share the following characteristic features; remarkable capacity for self-renewal, differentiation, and trans differentiation (plasticity). Self-renewal capacity is remarkably important since they need to keep generating undifferentiated daughter cells (Gonzalez & Bernad, 2012; Shenghui et al., 2009). Differentiation ability provides development, maintenance, or repair. Based on their differentiation capacities, stem cells are classified into four main groups including totipotent, pluripotent, multipotent, and unipotent. Totipotent stem cells such as fertilized egg (zygote) and derived from the early stage of blastomere are capable of differentiation into all cell types (Gupta et al., 2017). Pluripotent stem cells can differentiate into the cells from all three germ layers (endoderm, mesoderm, and ectoderm). Embryonic stem cells (ESCs) which are isolated from the inner cell mass of the blastocyst and induced pluripotent stem cells (iPSCs) which are genetically manipulated adult somatic cells by transduction with specific transcription factors are types of pluripotent stem cells (Takahashi & Yamanaka, 2006). Multipotent stem cells also called adult stem cells (ASCs) can only differentiate into a closely related family of cells in organs or tissue. ASCs are divided into sub-populations as Mesenchymal stem cells (MSCs) that are isolated from

several parts of the body, Hematopoietic stem cells (HSCs) that are generally found in the bone marrow and can transform into all types of blood cells, and neural stem cells (Robinson, 2001; Sobhani et al., 2017). ASCs display remarkable roles for homeostasis and regeneration. Unipotent stem cells or monopotent cells have the most limited differentiation capacity among all the stem cell types, thus they can only turn into one specific cell type.

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) which are a type of ASCs and also known as mesenchymal stromal cells, multipotential stromal cells, and mesenchymal progenitor cells, were initially isolated from bone marrow mononuclear cells as colony-forming unit fibroblasts (CFU-Fs) by Friedenstein and colleagues (Friedenstein et al., 1970; Pittenger et al., 2019). MSCs are easily isolated, cultured, and expanded *in vitro* and due to their multipotency, they can differentiate into limited cell types such as adipocytes, osteocytes, and chondrocytes (Dominici et al., 2006). Moreover, MSCs can also differentiate into myocytes, astrocytes, endothelial and neuronal cells under convenient conditions and start to express characteristic genes of cell types (Azizi et al., 1998; Pittenger et al., 1999; Wakitani et al., 1995; Woodbury et al., 2000). They characteristically exhibit fibroblast cell-like morphology and plastic-adherent under *in vitro* culture conditions and commonly express some surface markers such as CD29, CD44, CD73, CD90, and CD105 and lack expression of hematopoietic surface markers such as CD45, CD34, CD14 or CD11b, CD79a, CD19, and HLA class

II (Conget & Minguell, 1999; Galmiche et al., 1993; Haynesworth et al., 1992; Le Blanc et al., 2003; Pittenger et al., 1999; Sordi et al., 2005). MCSs are present throughout the body. Although bone marrow is considered as a rich source of MSCs (Haynesworth et al., 1992; Pittenger et al., 1999), they have been isolated from adipose tissue (Eirin et al., 2012) , placenta (In't Anker et al., 2004), umbilical cord (Romanov et al., 2003), dental tissue (G.-J. Huang et al., 2009), muscle connective tissue/synovial membrane (Hermida-Gómez et al., 2011), amniotic fluid (int Anker et al., 2003).

MSCs play beneficial roles in tissue repair, maintenance, and regeneration, neuroprotection, and immunosuppression (Pittenger et al., 2019; Samsonraj et al., 2017). MSCs promote tissue repair via two different ways (i) producing several growth factors, chemokines, and cytokines (ii) differentiating into related cell types (Prockop, 2007; Samsonraj et al., 2017). MSCs provide an inductive microenvironment for damaged tissues and promote regeneration by secreting bioactive factors (Caplan, 2007; Caplan & Dennis, 2006). Previous studies have shown that MSCs-derived secretomes exhibit anti-tumoral, anti-microbial, pro-angiogenic, and immunoregulatory activities (Alcayaga-Miranda et al., 2017; Vizoso et al., 2019; B. Zhang et al., 2015). MSCs-derived secretomes consist of various protein compositions and these different compositions decide the fate of their environment (Tachida et al., 2015). For example, the secretomes of MSCs isolated from adipose tissue enhance adipogenic properties while the secretomes of Wharton's jelly-derived MSCs excrete high amounts of growth factor and pro-inflammatory proteins

(Amable et al., 2014). Besides, MSC derived extracellular vesicles (MSCs-derived EVs) such as exosomes and microvesicles may contain cytokines, growth factors, and other bioactive factors that can act in a paracrine manner (Phinney & Pittenger, 2017).

MSCs play crucial roles in the maintenance of tissue homeostasis and enhancement of healing in damaged tissues. Their self-renewal capacities have a great impact on achieving tissue homeostasis by controlling cell turnover and repair. Additionally, MSCs can provide homeostasis through the promotion of proliferation, angiogenesis, differentiation, and vasculogenesis (Vizoso et al., 2019). Unfortunately, the enormous potential and pool of the MSC decrease with aging. Researchers have indicated that MSCs-derived from old cells have less immune-modulatory properties when compared to MSCs derived from young cells. Deficiency and depletion of MSCs may cause dysfunction in several organs and serious diseases such as diabetes, lupus, psoriasis, and rheumatoid arthritis (Vizoso et al., 2019).

Hematopoietic Stem Cells (HSCs)

Hematopoietic Stem Cells (HSCs) are multipotent adult stem cells that play pivotal roles in the production of blood and immune cells throughout the lifetime (X. Huang et al., 2007). HSCs mainly produce all blood cell types such as red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages, and platelets (Wilson & Trumpp, 2006).

Like MSCs, HSCs can also differentiate into various cell types such as neuronal cells (Hao et al., 2003) and liver cells (Lagasse et al., 2000).

HSCs can be isolated from different sources of the body such as bone marrow, mobilized peripheral blood, umbilical cord blood, and fetal liver. Bone marrow is the main source of HSCs and they reside in the specific niche of relative hypoxia in the endosteum (Morrison & Scadden, 2014). In adults, HSCs may be released into peripheral blood that makes the mobilized peripheral blood (Karpova et al., 2019). HSCs are highly found in umbilical cord blood and the placenta are significant sources of the fetal hematopoietic system. One of the most important characteristics of stem cells that originated from the umbilical cord blood is their potential for pluripotency. However, the components of the fetal hematopoietic system are discarded after birth (Dzierzak & Robin, 2010).

The tissue and organ homeostasis is mediated by tissue-specific stem cells and progenitor cells (Bryder et al., 2006). HSCs are primarily responsible for maintaining, renewal, and repairing immune and hematopoietic system cells (Masson et al., 2004). HSCs regulate the production of blood components for different purposes such as regulation of clotting, oxygen carriage, and immune system functions (Ng & Alexander, 2017). To sustain the homeostasis of the hematopoietic system, HSCs continually produce new blood cells and regulate programmed cell death known as apoptosis (Masson et al., 2004). Thereby, transplantation of HSCs is used for the treatment of

blood and immune systems related diseases such as leukemia (Weissman, 2000). HSCs are not just responsible for forming blood cells. Several studies have shown that HSCs may carry out regeneration of different tissues rather than blood tissues under appropriate conditions (Krause et al., 2001; S.-G. Lee et al., 2015). Moreover, HSCs can play important roles in tissue repairing and replenishing the cells that are lost after injury (Bryder et al., 2006).

Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are directly generated from somatic cells by nuclear reprogramming to behave like pluripotent stem cells. After the induction of nuclear reprogramming, iPSCs show characteristic features similar to ESCs such as cell morphology, surface antigens, telomerase activity, and transcriptional and epigenetic patterns (Gupta et al., 2017; Takahashi & Yamanaka, 2006).

Yamanaka et al. identified four transcription factors octamer-binding transcription factor (Oct) 3/4, SRY box-containing gene 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Myc among many candidates that enable the induction of pluripotency and cellular behaviors similar to ESCs by using retrovirus transduction. In 2006, Yamanaka and Takahashi described that mouse fibroblast cells turn into embryonic stem cell-like state by using Oct3/4, Sox2, Klf4, and c-Myc (Takahashi & Yamanaka, 2006). This method successfully transformed mouse and human somatic cells into iPSCs and granted the Nobel prize in Physiology or Medicine (2012) (Takahashi et al.,

2007; Takahashi & Yamanaka, 2006). This breakthrough of iPSC-technology has overcome ethical concerns and immune rejection regarding the use of ESCs (Rao et al., 2013).

Yu et al. also succeeded to reprogram human somatic cells using slightly different transcription factors (Oct4, Sox2, Nanog, and Lin28) (Yu et al., 2007). They preferred Lin28 as a useful alternative to c-myc in nuclear reprogramming, due to the possibility of retrovirus reactivation and the risk of tumor growth. However, the viral transduction method for nuclear reprogramming limits the use of iPSCs since this method has low efficiency and mutation induction possibility during viral integration (Takahashi et al., 2007; Yu et al., 2007). Instead of viral integration, many alternative techniques being used to induce pluripotency such as cDNA containing expression plasmids (Stadtfeld et al., 2008), recombinant reprogramming proteins (D. Kim et al., 2009; Zhou et al., 2009), different RNA molecules (Judson et al., 2009; Warren et al., 2010), or using small molecule-compounds (Hou et al., 2013; Huangfu et al., 2008; Shi et al., 2008).

Therapeutic Applications of MSCs

MSCs are ideal candidates for regenerative medicine due to their unique properties. MSCs which have robust proliferative, multilinear differentiation, and immunotolerance capacity, are obtained from a patient with easy surgical procedure. MSCs have various surface markers with a special distribution that provides unique immune

tolerant properties. The limited lifespan and senescence of MSCs are a double-edged sword from the viewpoint of clinical use (H. J. Kim & Park, 2017). MSCs do not form malignant tissue after transplantation, while they lose their critical properties for regeneration such as proliferation, differentiation, migration, and immunomodulatory. In addition to MSCs, their secreted molecules such as growth factors, cytokines, and exosomes also provide a regenerative potential (Marote et al., 2016). Many preclinical and clinical studies have investigated the effects of MSCs–based therapy in regenerative medicine. The preclinical and clinical studies have proven the safety and efficacy of the MSCs-based therapy (J. Wang et al., 2020).

Adipose-derived stem cells (ADSCs) -a type of MSC and derived from human lipoaspirate- were used in different animal models. In a Spraque Dawley rat model of Achilles tendon injury, Lee et al. demonstrated that ADSCs provided morphological and biomechanical recovery and overexpression of the human specific-genes including type 1 collagen and tenascin-1. Also, it has been observed that ADSCs tried to help the therapeutic healing process by secreting proteins (S. Y. Lee et al., 2017). According to another study with F344/NSIc rats having tendinopathy due to injection of collagenase in their Achilles tendon, administrations of ADSCs have been shown to ameliorate tendon regeneration and tendinopathy-related pathological problems (Oshita et al., 2016). ADSCs were also used for the treatment of collagenase-induced rotator cuff injury in a rat model and they have been shown to improve fiber arrangement, tendon organization and

decrease inflammatory cells (Chen et al., 2015). Moreover, Toghraie et al. showed that ADSCs were used in a model of osteoarthritis (OA) in the rabbit knee and displayed positive effects for delaying the progression of OA changes such as cartilage thinning, osteophyte formation, and subchondral sclerosis in the joint (Toghraie et al., 2011). In a previous study, ADSCs were labeled and applied to knee OA in New Zealand rabbits. After 3 and 20 days, they were observed in the synovial membrane and medial meniscus but not in cartilage tissue. However, ADSCs application has been shown to significantly decrease the progression of OA and increase cartilage thickness (Desando et al., 2013). Moreover, many clinical studies are using ADSCs for the therapy of orthopedic defects. The application of ADSCs is an innovative, useful, and effective treatment options without causing adverse effects. ADSCs lead to the improvement of clinical/functional scores and the quality of life and the reduction of pain (Kunze et al., 2020).

