INTERFERON-ALPHA SIGNALING PATHWAY IN THE SENSORY AUDITORY NEUROEPITHELIAL CELLS

by

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Abstract

The current study investigated the effect of interferon-α (IFN-α) on the cochlear cell line to shed light on the mechanisms by which interferon alpha may affect hearing. HEI-OC1 cell line and real time-PCR were used to determine the expression of those genes that might be involved in these mechanisms. Dose- (20, 40, & 80 U/ml) and time-dependent experiment-1 did not show significant alteration in gene expression associated with the stimulations of the IFN-α receptors. Therefore, a second experiment was planned. A 3 X 4 factorial design, consisting of three treatment conditions (0, 200 & 2000U/ml) and four time-points (6, 12, 24 & 48 Hrs), was employed. The results of experiment-2 revealed that significant differential expression of inflammatory genes, immune response genes and apoptotic genes is found in a dose- and time-dependent manner. This outcome indicates that IFN-α treatment led to initiation of an inflammatory response, an immune response and apoptosis of the cochlear cells, which was confirmed by a reduction in cell viability. The immune response was the most pronounced response; whereas inflammatory the apoptotic responses were transient.

Therefore, the current in-vitro study indicates that the inflammatory response, the immune response and apoptosis might be the underlying mechanisms involved in the hearing impairment previously reported in patients under IFN-α therapy. These results imply that pre-

treatment hearing evaluation as well as close monitoring of hearing function in patients undergoing long-term high-dose of IFN-α therapy are necessary to avoid or to minimize its adverse effect on hearing. The results also indicate that there is a need for further investigation of other markers that might be involved in signaling pathways of IFN-α, including markers for intrinsic pathway of apoptosis and antiapoptotic markers as well as markers for necrosis. This information might open an avenue for therapeutic intervention that can protect the inner ear from the ototoxic effect of some medications in general and IFN-α in particular and treat some immune-mediated inner ear disorders. In addition, this information might help in identifying novel diagnostic markers for vulnerability, severity, and outcomes of any cochlear pathology.

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1.0 CHAPTER I: INTRODUCTION

Interferons (IFNs) are a family of natural proteins secreted in small amounts mainly by the cells of the immune system in response to viral infections, bacterial infections and tumors. There are many types of IFNs; however, the most common in the clinical field are interferon-alpha (IFN-α), interferon-beta (IFN-β) and interferon-gama (IFN-γ). Interferons have many biological properties such as modification of inflammatory response, modification of immune response, and inhibition of cell proliferation (they stop cell growth or lead to cell death, apoptosis). Therefore, they are widley used clinically in the treatment of many diseases. For example, they are used in treatment of viral hepatitis C and hepatitis B, multiple sclerosis and regression of a number of malignant tumors.

Although IFNs have been successful as potent antivirals, immunomodulators and anticancer agents, treatment with IFN carries significant risks (Heathcote & Main, 2005). Many side effects have been reported with IFN therapy, including flu-like symptoms, depression, autoimmune reactions (Benelhadj, Marcellin, Castelnau, et al., 1997), and some blood disorders (Poynard, Bedossa, Chevallier, et al., 1995).

Recently, there are a considerable number of studies that have found an association between hearing impairment and IFN therapy, in particular with IFN-α (Atug, Akin, Yilmaz, Sari, Tozun (2009); Cadoni, Marinelli, De Santis, Romito, Manna, et al., 1998; Elloumi,

Houissa, Bel Hadj, Gargouri, Romani, Kharrat, & Ghorbel, 2007; Formann, Stauber, Denk, Jessner, Zollner, et al., 2004; Johnson, Sargent, Galizio, and Ubogu, 2008; Kanda, Shigeno, Kinoshita, Nakao, Yano, et al.,1994; Okanoue, Sakamoto, Itoh, Masahito, Yasui, et al., 1996; Piekarska; Jozefowicz-Korczynska; Wojcik; & Berkan; 2007; Sachar and Polio, 2004; Salkic, Zerem, Zildzic, & Basic, 2009; Wong, Cheong-Lee, Ford, Yoshida, 2005). The incidence of hearing impairment due to IFN was reported more frequently in patients with hepatitis C than in patients receiving IFN for other diseases.

On the other hand, a few studies showed no affect of IFN on hearing. For example, Kaygusuz, Ozturk Kaygusuz, Ozturk, Kilic, et al., (2004) found no effect of IFN-α on the hearing threshold of patients with chronic active hepatitis. In addition, Akyol, Sarac, Akyol, et al., (2001) found no histopathological changes in the organ of Corti of the cochlea of albino Swiss mice treated with a single dose of IFN; yet the auditory brainstem showed significant changes.