The nervous system with limited regenerative capacity plays a vital role in the body. Therefore, occurring any dysfunctionalities of this system cause fatal consequences. There is an urgent need to develop new therapeutic strategies. There are many promising approaches such as MSC derived EVs, for the treatment of injuries/damages in the nervous system (Tsiapalis & O'Driscoll, 2020). Recent studies have shown that rat MSCs, rat ADSCs, and human umbilical cord MSCs-derived EVs promote peripheral nerve recovery in rat models (Bucan et al., 2019; Ma et al., 2017, 2019). Zhang et al. demonstrated that rat

MSCs-derived EVs increased the number of mature/immature neurons and endothelial cells to promote the recovery of brain function using after traumatic brain injury in rats (Y. Zhang et al., 2015). In a mouse model of multiple sclerosis which is a serious nervous system-related disease, Clark et al. demonstrated that human placenta MSCs-derived EVs induce myelination in the spinal cord and ameliorate motor function outcomes (Clark et al., 2019). Moreover, MSCs-derived EVs have been used for Alzheimer's treatment in a mouse model, positive outcomes such as induction of neurogenesis and recovery of the cognitive function were obtained (Reza-Zaldivar et al., 2019).

Several studies have focused on the cardiac system due to insufficient endogenous myocardial repair mechanism in the case of any damage. MSC-based cell therapy is a promising approach for the treatment of cardiac disease. Numerous studies have investigated the therapeutic effects of cell-based therapy using MSCs for cardiac disease models in mice (M. Cai et al., 2016; Ghadrdoost et al., 2018; Peng et al., 2019; Salim et al., 2018; Sun et al., 2016). As a cell-free approach, MSC-derived EVs have been used to help to enhance regeneration. It has been reported that human MSC-derived EVs used to prevent the adverse effects of myocardial ischemia decreased infarct size, and unexpected remodeling while increased myocardial viability (Lai et al., 2010). In some studies, MSCs and MSCs-derived EVs have been used alone and in combination. As a result, combined treatment has been shown to be more beneficial than either application for improving cardiac function (P. Huang et al., 2019).

MSCs are clinically reliable cells for the treatment of bone-related diseases due to their versatile potential. Besides, MSC-derived EVs have been used as a new promising tool for bone regeneration studies. Recent studies have shown that human MSC-derived EVs and modified MSC-derived EVs with dimethyloxaloylglycin triggered angiogenesis stimulation and bone formation in the critical-sized calvarial defect in rats (Liang et al., 2019). In another study, exosomes derived from MSCs have accelerated the fracture healing process in a femur fracture model of CD9−/− mice (Furuta et al., 2016). The focus of many studies on using EVs in regenerative medicine is not only the treatment of bone-related disease but also dental tissue regeneration. Periodontitis is a common inflammatory disease characterized by the destruction of tooth-supporting tissues. If periodontal defects are not treated, may cause irreversible situations such as tooth loss (Zheng et al., 2019). Therefore, several studies have been conducted to investigate the effects of MSCs-based therapy. Chew et al reported that MSC-derived EVs displayed efficient regeneration potential in periodontal tissue in rats. Besides, EVs application enhanced the proliferation and migration capacities of the Periodontal ligament cells (Chew et al., 2019).

Cartilage defects are another challenging issue because of limited regenerative capacity. Functional problems may remain at a high rate after current therapies. Therefore, the researchers try to new candidates such as secreted molecules from MSCs. A recent study conducted by Vonk and colleagues showed that human MSC-derived

EVs triggered cartilage regeneration through the production of important extracellular matrix (ECM) elements to cartilage repair including collagen type II and proteoglycans in chondrocytes obtained from OA patients (Vonk et al., 2018). Similarly, it has been reported that human MSC-derived EVs induce chondrocyte proliferation, inhibit apoptosis, regulate immune response and matrix balance in rat and mouse models with osteochondral defects (Y. Wang et al., 2017; S Zhang et al., 2016; Shipin Zhang et al., 2018, 2019). Besides, human MSCs-derived EVs were also tested for muscle regeneration. Treatment with MSC-derived EVs has been shown to increase myogenic differentiation and vessel formation in *vitro* (Nakamura et al., 2015). In another study, amniotic fluid MSC-derived EVs have demonstrated muscle regeneration due to their protein and miRNA content which achieve to regulate angiogenesis and inflammation (Mellows et al., 2017). There are many studies related to the treatment of acute kidney injuries. Previous studies have shown that human MSCs-derived EVs or mouse MSCs-derived EVs elevated tubular epithelial cell proliferation while reduced cell apoptosis (Gatti et al., 2011; Shen et al., 2016). Different human MSCs-derived EVs were also used to treat chronic kidney disease, which is another common disease. The application of human MSCs-EVs has been shown to reduce disease progression (Jiang et al., 2016).

Therapeutic Applications of HSCs

HSCs localized in bone marrow, are responsible for the production of both the myeloid and lymphoid lineages of blood cells and routinely used in clinical. Hematopoietic cancers or cancers in other organs that are treated with high-dose chemotherapy, and various genetic and acquired diseases such as thalassemia, sickle cell anemia, autoimmune diseases, and aplastic anemia necessitate hematopoietic system transplantation (Domen et al., 2006). There are two types of transplantation; autologous and allogeneic. In autologous transplantation, the patient's own HSCs are used and possible immune system response is prevented however, allogenic transplantation is carried out between two different persons who are not genetically identical. The immune system response is decided with leukocyte antigens expressions produced by the recipient. If there is no genetic matching between donor and recipient, the transplantation fails (Ferrara et al., 1999).

Autologous transplantation is generally preferred for the treatment of multiple myeloma or various forms of non-Hodgkin's lymphoma (NHL). Actually, autologous transplantation has been developed to remedy for the lack of a suitable donor (Majhail et al., 2015). This therapy has also been used in pediatric or germ cell tumors and otoimmün diseases that is originated from lymphocyte depletion (Einhorn et al., 2007). Allogeneic transplantation provides complete immune system changing along with the hematopoietic system and can fight against leukemia. Negatively, even if there is not a complete

match of the major HLA antigens between host and donor, a high rate accord is necessary for transplantation (Navarro et al., 2013; Thomas, 1999). In addition to reconstituting the hematopoietic system, HSCs transplantation is also used for the regeneration of non-hematopoietic tissues such as heart, liver, and neurologic tissue (Müller et al., 2016).

Therapeutic Applications of iPSCs

The Discovery of the iPSCs provides new opportunities to understand more about epigenetic remodeling, reprogramming, differentiation, and cellular development. iPSCs are used in many research/clinical studies including regenerative medicine, drug screening, and disease modeling. Disease modeling is very open to failure due to limited mimicking capacity and efficiency with the existing techniques, therefore using iPSCs which are obtained from the somatic cells of the patients, are more convenient (Park et al., 2008). iPSCs are used in various disease modeling studies such as neurological, hematological, and neuromuscular diseases (Dimos et al., 2008; Filareto et al., 2013; Marchetto et al., 2010; Narsinh et al., 2011; Raya et al., 2009). Disease-causing mutations can be eliminated by using patient-derived iPSCs through gene editing (Hanna et al., 2007; Xu et al., 2009).

Similarly, iPSCs are widely utilized for the regeneration of tissue-specific cells in patients with various degenerative diseases and injuries and can avoid immune rejection when transplanted (Hanna et al., 2007; Li et al., 2012; H. Liu et al., 2011; Nori et al., 2011; Xu et al., 2009). Also, iPSCs have been used in drug discovery studies which imply screening small molecules and chemicals to identify

patient-specific drug and toxicity testing for assessment of safety (Ebert et al., 2009; G. Lee et al., 2009; Sayed et al., 2016). iPSC technology may provide save time and money by reducing the clinical studies of inefficient and toxic drugs (Chuang et al., 2017; Ebert et al., 2009; G. Lee et al., 2009; Sayed et al., 2016).

Various inherited and acquired diseases such as Parkinson's disease, amyotrophic lateral sclerosis, spinal cord injury and, spinal muscular atrophy are successfully treated by using iPSCs. It has been reported that mouse iPSCs differentiated into dopaminergic neurons are used for the treatment of Parkinson's disease in a rat model and they improve the clinical symptoms (Wernig et al., 2008). In another study, human iPSCs cells were transplanted into the rodent heart which was consciously injured and the application has been shown to trigger healing up to a certain degree for a short time (Nelson et al., 2009; J. Zhang et al., 2009). Recent studies have shown that insulin-producing cells, anterior foregut endoderm, intestinal cells, and liver cells were successfully produced from human iPSCs (Wu & Hochedlinger, 2011).

Moreover, researchers have tried to produce retinal cells from murine and human iPSCs. It has been reported that human iPSCs spontaneously differentiated into retinal pigment epithelium cells *in vitro* (Buchholz et al., 2009). In other studies, functional and characteristic cardiomyocytes (CMs) have been created by using both murine and human iPSCs. The produced cells have exhibit similar morphology, marker gene expression, chemical sensitivity, and

electrophysiological features to CMs of the cardiac muscle. The results indicated iPSCs derived from murine improve muscle and endothelial cardiac tissue damage resulting from cardiac infarction (Kuzmenkin et al., 2009; Medvedev et al., 2010; Nelson et al., 2009).

Recently, researchers have frequently preferred iPSCs technologies for the regeneration of various tissues. Alaa et al. showed that iPSCs treat salivary glands' diseases in mice. They increased gene expression of α-amylase for improving salivary gland function, but could not restrain malignant progression of the glands (Alaa El-Din et al., 2019). Besides, researchers have investigated the differentiation capacity of the iPSCs into ameloblast and odontoblast. In a recent study, mouse iPSCs were co-cultured with epithelial and mesenchymal cells and implanted into a sub-renal mouse capsule. Researchers have reported that the transplanted cells formed bone, dental pulp-like, and tooth-like structure after four weeks and caused the expression of the Osteopontin gene in the tooth-like structure. However, the application of the iPSCs without co-culture with epithelial and mesenchymal cells did not exhibit a similar effect (J. Cai et al., 2013; L. Liu et al., 2016; Wen et al., 2012). In another study, iPSCs derived from urine cells were transformed into epithelial cells and combined with dental mesenchyme from the mouse. Thus, iPSCs have been shown to induce the ability the formation of enamel, dentin, and pulp with physical and chemical properties similar to human teeth (J. Cai et al., 2013). Extracellular vesicles released from iPSCs which have unique properties may also be promising agents in regenerative medicine. The

molecules such as protein, mRNA, and miRNA which are transported in extracellular vesicles can be used for the treatment of several diseases, but the tumorigenic abilities of these stimulators should be avoided. The paracrine roles of the cells were proven in the study of salivary gland regeneration which was using iPSCs with embryonic submandibular gland cells (Ono et al., 2015).

CONCLUSION

As reviewed here, stem cells are divided into different groups according to their differentiation capacities and sources. They have various unique properties and play critical roles in the human lifespan. Many characteristic features of the cells are well known and their therapeutic properties are promising for the treatment of several diseases. There is no doubt that usage of stem cell-based therapies are the future of medicinal applications.

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CHAPTER 2

RELATIONSHIP BETWEEN MECHANICAL FORCE, ERYTHROCYTE AND NITRIC OXIDE

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INTRODUCTION

Erythrocytes are highly specialized cells in vertebrae to carry oxygen. Mammalian erythrocytes, in particular, become an extremely simple cell at the end of their maturation process, and with their hemoglobin content approaching ninety percent of their dry weight, they become almost perfect as structures specific to carrying oxygen. As a result of this highly specialized process, erythrocytes have sacrificed their nuclei and organelles to make more room for hemoglobin for their oxygen transport function. This sacrifice also affected the mechanical properties of erythrocytes in such a way that they allow them to change shape in order to pass through the capillary vascular bed, which has diameters smaller than one-third of their own diameters in places (Baskurt & Meiselman, 2003; Chien, 1987). The special biconcave-discoid forms of erythrocytes have also emerged as a result of this advanced degree of specialization. This shape is the most suitable geometric structure for both the deformation ability of erythrocytes and the transfer of respiratory gases between tissues (Chien, 1987).

Although erythrocytes are among the first cells observed by scientists historically, until a few decades ago they were hardly subject to a physiological evaluation beyond the function-structure relationship described in the first paragraph. However, it has been observed that many of the control mechanisms and intracellular communication elements introduced by modern cellular science are also involved in erythrocytes (Minetti & Low, 1997). These findings are often included

in the literature without comment. Observations that came to the agenda in the last quarter of the twentieth century began to suggest that erythrocytes should be evaluated as structures other than just hemoglobin-carrying sacs (Barvitenko, Adragna & Weber, 2005).

1. SIGNAL TRANSDUCTION MECHANISMS OF ERYTHROCYTES

Erythrocytes have many receptor and intracellular signal transduction mechanism elements that have well-defined functions in other mammalian cells (Barvitenko et al., 2005; Minetti & Low, 1997). The purpose of hormone and cytokine receptors expressed on the surface of the erythrocyte membrane and the signal transduction mechanisms to be associated with them in these simple cells has long been a matter of debate. Some scientists have suggested that these biological elements have certain functions in the precursors of erythrocytes in the hematopoietic process, while they do not have any biological functions in mature erythrocytes (Minetti & Low, 1997). As an alternative to this view, the hypothesis that signal transduction mechanisms, which have been shown to be active, may play a role in regulating the functions of erythrocytes is also supported (Barvitenko et al., 2005). Moreover, it is argued that erythrocytes not only regulate their own functions by these biological response mechanisms, but can also affect the functions of other cells (other blood cells and endothelial cells) with which they are in close contact (Allen & Piantadosi, 2006; Barvitenko et al., 2005; Baskurt, Uyuklu &

Meiselman, 2004; Bor-Kucukatay, Wenby, Meiselman & Baskurt, 2003; Ellsworth, Forrester, Ellis & Dietrich, 1995).

Among the intracellular signal transduction mechanisms shown to be active in erythrocytes, calcium-calmodulin dependent pathways (Tanaka, Kadowaki, Lazarides & Sobue, 1991), cyclic AMP (Tuvia, Moses, Gulayev, Levin & Korenstein, 1999), and cyclic GMP (Bor-Kucukatay et al., 2003; Zamir, Tuvia, Riven-Kreitman, Levin & Korenstein, 1992) mediated pathways, phosphoinoside cascade (Davis, Davis, Blas & Gombas, 1995; Shiffer, Rood, Emerson & Kuypers, 1998), their relationship with membrane ion transport. It has been examined to cover (Barvitenko et al., 2005). Detailed studies have revealed that some of these pathways are affected by hemoglobin oxygen saturation (Gibson, Seligman, & Davis, 1946). The relationship of hemoglobin with erythrocyte membrane skeleton proteins is also affected by the oxygenation-deoxygenation cycle (Barvitenko et al., 2005). The information on the subject in the literature is not sufficient for the design of a control system in which the mechanisms summarized above under the main headings are at the center. The most important deficiencies in constructing such a system are related to the effectors that will activate the said intracellular signal transduction mechanisms and the responses to be given at the end of this activation are not clearly defined.

2. EFFECTS OF MECHANICAL FORCES ON ERYTHROCYTES

Erythrocytes are among the cells of the mammalian organism that are most affected by mechanical forces and are forced to change shape. The source of this mechanical effect is mainly fluid shear forces associated with blood flow. In addition, erythrocytes are forced to pass through regions of flow where a wide variety of hydrodynamic conditions prevail, including capillary vessels with diameters down to 3 micrometers in places. The effects of mechanical forces, especially shear forces, on various cells have begun to be understood with their molecular details. Endothelial cells are among the cells in which these mechanisms are best studied (Chien, 2007; Traub & Berk, 1998). In erythrocytes, it has been determined that mechanical forces affect a number of intracellular communication mechanism elements and as a result, cation permeability changes (Baskurt et al., 2004; Larsen, Katz, Roufogalis & Brooks, 1981; Ney, Christopher & Hebbel, 1990; Oonishi, Sakashita & Uyesaka, 1997) and ATP outflow increases (Sprague, Ellsworth, Stephenson, Kleinhenz & Lonigro, 1998; Sprague, Ellsworth, Stephenson & Lonigro, 1996).

Increasing permeability to both monovalent cations and calcium under the influence of mechanical forces may affect the mechanical properties of erythrocytes, especially (Ney et al., 1990). Increased intracellular calcium concentration increases membrane rigidity by changing protein relationships within the membrane skeleton (Clark, Mohandas, Feo, Jacobs & Shohet, 1981; Mohandas & Chasis, 1993).

It has been observed that increased potassium permeability under mechanical stress may also be responsible for changes in erythrocyte deformability (Baskurt et al., 2004). Elsworth et al. investigated the roles of erythrocytes in vasomotor control with a series of experimental studies (Ellsworth et al., 1995; Sprague et al., 1998; Sprague et al., 1996; Sprague, Olearczyk, Stephenson & Lonigro, 2004; Sprague et al., 1995). The results of these studies revealed that ATP outflow increased in erythrocytes under both hypoxic and acidic conditions and mechanical stress. Molecular details of the mechanical stress-ATP release relationship were also investigated in the studies of this group (Sprague et al., 1998). It is known that ATP, which comes out of the erythrocyte, accelerates the synthesis of nitric oxide (NO) and prostaglandin I_2 (PGI_2) by binding to P_{2y} receptors on the endothelial surface and ultimately causes vasodilation (Ellsworth et al., 1995). Interestingly, NO inhibits ATP release by acting through the heteromeric G protein Gi in erythrocytes (Olearczyk, Stephenson, Lonigro & Sprague, 2004). These studies by Elsworth et al. are the most remarkable series regarding the fact that erythrocytes are not only cell-specific to carry oxygen but can also assume a regulatory function related to tissue perfusion and the role of mechanical stress in this relationship.

3. NITRIC OXIDE

Nitric oxide is a molecule with a very short half-life (3-5 seconds) that has an important role in many physiological events. With the demonstration that NO can be produced in human and other

mammalian organisms, the role of NO in physiological and pathological events was understood until 1987, with little known about its reason and metabolism in the body, and the molecule of the year was selected in 1992 (Koshland, 1992; Shinde, Mehta & Goyal, 2000). In 1980, Dr. Robert Furchgott demonstrated that when endothelial muscarinic receptors are activated by acetylcholine, an endothelial-derived relaxing factor (EDRF) is released. Then, in 1987, when Moncada and Ignarro obtained evidence that EDRF could be NO, it was understood that the answer to many questions in science was NO. Furchgott, Ignarro, and Moncada were awarded the Nobel Prize in Medicine and Physiology in 1998 for their work on the role of NO as a signaling molecule in the cardiovascular system (Janero, 2000; Shinde et al., 2000). These studies suddenly changed the fate of NO, which is reported to pollute the atmosphere, pierce the ozone layer and cause acid rain.

NO is a nonpolar, colorless gas (Marin & RodriguezMartinez, 1997). Nitric oxide, a poorly water-soluble, lipophilic molecule, easily passes through the cell membrane. The fact that nitric oxide is an uncharged molecule allows it to easily pass from cell to cell without encountering any barrier since it carries unpaired electrons (Marin & RodriguezMartinez, 1997; Osorio & Recchia, 2000). At the same time, NO can be called a radical molecule because of the unpaired electron it carries. While other free radicals are generally harmful to cells, NO plays a role in very important physiological functions at low concentrations. However, excessive and uncontrolled NO synthesis

can be detrimental to cells (Lowenstein, Dinerman & Snyder, 1994). With these properties, NO gains an ideal physiological messenger molecule. NO shows different effects at acidic, neutral and basic pHs, it is cell-protective when produced in small amounts (nanomolar concentrations), and toxic when it increases to millimolar concentrations. Excessive production or suppression of NO may cause harmful effects.

3.1. Functions of Nitric Oxide

Studies with NO synthesis inhibitors have found that the absence of NO causes an increase in vascular resistance and an increase in blood pressure (Shinde et al., 2000; Vallance & Hingorani, 1999). These studies show that NO has a major homeostatic role in balancing vascular resistance. While some of the nitric oxide formed in the endothelial cell diffuses into the vascular smooth muscle cell, the remaining part passes into the blood and acts on neighboring circulating cells (leukocytes, platelets) (Ignarro, 1993). It can also bind to hemoglobin by diffusing into erythrocytes (Mizutani & Layon, 1996). After NO diffuses into the smooth muscle cell, it increases cGMP and causes smooth muscle relaxation. The increase in cGMP can cause relaxation by 6 different mechanisms. These mechanisms are as follows (Marin & RodriguezMartinez, 1997): Decrease of intracellular Ca^{2+} concentration by activation of Ca^{2+}-ATPase in the sarcoplasmic reticulum; dephosphorylation of myosin light chain; Inhibition of receptor-mediated Ca^{2+} channels in smooth muscle cell membranes; Ca^{2+} transporters, G proteins, receptors that decrease

intracellular Ca^{2+} and phosphorylation of channel proteins; stimulation of Ca^{2+}-ATPase in the membrane; hyperpolarization by increasing potassium passage through potassium channels.

NO has many physiological effects on the gastrointestinal system. It has been shown to play a role in secretion and motility, blood flow, electrolyte and water absorption, mucosal protection, and inflammation (Martin, Jimenez, & Motilva, 2001). NO increases stomach blood flow. NO donors can reduce vagal stimulation or histamine-induced acid secretion (Bogle, Baydoun, Pearson, & Mann, 1996). It suppresses stomach tone and motility, NO donors provide mucosal protection against gastric acid by increasing duodenal mucus secretion. NO functions as a neurotransmitter in the central nervous system, supporting many functions such as memory formation, balance, and olfactory perception (Dawson, Dawson & Snyder, 1992; Ignarro, 1999; Marin & RodriguezMartinez, 1997). In the peripheral nervous system, it affects the nonadrenergic noncholinergic nervous system, contributing to vasodilation, respiratory, gastric and intestinal functions (Tottrup, Glavind & Svane, 1992).

Nitric oxide is the most important paracrine modulator and mediator in the control of renal functions such as renal blood flow, renal autoregulation, glomerular filtration, renin secretion, and salt excretion. Glomerular and medullary microcirculation in the kidney is regulated by endogenous NO (Brezis, Heyman, Dinour, Epstein & Rosen, 1991; Zatz & Denucci, 1991). Tubuloglomerular feedback is partially regulated by NO release (Brezis et al., 1991).

Proinflammatory cytokines increase NO production by stimulating iNOS. It has been reported that NO mediates the effects of macrophages in eliminating intracellular and extracellular pathogens, however, the cytotoxic effect is not the form of NO but peroxynitrite, which is formed by its interaction with O_2-. In addition, inhibitory effects of macrophage-derived NO are seen on lymphocytes as well as on tumor cells (Nathan & Hibbs, 1991). The suppressive effect of activated macrophages on the proliferative response of lymphocytes to mitogens or antigens is partly attributed to NO (Albina, Abate & Henry, 1991; Hoffman, Langrehr, Billiar, Curran & Simmons, 1990; Mills, 1991).

3.2. Nitric Oxide Production

Nitric oxide is synthesized by the nitric oxide synthase (NOS) enzyme. Functionally defined by Bult et al. in 1990, primary structures of NOS have high homology, and there are three different isoforms: 1) neuronal NOS (NOS I or nNOS); 2) inducible NOS (NOS II or iNOS); 3) endothelial NOS (NOS III or eNOS) (Govers & Rabelink, 2001; Groves & Wang, 2000; Pfeilschifter, 2000; Stuehr, 1997). While two isoforms are constitutively expressed (NOS 1, NOS 3) the other isoform is non-structural and can be induced by various cytokines (NOS 2). Nitric oxide is formed by the oxidation of L-arginine by the catalysis of NOS enzymes.