The inconsistency in findings was not limited to the absence of the affect of IFN on hearing. There is also inconsistency with regard to effectiveness of IFNs to treat sensorineural hearing loss (SNHL), especially that of viral or autoimmune origin (Kanemaru, Fukushima, Nakamura, et al., (1997).

The current study was designed to evaluate the incocsistency in the effect of IFN-α on the auditory system. This incosistency among the findings implies the need for a well-controlled study at the cellular level (using methods of molecular biology) to determine if this contradiction is a matter of mechanism(s) or simply dose-dependent effects. In particular, there is a need to know when IFN can be pathologic to the organ of hearing rather than (potentially) protective or therapeutic. Second, from a clinical point of view, because IFNs are widely used today in the

clinical arena, it is important to ensure the safety of such medications in order to avoid subsequent handicap (in this case hearing impairment). Regular monitoring of the hearing function might be required in those patients who are receiving IFNs, but the underlying mechanisms first must be explored.

In general, understanding the molecular and cellular signals that regulate and control the effect of IFNs on the cochlear structures will help in understanding the origin and mechanism of many inner ear diseases, especially that of autoimmune origin. It also might lead to developing effective methods for preventing unwanted side effects on the inner ear and opening an avenue for new therapeutic agents to manage many inner ear disorders. In addition, it might help in identifying novel diagnostic markers for vulnerability, severity, and outcomes of any cochlear pathology.

2.0 CHAPTER II: REVIEW OF THE LITERATURE

2.1 BACKGROUND

Interferons (IFNs) are members of the cytokines' family, a large group of proteins secreted by the cells of the immune system (Isaacs and Lindenmann, 1957). Cytokines, including IFNs, form a communication network to help the cells of the immune system perform their functions. Cells of the immune system are not localized in a specific organ; rather, they are peripatetic. In other words, within the immune system, there are cells that first detect inflammatory stimuli, cells that present antigen to lymphocytes, and cells that clear the antigen and repair tissue damage. In addition, the lymphocytes sometimes wander the body in the blood stream, sometimes traffic through the organs and tissues, and sometimes are localized in the lymphoid tissues. This dynamic feature of the immune system cells makes it difficult for them to communicate and to perform their function in developing a rapidly integrated response to any antigen in any part of the body. Therefore, cytokines, by their unique characteristics, can help these cells to act locally or remotely, specifically or globally, and transiently or continuously. The unique characteristics of cytokines that make them a perfect communication network for the cells of the immune system can be summarized as follows:

- Most cytokines are small-sized molecules made of polypeptides or glycoproteins.
- The same cytokine may be made by a number of different cells.

- Likewise, the same cytokine may act on different cells (pleiotropic actions), including cells of the immune system and inflammatory-modulating cells.
- Cytokines have considerable redundancy in that many of them can share similar functions.
- Cytokines often are produced in a cascade (sequence of events with one event leading to another), as one cytokine stimulates its target cells to make additional cytokines.
- Cytokines act synergistically (which means that two or more cytokines act together) or antagonistically (which means that cytokines are working in opposition to one another).

Since cytokines in general and interferons in particular originate from, exert their effects on, and communicate between the cells of the immune system, it is helpful to give an overview of some of the important points about the immune system, its cellular components, and its function.

2.1.1 The immune system

The immune system is considered a part of the hematopoietic system, in which the cellular components of the blood are manufactured and differentiated. Briefly, all blood cells originate from a pluripotent stem cell (i.e., a cell that has multiple potentials to differentiate to other stem cells or progenitors) in bone marrow (Figure 1). These progenitors include the erythroid progenitor, the myeloid progenitor and the lymphoid progenitor.

While the erythroid progenitor differentiates to form red blood cells and blood platelets, the myeloid progenitor differentiates to form the myeloid linage of white blood cells or leukocytes (Figure 2.2), which include the following cells:

- Neutrophils that are specialized to engulf and kill microorganisms through a process called phagocytosis.
- Eosinophils that defend against parasites.
- Basophiles that help to alleviate some allergic reactions.
- Monocytes that circulate in the blood or migrate to tissue where they form tissue macrophages, the main phagocytic cells of the immune system in case of infection or inflammation.
- Dendritic cells (DCs or antigen presenting cells APCs) that present degraded or intact antigen to cells that are specified to kill or engulf that antigen.
- Mast cells that make major contributions to hypersensitivity and inflammatory reactions.

The lymphoid progenitor (Figure 2) gives rise to form the lymphoid linage of white blood cells, which constitute the main cells of the immune system. There are three distinguished populations of lymphocytes:

- Natural killer cells (NK).
- B- Lymphocytes (B-cells).
- T- Lymphocytes (T-cells).