The subcellular localization of these three NOS isoforms also varies. nNOS and iNOS are soluble cytosolic proteins. eNOS is found in the particulate subcellular fraction in the cellular membrane, particularly in the plasmalemmal caveola (Michel & Feron, 1997). Caveolin-1, which is bound to eNOS in endothelial cells; suppresses eNOS activity. The increase in intracellular Ca^{+2} concentration after the activation of the agonist causes the calmodulin to bind to eNOS and enables eNOS to be separated from the caveolin. eNOS-calmodulin complex synthesizes NO until the intracellular Ca^{+2} concentration decreases. As the Ca^{+2} concentration decreases, the inhibitory eNOS-caveolin complex is then formed again (Michel & Feron, 1997).

3.3. Nitric Oxide and Erythrocyte

The relationship of NO with erythrocytes has come to the fore primarily because of the high affinity of hemoglobin to this molecule (Han, Hyduke, Vaughn, Fukuto & Liao, 2002). The role of erythrocytes in the control of vascular smooth muscle tone has been found to be largely related to their effect on local NO levels (Allen & Piantadosi, 2006). The role of consuming NO of erythrocytes has taken its place as the basic element of vascular control models in which this molecule is at the center (Allen & Piantadosi, 2006; Gladwin, 2006; Gladwin, Crawford & Patel, 2004; Vaughn, Kuo & Liao, 1998). Accordingly, this effect of erythrocytes is one of the factors that determine the intensity of NO synthesized in the endothelium to reach vascular smooth muscle. The NO-consuming effect of erythrocytes decreases with the increase of blood flow (Liao,

Hein, Vaughn, Huang & Kuo, 1999). If the hemoglobin is not in the erythrocytes but in the free state in the environment, the NO consumer effect is 600 times higher (Gladwin et al., 2004). As with many other molecular events in erythrocytes (Barvitenko et al., 2005), the oxygen content of hemoglobin plays an important role in NO-related interactions. It is known that the NO affinity of hemoglobin is high at high oxygen partial pressure and low at low values (Stamler et al., 1997).

On the other hand, Jia et al. first suggested in 1995 that NO can react with hemoglobin to form S-nitrosohemoglobin, and NO can be separated from this structure and sent out of the cell under appropriate conditions, in other words, erythrocytes can also be seen as NO carriers (Jia, Bonaventura, Bonaventura, & Stamler, 1996). This concept was later developed by other researchers and it was emphasized that NO synthesized in the lungs can be transported by erythrocytes to other parts of the vascular system (Allen & Piantadosi, 2006; Stamler et al., 1997). In relation to this function, it has been reported that Band 3 (anion exchanger) proteins may play a role in the transport of NO bioactivity outside the erythrocyte (Pawloski, 2005; Pawloski, Hess & Stamler, 2001). It has been reported that erythrocyte-derived NO can also be formed by reduction of NO_2- by deoxyhemoglobin or by xanthine oxidoreductase and nitric oxide synthase in the membrane (Cosby et al., 2003; Rathod, Webb, Lovell, Lecomte & Ahluwalia, 2006). However, it is still discussed in the literature whether this mechanism plays a role in erythrocyte mediated

vasodilation under hypoxic conditions, and it is suggested that the mechanism related to S-nitrosohemoglobin may be more effective in this control (Allen & Piantadosi, 2006).

In addition, erythrocytes have been found to have all molecular mechanisms related to NO production and the cellular effects of this important molecule (Bor-Kucukatay et al., 2003; Kleinbongard et al., 2006). Kleinbongard et al. demonstrated in their extensive studies that a protein with eNOS immunoreactivity is located both in the cytosol of erythrocytes and in the interior of the cell membrane (Kleinbongard et al., 2006). In the same study, the presence of NOS activity in the erythrocyte membranes was also clearly demonstrated (Kleinbongard et al., 2006). It has been observed that NO synthesized by both external sources and erythrocytes affects the erythrocyte mechanical properties (Bor-Kucukatay et al., 2003; Kleinbongard et al., 2006). Evidence has been obtained that NO must be in a certain concentration range in the environment in order to maintain normal mechanical properties (Bor-Kucukatay et al., 2003). Although these studies have shown that erythrocytes have an NO synthesis mechanism whose efficiency can be controlled (Kleinbongard et al., 2006), a valid opinion on what these control mechanisms mean in physiological conditions has not been included in the literature to date.

3.4. Control of NO Synthesis in Erythrocytes

Carvalho et al. reported that acetylcholine and choline increase erythrocyte nitrite and nitrate levels through M1 receptors and

proposed a protein tyrosine kinase-mediated signal transduction mechanism for this effect (Carvalho, Mesquita, Martins-Silva & Saldanha, 2004). Bhattacharya et al. isolated a protein stimulated by insulin from the erythrocyte membrane with NO synthase activity (Bhattacharya, Chakraborty Patra, Basu Roy, Kahn & Sinha, 2001). As far as is known, studies on the regulation of NO-related mechanisms in erythrocytes consist of these. It can be thought that the presence of erythrocytes under the influence of large-scale mechanical forces and reversibly significant shape changes and intracellular communication mechanisms that have been shown to be affected by these changes can control NO-related functions.

In recent years, it has been stated that NO from erythrocyte can have an important place in vasomotor control, especially in hypoxic conditions (Allen & Piantadosi, 2006; Cosby et al., 2003; Pawloski et al., 2004; Stamler et al., 1997). The basis of this view, which will have an important place in the regulation of the circulatory system and can find a therapeutic application area, is the exit of NO to the vascular lumen and the vascular smooth muscle tone, which is carried by the erythrocytes in the microcirculation region. However, this hypothesis is based almost entirely on NO, which is acquired by erythrocytes during lung passage and "stored" in the form of S-Nitrosohemoglobin and carried into the microcirculation (Allen & Piantadosi, 2006; Jia et al., 1996; Pawloski et al., 2004; Stamler et al., 1997). Within the scope of these views, NO synthesis mechanisms (Kleinbongard et al., 2006),

whose presence and functionality in erythrocytes have been clearly demonstrated, are not taken into consideration.

CONCLUSION

In physiological conditions, the microcirculation bed where the erythrocytes are under the highest degree of mechanical stress also corresponds to the circulation region where the oxygen due to hemoglobin is transferred to the tissues, that is, the deoxyhemoglobin content is increased. These hypoxic conditions facilitate the sending of NO produced in the erythrocyte to the outside of the cell via the aforementioned pathways, for example through the anion exchanger (band 3) (Pawloski et al., 2001), instead of being consumed by hemoglobin (Barvitenko et al., 2005). On the other hand, in the microcirculation region where NO of erythrocyte origin is expected to exit into the vessel lumen, erythrocytes undergo significant shape changes under the effect of relatively large mechanical forces. It is possible that these changes stimulate NO synthesis mechanisms and increase intra-erythrocyte NO synthesis through the signal transduction mechanisms discussed above.

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CHAPTER 3

FEMALE REPRODUCTIVE SYSTEM IN INSECTS

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INTRODUCTION

Insects constitute an important group due to the large variety of species and their wide distribution areas in the ecosystem. Therefore, they have been the subject of many studies systematically, morphologically and as reproductive biology (Vitale et al., 2011, 2015; Pappalardo et al., 2016). They can cause significant damages to the cultivated areas by means of the ability to increase their numbers quickly. For this purpose, the biology of the insect must be known well for the studies about pest management. Based on this idea, general information about the female reproductive systems in insects has been given in this section.

1. GENERAL INFORMATION ABOUT THE FEMALE REPRODUCTIVE SYSTEM

The main functions of the female reproductive system in insects are egg production and storage and protection of male spermatozoa until eggs are ready for fertilization. The controlled transfer of spermatozoa to the female reproductive system is arranged by the female insect (Gullan and Cranston, 2014; Amutkan Mutlu, 2020). The main components of the female reproductive system in insects are a pair of ovaries associated with a pair of lateral oviducts. The lateral oviducts are connected to the genital chamber via the common oviduct (Chapman, 2013; Gullan and Cranston, 2014; Amutkan Mutlu, 2020). There are two ectodermal glands opening into the genital chamber. One of them is the spermatheca, which stores the spermatozoa needed

for egg fertilization. The spermatheca is typically a single, pouch-like structure, usually with a diverticulum that forms a tubular spermathecal gland. The secretory cells or glands in the storage part of the spermatheca provide nourishment to the spermatozoa it contains. The second type of ectodermal gland, known as the accessory gland, is a gland that opens to the posterior of the genital chamber, maintaining functions such as producing various substances in the transmission of sperm, fertilization, and protecting the egg (Gillott, 2005; Resh and Cardé, 2009; Gullan and Cranston, 2014; Amutkan Mutlu, 2020).

A pair of ovaries is located in the abdomen on both sides of the digestive system. Each ovary consists of multiple ovarioles, occurs from a terminal filament, a germarium, a vitellarium, and a pedicel (Figure 1A, 1B). Each ovariole is corralled in a peritoneal sheath covered with trachea (Heming, 2018). Terminal filaments from all ovarioles fuse with each other and connect to the body wall (Amutkan Mutlu, 2020). The germarium is the region where the primary oocytes divide by mitosis. The vitellarium is the region where the oocytes mature in a process known as vitellogenesis. Oocytes develop within the ovarioles (Chapman, 2013; Gullan and Cranston, 2014; Amutkan Mutlu, 2020). Each ovary contains a series of developing oocytes, each surrounded by a follicular cell layer. The youngest oocytes occur at the apical side of the germarium region, while the most mature oocytes are located close to the pedicel (Resh and Cardé, 2009; Gullan and Cranston, 2014; Amutkan Mutlu, 2020).

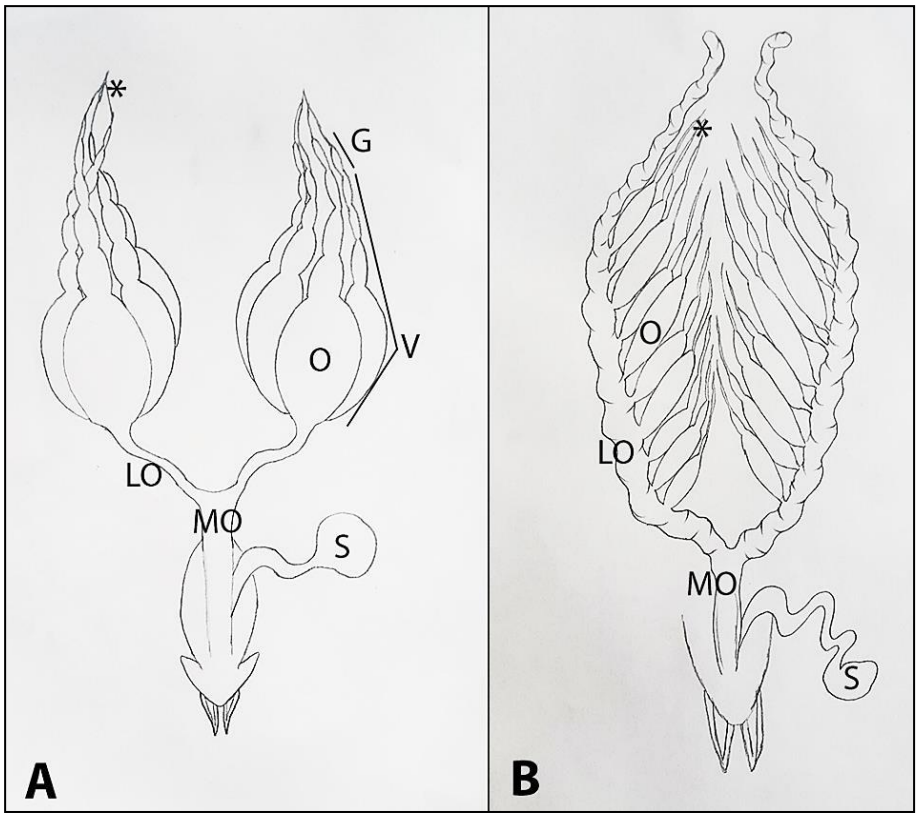

Figure 1: A. Schematic drawing of the female reproductive system of a species in Hemiptera order. B. Schematic drawing of the female reproductive system of a species in Acrididae family (Orthoptera order). *: Terminal filament, G: Germarium, V: Vitellarium, O: Ovariole, LO: Lateral oviduct, MO: Median oviduct, S: Spermatheca. The figure is drawn by Damla Amutkan Mutlu

2. STRUCTURE OF THE OVARY

The number of ovarioles in one ovary differs according to the insect species, insect taxonomic position, or the age and the lifestyle of the insect. As a general rule, adult species have more ovarioles than non-mature species; therefore, immature locusts generally only have four ovarioles per ovary, whereas this number may be more than 100 in adult individuals (Chapman, 2013; Amutkan Mutlu, 2020).

Insects have adapted to produce numerous oocytes within the ovary (Leather and Hardie, 2018). Three different types of ovarioles have been described among insects (Figure 2A, 2B, 2C). One of them is the panoistic type ovariole seen in Thysanura, Paleoptera, Orthoptera, and Siphonaptera orders (Figure 2A). The specialized nutritive cells which are called trophocytes are not found in the panoistic type ovariole. The oocytes are surrounded by a follicular epithelium (Heming, 2018). Therefore, the necessary nutrients are transferred from the hemolymph through the follicle epithelium, and the oocytes are nourished. Each ovariole consists of the terminal filament where the flattened cells are made up, the germarium where the germ cells are undergoing divisions, and the vitellarium where the developmental stage of the oocytes occurs (Leather and Hardie, 2018).

The other two types of ovarioles that are the telotrophic type (Figure 2B) and the polytrophic type ovary (Figure 2C) which are classified within the meroistic type ovariole, contain trophocytes (nurse cells) that contribute to the nourishment of developing oocytes. In telotrophic type ovariole seen in Hemiptera and Coleoptera orders, the trophocytes are located in the germarium and while the oocyte moves from the germarium to the pedicel, the oocyte is nourished through a cytoplasmic channel (cytoplasmic cord). The trophocytes connect to the oocytes by cytoplasmic channel (Gullan and Cranston, 2014). In the polytrophic type ovariole, the trophocytes and the oocytes are lined up one after the other. So, this type of ovariole contains an

alternating succession of the trophocytes and the oocytes (Heming, 2018). This type of ovariole is seen in Dermaptera, Psocoptera, and Phthiraptera orders (Gillott, 2005; Resh and Cardé, 2009; Chapman, 2013; Gullan and Cranston, 2014; Amutkan Mutlu, 2020). Insects belonging to different orders have only one of these three types of ovaries (Figure 2A, 2B, 2C).

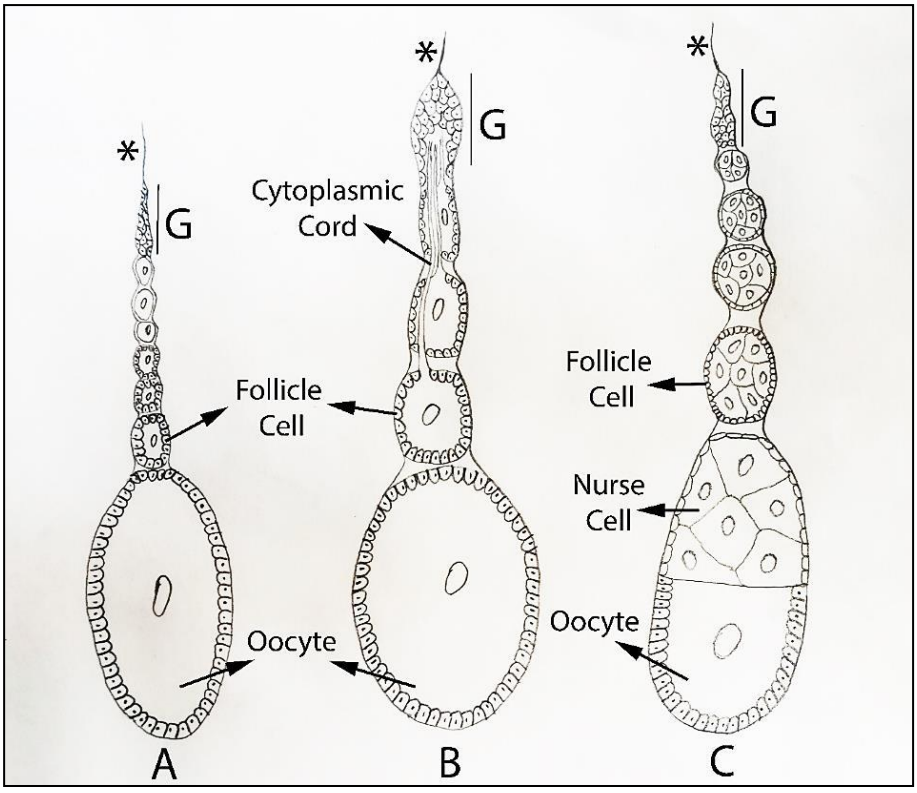

Figure 2: Ovariole types in the female reproduction system of insects. A. Panoistic type ovariole. B. Telotrophic type ovariole. C. Polytrophic type ovariole. *: Terminal filament, G: Germarium. The figure is drawn by Damla Amutkan Mutlu

Each ovary in the female reproductive system varies in number depending on the insect orders (Chapman, 2013; Heming, 2018). It has approximately 150-300 ovarioles in each ovary in *Ecdyonurus*

venosus (Ephemeroptera, Heptageniidae) (Gaino and Rebora, 2003). There are about 3000 ovarioles in *Parthenolecanium pomeranicum* (Hemiptera, Coccidae) (Liu et al., 2014). In *Zorotypus hubbardi* (Zoroptera, Zorotypidae), this number ranges from four to six (Dallai et al., 2012). The number of ovarioles can be changed depending on their families in the species in the Orthoptera order. There are about five to 10 ovarioles in species in the Acrididae family. This number varies between 15-30 in species in the Tettigoniidae family and between 150-170 in species in the Gryllidae family (Leather and Hardie, 2018; Amutkan Mutlu, 2020).

3. OTHER STRUCTURES OF THE FEMALE REPRODUCTIVE SYSTEM

Each ovary is connected to the lateral oviduct, and these two lateral oviducts open to the common or median oviduct, which allows the mature eggs to pass outside (Amutkan Mutlu, 2020). Although the lateral oviduct is usually short (Figure 1A), it is quite long in some species (Figure 1B). In species in Ephemeroptera order, each ovary is attached obliquely to the lateral oviduct. Therefore, they have a very long lateral oviduct (Soldan, 1979; Gaino and Mazzini, 1990). A similar morphology is also stated in *Locusta migratoria* (Orthoptera, Acrididae), *Baeacris punctulatus* (Orthoptera, Acrididae), and *Pseudochorthippus parallelus parallelus* (Orthoptera, Acrididae) (Michel and Terán, 2005; Lange and da Silva, 2007; Lange, 2009; Amutkan Mutlu, 2021).

The spermatheca, which serves for the storage of sperm in female individuals, differs in number between species (Chapman, 2013). There is one spermatheca in *Campodea* sp. (Diplura) (Pascini and Martins, 2017). While there are two spermathecas in *Blaps* sp. (Coleoptera), *Phlebotomus* sp. (Diptera), and *Drosophila melanogaster* (Diptera), there are 10 spermathecas in *Diplatys* sp. (Dermaptera) (Chapman, 2013; Hopkins et al., 2019). It was reported that one spermatheca was found in *B. punctulatus* (Acrididae), *L. migratoria* (Acrididae*), Eupholidoptera chabrieri bimucronata* (Tettigoniidae), and *Uromenus brevicollis trinacriae* (Tettigoniidae) from species in the order Orthoptera (Clark and Lange, 2000; Michel and Terán, 2005; Viscuso et al., 2015; Pascini and Martins, 2017). On the other hand, since females of *Telmatoscopus albipunctatus* (Diptera, Psychodidae) do not have spermatheca, they have to convey the sperm of male individuals up to the lateral oviduct of the female (Burrini and Dallai, 1975; Pascini and Martins, 2017). It has been stated that in the absence of spermatheca, sperm can be stored in analogous sperm-storing organs such as mesodermal pseudospermatheca or other parts of the female reproductive system such as lateral oviducts (Marchini et al., 2010; Pascini and Martins, 2017).

4. OOGENESIS IN INSECTS

One of the evolutionary successes of insects can reproduce a great number of progeny. They can remarkably increase their population. Since most insects reproduce sexually, a process in females called oogenesis happens (Heming, 2018). The germarium region of the ovarioles contains undifferentiated germ cells called oogonia. The oogonia are generally small cells and large nuclei (Raven, 2013). The oogonia start the division process and the amount of the oocytes increases. Those that will form the egg cell from oogonia are called primary oocytes. Primary oocytes undergo two successive divisions. The cells that divide into two cells by the first meiosis are not equal in size. One of them has a large shape and abundant cytoplasm. This is a secondary oocyte. The other one is the first pole cell with a small size. The secondary oocyte also divides unequally with meiosis. Ootid with large cytoplasm and secondary pole cell of small size are formed. As a result of this, four cells are formed by meiosis (Cummings and King, 2005; Raven, 2013; Heming, 2018).

Finally, the secondary oogonia now transform into oocytes, which begin to grow. Generally, the period of development of the oocyte involves three phases: previtellogenesis, vitellogenesis, and choriogenesis (Żelazowska and Fopp-Bayat, 2017; Heming, 2018). In the previtellogenesis stage, the follicular epithelium is single-layered and the nucleus of the oocyte is pretty large (Szymanska et al., 2001). In the vitellogenesis stage, the accumulation of yolk granules occurs. The content of these yolk granules can be carbohydrates, proteins, or

lipids. The ooplasm becomes loaded during this stage. In choriogenesis which is the last stage, egg envelopes on the oocyte surface become formed (Tworzydło and Biliński, 2008).

CONCLUSION

In conclusion, the general information about the female reproductive system in insects is given in this section. It is thought that this information will contribute to further studies about the female reproductive system in insects.

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CHAPTER 4

TYPES OF SECONDARY METABOLITES, THEIR DUTIES AND THEIR BENEFITS

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INTRODUCTION

1. What are Primary and Secondary Metabolite?

As long as plants remain alive, they produce a large number and variety of metabolites. Some of these synthesized organic compounds are used for the plant to continue its vital activities. Other metabolism products that cannot be used accumulate in various organs of the plant as waste material or later as a storage product to fulfill various tasks.

These compounds, which the plant produces for the purpose of creating structural material and providing energy, and that have to be present in its body for a healthy life are called "primary metabolites". These products; organic substances such as nucleotides, amino acids, carbohydrates. Plants, of course, like all other living things, need these products in order to survive. However, for the "secondary metabolites" that plants create as waste or storage products, they do not necessarily have such a primary degree in order to ensure the life continuity of the plants. Despite that; Plants also need secondary metabolites in their life cycle in order to facilitate their life. According to (Gueven and Knorr, 2011), secondary metabolites are not essential for the survival of the plant. However, especially when faced with stress factors such as ultraviolet and herbicides; they are chemical compounds with a complex structure that are beginning to be synthesized as a defense response. However, they also affect the metabolic activities that protect the plant during growth and accompany its development.

Not just against stress factors; plants are known to respond to infections caused by microorganisms and inhibit the growth of competitive plants with these secondary products.