While NK cells, along with the cells of the myeloid linage, play an important role in innate immunity, both B-cells and T-cells are responsible for adaptive immunity. Although both B and T cells originate from the lymphoid progenitor in the bone marrow, B cells complete their maturation there before entering the blood, but T cells migrate to the thymus where they complete their maturation (Mayer, 2006). The mature lymphocytes are able to differentiate the self antigen (the body's own tissue) from foreign antigen on subsequent exposure, and afterward they are allowed to leave the primary lymphatic organs (bone marrow and thymus) to enter the

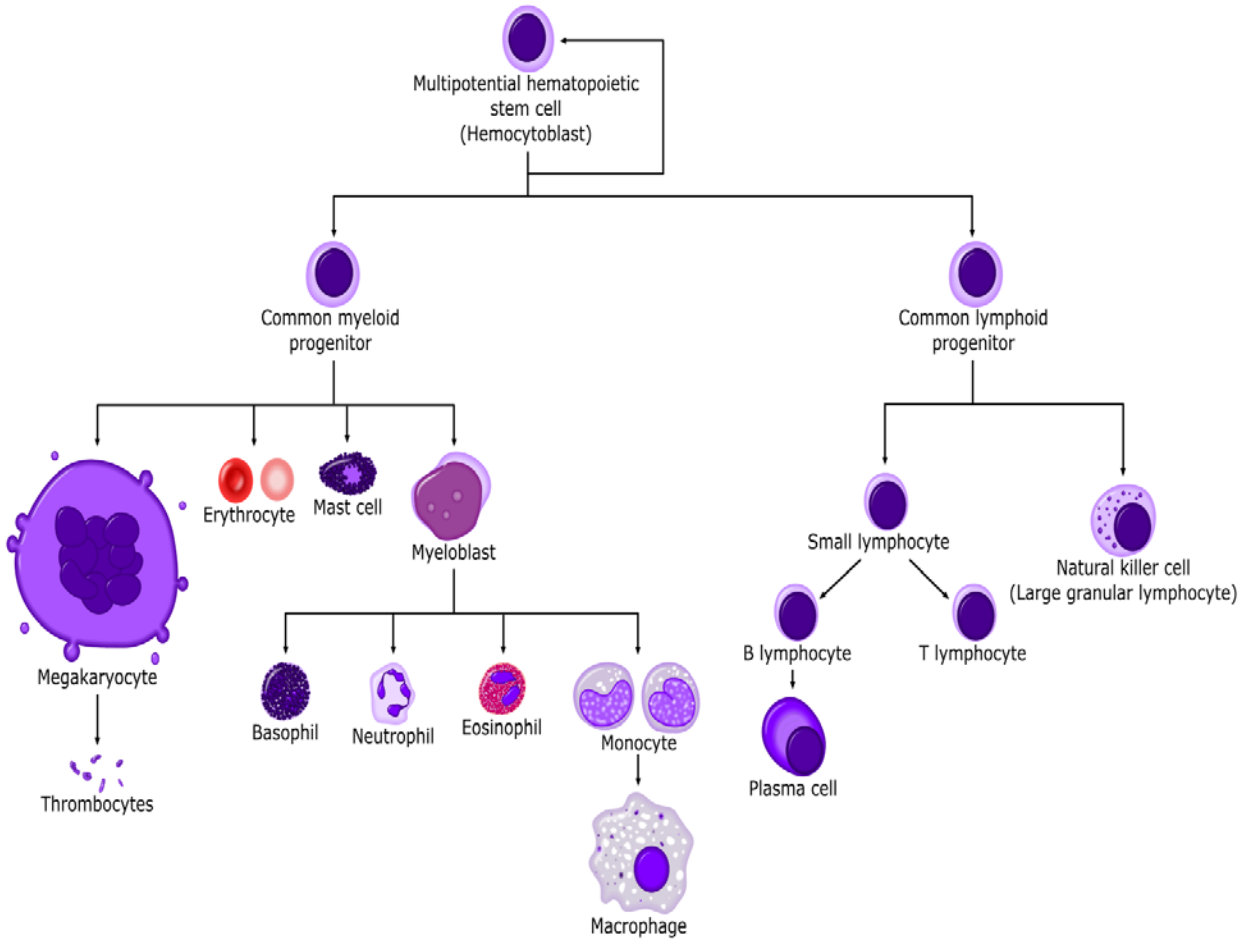

Figure 1: Development of the cells of the immune system

www.allthingsstemcell.com/tag/hematopoietic/

blood. Failure of lymphocytes in differentiating self antigens from foreign antigens will lead to a breakdown of the self tolerance and developing of autoimmune reaction to the body's own tissue. During their circulation in the blood and traveling into secondary lymphatic organs (spleen, lymph nodes), lymphocytes encounter foreign antigens brought from a site of infection.