Although it is not directly related to the basic vital functions of the plant; the products synthesized as a result of plant metabolism are called “secondary metabolites” that take part in protecting the plant in many different ways and help the defense system, contribute to the plant's reproduction and support its healthier growth and development. Since secondary metabolites do not affect the survival of the plant primarily; can be called "secondary metabolism products", "secondary by-products" or simply "secondary products".

Which biosynthesis path they have followed is not yet clear to all; with new researches, new information at both cellular and molecular level continues to be obtained. The aim should be to develop uninterrupted production methods of these products (Oskay and Oskay, 2009). For this purpose, using plant tissue culture methods is very advantageous in terms of time and money.

2. Locations of Secondary Metabolites in Plants:

Unlike primary metabolites, secondary metabolites are not found in all plants. Not all types are available in those found. It can be found in limited amounts and spread in plants.

According to (Tanker and Tanker, 1990), secondary metabolites are components that take part in plant defense and occur in special cells. In (avys.omu.edu.tr, 2021), it has been stated that secondary metabolites in many structures from feathers to vacuoles can be found in plants, but not in every cell, but in some special cells.

These secondary metabolites, which are chemically soluble in oil or water, and their locations in the plant (Wink, 2009) are listed below.

2.1. Types of secondary metabolites that dissolve in fat and the plant structures in which they are stored;

Fat Cells; Anthraquinone, terponoid, hypericin.

Resin Channels; Terpenoids, lipophilic flavonoids.

Cuticle; Terpenoids, lipophilic flavonoids, candles.

Feathers; Quinone, monoterpenes, sesquiterpenes.

Latissifers; Quinone, lipophilic flavonoids, diterpenes, polyterpenes.

Plastid membrane; Tetraterpenes, ubiquinone.

2.2. Types of secondary metabolites that dissolve in water and plant structures in which they are stored;

Vacuole; Most of the alkaloids, NPAA, glycosides, saponins, flavonoids, anthocyanins, tannins, amines, cyanogens, glycosinolates.

Cell wall; The tannins.

Latisifer; Some of the alkaloids (papaver, lobelia, chelidonium), NPAA, cyanogens, cardiac glycosides (nerum).

3. What are the Benefits of Secondary Metabolites to Plants?

In the life cycle of high-rise plants starting from the seed state until they form a seed again. They use some means to protect themselves. For example, they reduce their metabolic rate from time to time, especially in cold weather, and they resist adverse conditions by spending little energy until the ambient conditions recover again. In cases where the temperature is high, to prevent excessive water loss; they increase their survival probability with the changes they develop in their various organs, especially leaves.

Besides the ways of protecting plants like these from negative external factors; secondary metabolites also make great contributions to plants for this purpose. Plants with their herbal hormones and other secondary products; both in providing the integrity between their tissues; are also able to protect themselves against negative external factors by staying in contact with other plants.

According to (acikders.ankara.edu.tr, 2021); In protecting the plant, secondary metabolites accompany the plant as well as protective tissues. It has protective functions, especially against plant pathogens. Besides, secondary metabolites play an active role in seed formation by facilitating pollination. In addition, they also contribute to growth and development, especially thanks to some of the terpenes, a variety of secondary metabolites.

In the pollination event, by attracting the insects that will carry the seeds through these by-products; they are known to provide seed propagation.

Plants can respond to stress caused by almost all kinds of environmental changes and environmental conditions; in the case of injury, they have the ability to fight microorganism infections by synthesizing compounds called phytoalexins (Yazgan, 1976). When faced with a disease-causing fungal infection, secondary metabolite varieties called phytoalexins (formed by combining the Latin plant and protective words) are synthesized by plants for defense purposes (Cruickshank, 1963).

According to (Galston and Davies, 1970), they reported that phytoalexins are synthesized in the presence of pathogenic microorganisms. They stated that these substances, which have a lethal effect on fungi, are effective in preventing the development and reproduction of fungi.

4. What are the Benefits of Secondary Metabolites to Humans?

Besides the benefits of secondary metabolites to plants; what they provide to humanity is also undeniable. It is possible to evaluate these benefits in terms of industrial, human health, and food-nutrition. However, as mentioned before, these metabolites can be synthesized in plants in natural conditions in limited quantities. By using plant tissue cultures to increase their quantity; more production of these secondary metabolites is possible. According to (Güven and Gürsul,

2014); the use of secondary metabolites obtained in this way is very important, especially in medicine and pharmacy, in order to contribute to the improvement of health and development of people. In recent years, studies such as the synthesis of these compounds with tissue culture techniques and their production in fermenters have been accelerated.

It is disadvantageous in terms of product amount and time to obtain secondary metabolites that people will benefit from nature by classical methods. However, if it is obtained with tissue cultures, more desired products can be reached in short periods and under controlled conditions, as well as much more economically. In addition, the danger of extinction of some sensitive plant species is almost zero thanks to this method.

Due to the totipotency feature of plants, the target product can be obtained faster by developing it from the cell or tissue through tissue culture techniques (Güven and Gürsul, 2014).

Secondary metabolites are compounds used in many fields, especially in medicine and pharmacy, due to their bioactive properties. There are also uses in the food industry as an additive. They are effective food ingredients and are very valuable as a supplement to nutrients (Güven and Gürsul, 2014).

Secondary metabolites besides contributing to foods for humans; they also provide additives as medicinal active substance, drug, spice, stimulant drink, toxin, and fiber (Alaca and Arslan, 2012). They also

contribute as additives, nutritional supplements, and food components in the field of the food industry (Güven and Gürsul, 2014).

The secondary metabolites that are mostly researched today; terpenoids, phenolic compounds, alkaloids, tannins, saponins, anthocyanins, and essential oils. Synthesis materials obtained from these by-products are used in industry, mostly in the manufacture of paints, soaps, perfumes, plastics, adhesives, vegetable oil, and pesticides (bilgiustam.com, 2021).

Secondary metabolites are found in the structures of some known drugs, as they are effective in the treatment of diseases in humans. Secondary metabolites used extensively in the pharmaceutical industry are steroids and alkaloids. For example, for this purpose, alkaloids are obtained from the plants; *Digitalis*, *Catharanthus*, *Atropa* (atropine, scopolamine and hyoscyamine), and *Opium* (papaverine, morphine and codeine). It has been reported that diterpene esters obtained from some *Euphorbia* species were used in chemical carcinogenesis studies. Rosmaric acid, also a polyphenolic compound, is one of the secondary metabolites used in cancer treatments. Secondary metabolites can be found in the softener used in laundry, creams that have healing and regenerative effects. The iridoids and secoiridoids found in some plants belonging to the Gentianaceae family; They are among the alkaloids used in appetizing and relieving stomach ailments (bilgiustam.com, 2021).

5. Secondary Metabolite Types:

Secondary metabolites are products with different chemical structures and are examined in three different groups.

1. Terponeids (terpenes),

2. Alkaloids (nitrogenous compounds),

3. Phenolics (phenolic compounds) (Morris and Robbins, 1997).

According to (Oskay and Oskay, 2009), (Morris and Robbins, 1997), the grouping of metabolites formed as a result of photosynthesis is shown in the following list in more detail.

* Primary Metabolism Products
    1. Proteins
    2. Fats
    3. Carbohydrates
* Secondary Metabolism Products
    1. Terponeids
    2. Alkaloids
    3. Phenolics
        3.1 Phenolic acid ⟶ Lignine
        3.2 Cumarins
        3.3 Ketones
            3.3.1 Flavons

3.3.2 Flavanones

- Isoflavones (fungicidal substances)
- Pterocarpanes (fungicidal substances)
- Koumestans (fungicidal substances)
- Dihydroflavones

1 Flavonoids (Yellow color substance in flowers)

2 Flavonois

- Condensed diagnoses
- Antocyanins (Red color substance in flowers)

5.1. Terponeids (Terpenes):

They are the most common group of secondary metabolites (food-info.net, 2021), (biyologlar.com, 2021). While they take on different tasks in animals and plants; they are important in terms of food flavor as additive. The aromas of citrus fruits, cinnamon, and some spices are caused by various terpenes. Limonene and citral in lemons, pinene in pine trees, geraniol in roses, and eugenol in cloves are among the most common and common terpenes. They are mostly found in essential oils (food-info.net, 2021).

Terpenes have been formed by the chemical structure with a certain number of isoprene units (food-info.net, 2021). They are formed by the combination of five-carbon isoprene units. They are also called isoprenes since they decompose into isoprenes that form when the

temperature is high. Generally, they are insoluble in water (biyologlar.com, 2021).

When classifying terpenes, their carbon numbers were taken into account (biyologlar.com, 2021), (Karahan, 2007). Their classification is shown in Table 1 below (Karahan, 2007).

Table 1: Classification of Terpenes According to Their Carbon Numbers

Isoprene Number	Carbon Number	Class
1	5	Hemiterpenes
2	10	Monoterpenes
3	15	Sesquiterpenes
4	20	Diterpenes
5	25	Sesterpenes
6	30	Triterpenes
8	40	Tetraterpenes (carotenoids)
N	$(5)_n$	Polyterpenes

Terpenes are examined in two groups according to their physical properties. Volatile terpenes; are small molecule monoterpenes and some sesquiterpenes. Non-volatile terpenes; large molecule sesterpenes are sesquiterpenes, diterpenes, triterpenes, and polyterpenes (Kılıç, 2002). Gibberellins, some of the important herbal hormones, are diterpenes (acikders.ankara.edu.tr, 2021).

Some of the terpenes take an active role in the growth and development of the plant. For example, the gibberellin hormones

known as plant growth regulators are diterpenes. Sterols, which are also the main components of plant cell membranes, are derived from triterpen. In addition, carotenoids that act as co-pigments in photosynthetic activities are also tetraterpenes. Abscisic acid, another herbal hormone, is also a sesquiterpen (biyologlar.com, 2021).

The aromatic scents emitted by the essential oils from plants generally indicate that they contain terpenes.

Due to their toxic effects, terpenes provide effective protection against some mammals and insects that feed on plants and threaten them. Pyrethroids, monoterpenes found in some *Chrysanthemum* (chrysanthemum) species, have insecticidal effects. And this natural or artificially derived active ingredient is used as an insecticide in the market, with commercial returns (biyologlar.com, 2021).

Again, some monoterpenes, especially in the pine family, protect the plants by having a lethal effect against bark beetles. The plant repellent effects of essential oils in the terpenes group against insects are well known. Another terpene used to repel insects is limonoid obtained from lemons. Essential oils are also important for their use as flavoring in foods and their commercial additives in the perfume industry (biyologlar.com, 2021).

Cardenolides obtained from *Digitalis* species are used in medicine for their effectiveness in slowing and strengthening the heart rate (biyologlar.com, 2021).

5.2. Alkaloids (Nitrogenous Compounds):

The name alkaloid was first proposed in 1805 by Saturner and then by Meissner, and it was stated that morphine has a base character. Among the most widely known examples; are quinine, nicotine, morphine, and ephedrine (bilgiler.gen.tr, 2021).

Many varieties can be obtained artificially. Because they have been needed and used frequently in medicine throughout history. They are still in demand. For example, the quinine alkaloid has long been used against malaria. Morphine has also been used to relieve strong pains. However, the issue to be considered is the high rate of using some of these products to make a habit. People have been known to use alkaloids as medicine or poison for many years.

Alkaloids are generally found in greater amounts in some organs of plants. For example, the poppy plant has alkaloids in its fruit but not in its seeds. It is also not seen in the seeds of tobacco while it is present in the leaves and flowers (Tanker and Tanker, 1990).

Alkaloids of generally vegetable origin (few animal origins); have weak base character. And they can form salt by reacting with acids. Already the word alkaloid means alkaline. Because it contains nitrogen, it is also called nitrogenous compounds. Most of them are colorless, crystalline, and toxic. Some of the plant families known to have alkaloids are Rosaceae, Soeleneceae, and Graminaceae (bilgiler.gen.tr, 2021).

Today around 12000 alkaloids are known to exist. They are found in different numbers and varieties in every plant. For example, more than 10 alkaloids have been detected in coca trees and 60 in propeller flowers. It belongs to the alkaloid group, and it is possible to list some of the leading ones, especially according to their intended use in medical fields, as follows:

Those with antitumor properties; Vincristine, vinblastine,

Those with vascular constricting properties; Pilocarpine, physostigmine,

Those with vasodilating properties; Atropine,

Pain relievers: Morphine, codeine,

Those that stimulate cardiovascular work and increase blood pressure: epinephrine, ephedrine,

Those which lower blood pressure; Protroveratrin A, reserpine,

Those that heal the central nervous system; Brutsine, strikinin,

As a sedative preparation; Scopolamine,

Against morphine and phosphoorganic insecticides; Atropine,

Against the malaria parasite factor; quinine is used (avys.omu.edu.tr, 2021).

It is possible to classify alkaloids according to the basic compounds from which they are synthesized. According to this;

Alkaloids, which are lysine derivatives, are morphine; Codeine, lobeline, cone, nadorine, piperidine,

Ornithine derivative alkaloids; Nicotine, atropine, cocaine,

Alkaloids which are tryptophan derivatives; Vinblastine,

Histidine derivative alkaloids; Caffeine, theobromine, pylorcarpine, theophylline,

Phenylalanine, Tyrosine, or Anthranilic acid type alkaloids; Quinine, ephedrine, lycorine, mescaline, pellotin,

Alkaloids synthesized by the isoprenoid route; Colchicine (avys.omu.edu.tr, 2021).

It is possible to say that alkaloids act as a nitrogen source for plants due to their nitrogen transport. In addition, because of their poisonous nature, these metabolites also fulfill the duty of protecting the plants they are found by preventing animals from eating. In medicine; it is known that a large number of alkaloids are used as pain relievers, regulating the work of the heart, as a respiratory stimulant, as a constrictor or dilator of blood vessels, as a cure for cold, and as muscle relaxants.

5.3. Phenolics (Phenolic Compounds):

There are over 30,000 phenolic compounds naturally produced by plants. Scientific studies have revealed that many of them have antiallergic, antidiabetic and anti-inflammatory properties (Atak and

Uslu, 2018). These secondary products, which are mostly found in plants are frequently seen in tea, coffee, legumes, fruits, and vegetables (Dai and Mumper, 2010). They play an active role in the fight against parasites and viruses of plants (Karabulut and Yemiş, 2019).

According to (Kolaç et al, 2017); phenolic compounds are divided into five main groups according to their cyclic structure. Thet are phenolic acids, tannins, flavonoids, lignans and stilbenes.

According to (Shahidi and Yeo, 2016), phenolic compounds are grouped as follows.

* Non-flavonoids

1. Phenolic acids; Cinnamic acids (coumaric acid, ferulic acid, caffeic acid), benzoic acids (gallic acid),

2. Lignans; Pinoresinol,

3. Stilbenes; Resveratrol, Pikeid,

* Flavonoids

1. Flavones; Routine, Apigerin,

2.Flavanols; Catechin, Epicatechin, Epigallocatechin,

3. Flavanones; Naringenin, Hesperidin,

4. Anthocyanidins; Malvidin, Cyanidine, Delfinidine,

5. Isoflavones; Genstein,

6. Flavonols; Caemferol is quercetin.

There are dense amounts of phenolic substances in fruits and vegetables from plant foods (Yıldız and Baysal, 2003). The reason why herbal foods leave a bitter taste in the mouth is phenolic substances (Shahidi and Naczk, 1995). It is known that other phenolic substances are derived from benzene, the simplest phenolic substance (Cemeroğlu and Acar, 1986).

It has been determined that the tannins in strawberries have antiviral effects against Herpes, Polio and Enteric viruses (Shahidi and Naczk, 1995). In addition, phenolic substances also have an anti-edematous and anti-allergic effect (Hertog et al, 1993).

The source of antioxidant properties of fruits and vegetables; are flavonoids such as catechin, anthocyanin and flavone synthesized in their bodies (Wang et al, 1996).

Consumption of foods containing high amounts of phenolic substances is also effective in preventing many disorders in the body (Deveci et al, 2016).

CONCLUCSION

Although secondary metabolites are not essential for plants to survive, they are the metabolism products they need to make their lives easier. Although generally divided into three as terpenes, alkaloids, and phenolic compounds; tens of thousands of different types of secondary metabolites have been identified. Such a wide variety of active ingredients has also been used to increase people's living standards. However, their activities, synthesis paths, etc. There are many metabolites whose properties need to be studied.

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CHAPTER 5

PLANT-ASSOCIATED MICROBIAL BIOFILMS: FROM ECOLOGY TO MOLECULAR GENETICS

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INTRODUCTION

Biofilms are defined as the ensemble formed by microbial cells adhering to biotic and abiotic surfaces in a resistant extracellular polymeric matrix they produce. Biofilms can also be defined as a protective growth mode of various microorganisms to survive in unfavorable environments, establish new biofilm architectures, and spread into nutrient-rich environments (Hall-Stoodley et al., 2004).

Microorganisms, living in a cluster, that exhibit both beneficial and harmful activities in medical, industrial, and agricultural fields can grow naturally by attaching to living and non-living surfaces. Ramey et al. (2004) observed that phytopathogen *Agrobacterium tumefaciens* develops biofilms on both living tissues and non-living surfaces. In like manner, plant-associated soil bacteria *Sinorhizobium meliloti* and *Rhizobium leguminosarum* form biofilms on both abiotic and living surfaces (Fujishige et al., 2006). Biofilms formed by rhizobacteria facilitate the nutrient acquisition of plants, regulate plant hormone levels, prevent plant pathogens from reaching nutrients by storing nutrients, prevent phytopathogens from settling by colonizing root surfaces, and reduce various environmental stress factors (Ahemad and Kibret, 2014; Kimani et al., 2016; Lugtenberg and Kamilova, 2009). In addition to many benefits, biofilms have detrimental impacts on plants. Phytopathogenic microorganisms such as *Agrobacterium tumefaciens*, *Bacillus cereus*, *Clavibacter michiganensis*, *Pseudomonas aeruginosa, Ralstonia solanacearum* can cause various diseases by forming biofilms on plant surfaces (Velmourougane et al.,

2017). Biofilm mode of growth offers great advantages to biofilm-forming microorganisms. Therefore, a large number of microorganisms in nature prefer to live in groups as biofilms over planktonic lifestyle. It is important to consider in detail such an important lifestyle. Although the number of studies with biofilms has increased rapidly in recent years, there are limited studies on the biofilm formation of microorganisms related to plants. For this reason, some important microorganisms not related to plants are also included in this section.

1. STEPS OF BIOFILM DEVELOPMENT

The first stage of biofilm development is the attachment of a small number of microorganisms to a living/non-living surface. Microbial aggregation followed by mature three-dimensional biofilms consists of reversible and irreversible processes and involves a great number of species-specific factors (Kostakioti et al., 2013). Binding to abiotic and biotic surfaces is generally performed by non-specific interactions and specific molecules, respectively (Carpentier and Cerf, 1993). Initial binding is dynamic and reversible. Meanwhile, microorganisms can return to planktonic form depending on hydrodynamic forces (separating bacteria from the surface), driving forces, the presence of nutrients, and other environmental factors (Kostakioti et al., 2013).

If environmental conditions are suitable for biofilm formation, microorganisms remain irreversibly attached to the surface. Exopolysaccharide exhibits an important role in irreversible binding.

Increased production of the extracellular polymeric matrix surrounding microbial cells contributes greatly to a mature three-dimensional biofilm formation. At this stage, the production of cell-cell signaling molecules is important for biofilm-forming microorganisms to communicate with each other. A mobile community exists in mature biofilms. This community actively exchanges and shares substances showing key roles in biofilm architecture and offering an appropriate habitat for settled microorganisms (Kostakioti et al., 2013). This group of organisms is optimally clustered to take advantage of available nutrients. Depending on various factors such as the presence/absence of nutrients, changes in oxygen levels, the production of toxic substances, or other stress factors, cells in mature biofilms move to different environments to create new biofilm structures (Kostakioti et al., 2013).

2. BIOFILM MATRIX

The microbial community in biofilm accounts for approximately 10% of total biofilm mass, whereas the extracellular polymeric substance (EPS) that holds microorganisms together can make up more than 90% (Satpathy et al., 2016). Biofilm matrix may also be called the glycocalyx; extracellular matrix usually consists of complex polysaccharides, proteins, glycopeptides, lipids, DNA, water, and lipopolysaccharides (Zhang et al., 1998; Flemming and Wingender, 2010; Satpathy et al., 2016). Due to its cohesive and adhesive properties, EPS exhibits a major role during biofilm development and

irreversible adhesion to a surface. The greatest effect on the mechanical stability of biofilms is also provided by exopolysaccharides (Flemming and Wingender, 2010).

Biofilm-forming microorganisms produce various EPSs, including succinoglycan, galactoglucan, and cellulose. The composition of EPS can be varied greatly depending on the growth conditions, environmental factors, strains, and substrates used. *Bacillus subtilis* strains can secrete two different polymers such as EPS and poly-d-glutamate (PGA). Both of these molecules contribute to biofilm development differently depending on the strains and conditions studied (López et al., 2010). *Sinorhizobium meliloti* strains secrete two different kinds of exopolysaccharides known as succinoglycan and galactoglucan, and both have different functions in supporting symbiosis relationship and biofilm formation (Sorroche et al., 2012). In a study with *Agrobacterium tumefaciens* biofilms, normal exopolysaccharide secretion contributed to normal root attachment whereas an excessive amount of cellulose fibers produced denser biofilms (Matthysse et al., 2005). In this work, mutations produced in cellulose-secreting genes significantly reduced the attachment, root colonization, and biofilm development of the bacterium (Matthysse et al., 2005).

Attachment is a crucial step for effective root colonization. EPS is the most important factor for sticking to a surface. Mutational analysis demonstrated that exopolysaccharides and cellulose of *Rhizobium leguminosarum* have a critical effect on the attachment to root hair

and nodulation of legume plants (Laus et al., 2005). EPS also regulates the attachment and clustering of *Azospirillum brasilense* to plant root surfaces (Burdman et al., 2000). Mutations in EPS genes revealed that *Rhizobium meliloti* forms ineffective nodules not containing intracellular bacteria (Leigh et al., 1987).

3. FACTORS AFFECTING BIOFILM FORMATION

Biofilm development depends on a wide variety of factors such as temperature, pH, nutrients, salt concentration, water, oxygen, metal ion concentrations, antimicrobials, plant volatiles, and genetics (Velmourougane et al., 2017; Çam and Brinkmeyer, 2020a). The first important stage of biofilm development is the attachment to a biotic/abiotic surface. The ability of biofilm-forming microorganisms to adhere to a surface is influenced by temperature and pH changes during biofilm formation (Velmourougane et al., 2017). For this reason, temperature and pH appear to be the first essential factors for the initiation of biofilm formation. The temperature might have an increasing or decreasing effect on biofilm production. Since optimum temperature increases enzymatic activities and microorganism metabolism, more biofilm production might be expected (Stepanović et al., 2003). However, it was observed that some microorganisms produced more robust biofilms at lower temperatures (Townsley and Yildiz, 2015; Çam and Brinkmeyer, 2020a). More biofilm production at low temperatures is due to the fact that some microorganisms undergo a phenotypic change and develop a new variant that is more resistant to adverse environmental conditions. In studies on different

microorganisms, different interpretations have also been made such as that changes in temperature influence the viscosity of EPSs and subsequently attachment step and three-dimensional biofilm structure; temperature shifts affect the microbial appendages involved in adhesion; the decreased temperature reduces adhesive properties of bacteria; microbial polysaccharides are more stable at low temperatures, thus favors establishing denser biofilms (Velmourougane et al., 2017). In biofilms of *Sinorhizobium meliloti*, which create a symbiotic relationship with legumes and promote plant growth, nutrients increased biofilm formation while extreme temperatures showed negative effects on biofilm development (Rinaudi et al., 2006).

Scientists have reported that pH effect on biofilm formation by different microorganisms varies greatly (D'Urzo et al., 2014). The pH effect reveals great variation from one microorganism to another. pH changes in *Pseudomonas fluorescens* had no effect on biofilms (O'Toole and Kolter, 1998). Excessive pH changes adversely affected *Sinorhizobium meliloti* biofilms (Rinaudi et al., 2006). On the other hand, acidic pH increased the biofilm formation of *Vibrio vulnificus* (Çam and Brinkmeyer, 2020a). In this study, different strains of the same species produced varying levels of biofilm production at different pH values. Similarly, some strains of *Streptococcus agalactiae* formed more biofilms in an acidic environment than others (D'Urzo et al., 2014).

Another factor in biofilm formation is salt concentration. The preference of microorganisms for biofilm mode of growth under adverse conditions is an important survival strategy. Biofilm formation against salt stress increases the survival struggle of microorganisms by compensating for salt stress in the rhizosphere (Kasim et al., 2016). Similar to temperature and pH factors, the salt parameter also showed different effects on the ability of biofilm formation by different microorganisms (Lee et al., 2014; Qurashi and Sabri, 2012; Xu et al., 2010). Kasim et al. (2016) observed that some rhizobacteria increased biofilm production under increasing salt concentrations, but there was no difference in others. Similarly, significant increases in biofilm production of salt-resistant *Halomonas variabilis* and *Planococcus rifietoensis* species were reported against increased salt stress (Qurashi and Sabri, 2012). The positive effect of increasing salt concentration on biofilm formation was attributed to the increase in exopolysaccharide production, as it increases biofilm formation and protects the biofilm mechanism by forming a water layer around the cells (Qurashi and Sabri, 2012).

Microorganisms in terrestrial environments prefer to live in the biofilm mode of growth on unsaturated areas. Adaptation mechanisms of microorganisms in low osmolarity environments are different from those in habitats with high water content. Therefore, water limitation in the environment affects biofilm formation (Van De Mortel and Halverson, 2004). Oxygen availability might play an important role in microbial biofilms. A sudden decrease in oxygen levels affected the

dispersal of *Shewanella oneidensis* from biofilms individually (Thormann et al., 2005). Besides, a strong relationship was found between the iron levels in the bacterial culture media and biofilm formation (Çam and Brinkmeyer, 2020a).

Genetic factors also have an important effect on biofilm development. Biofilms have complex community interactions, genetic diversity, and structural changes. Different levels of biofilm formation can be observed between different isolates of the same species under the same conditions (Hoštacká et al., 2010). Microorganisms in biofilms are physiologically different from planktonic counterparts. Differences in mobility, matrix and protein production, and antibiotic tolerance levels were observed in biofilm-forming cells (Stewart and Franklin, 2008). A recent study has also shown that microorganisms exhibit different levels of gene expression in biofilms compared to planktonic forms (Çam and Brinkmeyer, 2020b). Even different cell groups in biofilms may exhibit physiologically and genetically distinct behaviors from adjacent cells (Stewart and Franklin, 2008).

4. IMPORTANCE OF BIOFILMS FOR PLANTS

Microorganisms that thrive in biofilms are more likely to survive in a competitive environment, as they are more resistant to adverse environmental conditions than planktonic living organisms. Therefore, rhizobacteria that form biofilms contribute more to plant growth. They can increase the plant growth-promoting properties by forming biofilms on the root surfaces or nodules of beneficial microorganisms

that colonize plant roots. *Azospirillum brasilense*, which has properties that stimulate plant growth such as nitrogenase activity, plant hormone levels, nitrate reduction, has effects that increase root development by forming biofilms on plant roots (Arruebarrena Di Palma et al., 2013; Jofré et al., 2004). *R. leguminosarum* and *S. meliloti* that form nodules in legume plants also develop biofilms on the roots (Janczarek et al., 2010; Sorroche et al., 2012). In this way, it promotes plant growth by converting atmospheric nitrogen into a usable form which could be easily absorbed by plants.

The ability to promote plant growth mediated by microorganisms is generally based on more than one promoting properties. For example, only an increase in phytohormone level may not contribute to plant growth sufficiently. Moreover, single-species biofilms might be weaker than multi-species microbial biofilms. Therefore, the ability of rhizobacteria to form biofilms with cyanobacteria and fungi has been investigated. Strains of cyanobacterium *Anabaena torulosa* were cultured together with agriculturally important rhizobacteria such as *A. chroococcum, Mesorhizobium ciceri,* and *Pseudomonas striata*, and their biofilm-forming properties were investigated (Prasanna et al., 2011). It has been suggested that newly established biofilms by these mixed species, which have agriculturally useful properties, would positively affect plant growth. Significant differences have been observed in the biofilms of *Trichoderma viride*, a fungal species, together with *Azotobacter chroococcum*, *Bacillus subtilis*, and *Pseudomonas fluorescens* species that promote plant growth (Triveni

et al., 2012). *Trichoderma-Bacillus* and *Trichoderma-Pseudomonas* biofilms produced more ammonia, indole acetic acid, and siderophore compared to other groups. The highest nitrogenase and ACC deaminase activity were observed among *Trichoderma-Azotobacter* biofilms (Triveni et al., 2013).

For sustainable and environmentally friendly agriculture, beneficial biofilms can be developed under *in vitro* conditions and utilized as biological fertilizers for plants when applied in large quantities. Biofilm biofertilizers applied to tea and rice plants reduced the recommended amount of chemical fertilizers by approximately 50% (Seneviratne et al., 2009). The use of improved microbial biofilms together with chemical nitrogen fertilizers was found to be effective in the regulation of degraded soils and the renewal of microorganisms in the rhizosphere by traditional agricultural practices (Seneviratne et al., 2011). Wheat growth, soil fertility, and nutrient uptake were increased by biofilm-based inoculants formed by different rhizobacteria with cyanobacterium *A. torulosa*. P uptake of the plants was increased by bio-fertilizers inoculated with *Anabaena-Pseudomonas* biofilms (Swarnalakshmi et al., 2013). Furthermore, *Trichoderma viride-Bradyrhizobium* biofilm applications increased the wet/dry weight of plants by 20-45% compared to other microbial applications, while the greatest dehydrogenase activity was observed among *T.viride-Azotobacter* biofilms (Prasanna et al., 2014).

Biofilm-forming microorganisms could be considered as an effective alternative to chemical drugs in biological control. Cyanobacterial biofilm inoculation has demonstrated effective biocontrol performance in paddy, wheat, legumes, and cotton (Babu et al., 2015). The effect of biofilm formulations of microorganisms varies from species to species in biological control. Combinations of bacterial or fungal antagonist microorganisms have greater biocontrol efficiency than single cultures (Triveni et al., 2015). The best biocontrol performance of cotton plant infected with a fungus was observed in *Anabaena-T. viride* biofilm formulations and plant mortality rates were 11.1% lower than commercial *Trichoderma* formulations (Prasanna et al., 2015). *Bacillus subtilis* biofilms are found to be an effective biocontrol agent against plant pathogen *Pseudomonas syringae* (Bais et al., 2004). The lowest mortality rate in cotton plants infected with plant fungal pathogen *Macrophomina phaseolina* was observed in seeds inoculated with *T. viride-B. subtilis* biofilms compared to dual and single culture biofilms (Triveni et al., 2015). *Paenibacillus polymyxa* biofilms might be used as biocontrol agents against phytopathogen *Ralstonia solanacearum* and *Fusarium graminearum* (Timmusk et al., 2019; Yi et al., 2019)

5. MOLECULAR MECHANISMS OF BIOFILM

Biofilm-forming bacteria use a signal communication system called quorum sensing (QS), which depends on population density, to communicate with each other and control gene expressions. Gram-negative bacteria use N-Acyl homoserine lactones (AHLs) as signal

molecules in this communication; Gram-positive bacteria, on the other hand, use specific peptides (Satpathy et al., 2016). QS systems in rhizobacteria regulate the levels of gene expression involved in virulence, colonization, EPS and biofilm production, and symbiotic interactions. Biofilm formation in *Sinorhizobium fredii*, *S.meliloti,* and *Bradyrhizobium japonicum* is very important for root colonization and symbiotic relationships and is mediated by QS mechanisms (Pérez-Montaño et al., 2014). *P. aeruginosa* employs las and rhl QS systems. In these systems, *las1* and *rhl1* genes encode enzymes that produce signaling molecules. These molecules play important roles during biofilm formation by regulating intercellular communication (De Kievit et al., 2001). *luxS* is an important regulatory gene of the QS systems in *Paenibacillus polymyxa.* This gene positively influences biofilm formation and increases biocontrol efficacy against *Ralstonia solanacearum* (Yi et al., 2019).

C-di-GMP is a second messenger, cellular signal molecule and plays an important role in various cellular functions such as biofilm formation. For example, c-di-GMP *cdgA* gene regulates biofilm and exopolysaccharide production in *A. brasilense* (Ramírez-Mata et al., 2016). *P. aeruginosa* employs this secondary communication system in regulating biofilm formation (Valentini and Filloux, 2016). Inorganic phosphate is an environmental signal that regulates the biofilm formation in *Pseudomonas fluorescens*. In the absence of inorganic phosphate, c-di-GMP loses its effect in *P. fluorescens* and causes the loss of adhesion protein LapA. Therefore, it decreases the

attachment of microorganisms to a surface and subsequently biofilm formation (Newell et al., 2011). Nitric oxide is the signaling molecule that controls various functions during rhizobacteria-plant interactions. Nitric oxide in *Shewanella oneidensis* stimulates biofilm formation by controlling bacterial c-di-GMP levels (Plate and Marletta, 2012).

Bacteria detect environmental changes through the two-component regulatory systems and give an appropriate adaptive response. ExoR and exoS-chvI, a two-component system, in *Sinorhizobium meliloti* regulates various functions, including symbiosis, mobility, and biofilm development (Wells et al., 2007). This regulatory system also plays a considerable role in the biofilm development of *B. licheniformis* and *B. subtilis* (Vlamakis et al., 2013; Wang et al., 2020). The production of extracellular EPSs involved in the attachment of *P. aeruginosa* to a surface is controlled by two-component systems (Mikkelsen et al., 2011). In another study, a new two-component BqsS-BqsR system was reported to regulate biofilm degradation in *P. aeruginosa*. Mutations in the *bqsS* or *bqsR* genes that regulate this system lead to a significant increase in biofilm development (Dong et al., 2008).

CONCLUSION

The rhizosphere is an important area between plants and soil where beneficial and harmful activities occur between plants and microorganisms. This area is not always suitable for microorganisms and environmental conditions tend to change constantly. For this reason, many rhizobacteria prefer a biofilm lifestyle to survive and escape from stress conditions. This way of life is considered a vital strategy for these microorganisms. It is known that beneficial microorganisms form biofilms and subsequently increase plant growth, the amount and quality of crops through biofilmed formulation. Biofilm formation is also important in combating phytopathogens. In previous studies, planktonic living microorganisms were preferred as biocontrol and biological fertilizers. In recent years, this situation has turned to microorganisms forming biofilms. Environmental and genetic conditions significantly affect the ability of microorganisms to form biofilms and reveal significant differences even among different strains of the same species. Small changes may lead to considerable differences in the behaviors and genetics of microorganisms. Even the interactions between microorganisms in biofilms might significantly affect the effects of biofilms on plants. Therefore, the responses of biofilm-forming beneficial microorganisms, which could be used as biocontrol and biofertilizer, to environmental and genetic parameters as well as their interactions with each other should be well analyzed and then applied.

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ISBN: 978-605-74646-3-7