On exposure to foreign antigen, the naïve B-cells differentiate to form plasma cells, which secrete numerous amounts of antibodies that are specific to that antigen. Likewise, on exposure to foreign antigen, the naïve T-cells will be activated into effector T-cells, namely T-helper lymphocytes (Th) or CD4+ T-cells and cytotoxic T-cells or CD8+ T-cells. T-helper lymphocyte (Th) or CD4+ cells, include T-helper-1(Th1) and T-helper-2 (Th2). They are called helper because they are specialized to help other cells of the immune system to respond to extracellular organisms. While Th1 cells activate macrophage to phagocyte and kill extracellular organisms and secrete cytokines such as IFN-gamma (IFN-γ); Th2 cells help B-lymphocytes to produce antibodies. The second type of the effector T-cells is the cytotoxic T-cells or CD8+ T-cells, which kill cells infected with viruses and other intracellular pathogens. The term "CD" stands for "clusters of differentiation". The "CD" glycoproteins present on the cell surface and act as coreceptors (molecules that activate the receptors).

The cells of the immune system interact with each other to provide different defense mechanisms against microorganisms or any foreign body; most of these interactions are accomplished via different members of cytokines. The following section will overview these defense mechanisms.

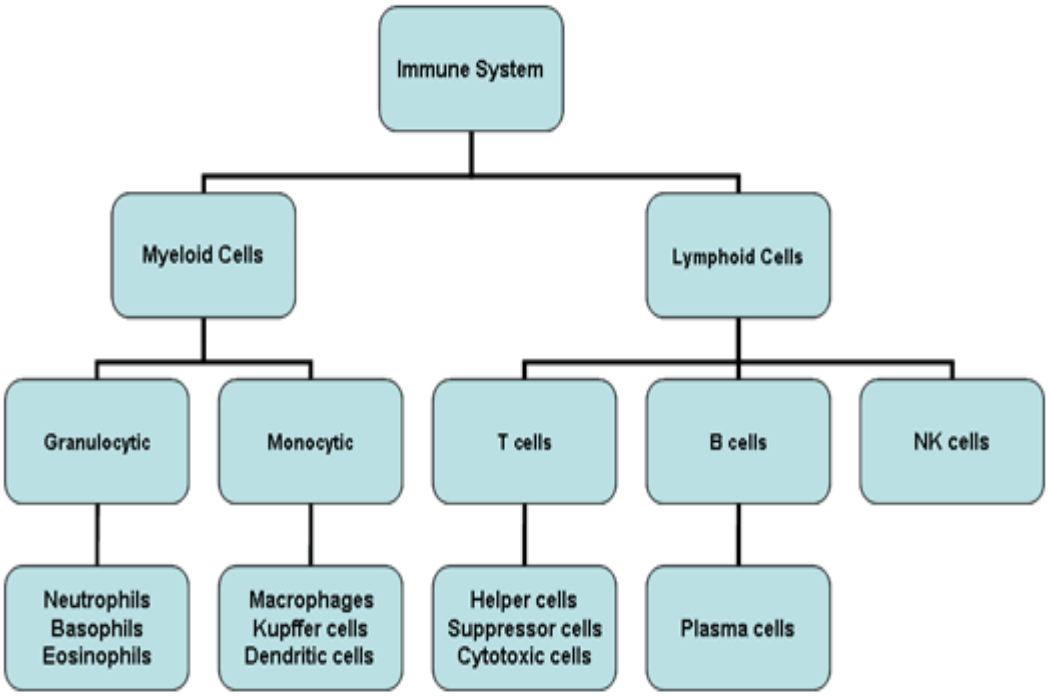

Figure 2: Cells of the immune system

2.1.2 Role of the immune system in the body's defense

In response to any microorganism or foreign antigen, the immune system has three layered lines of defense with increased selectivity, including the skin as a surface barrier of the body, the innate immunity and the adaptive immunity. Normally, the surface epithelia provide a barrier against entry of most of the pathogens to the interior of the body. However, if the pathogen is successful in penetrating the surface epithelia, it will be faced by the effector cells of the innate immunity; the mechanisms of which are determined entirely by the genes carried by the person. The response of the innate immunity is fixed, fast, and non-selective and it is usually effective in controlling most of the infection. An essential response to invasion by a foreign antigen is the inflammatory response. This response occurs in response to bacterial infection, and it is mediated by the cells of the innate immunity, which include phagocytes (macrophages, neutrophils, and DCs), mast cells, eosinophils, basophiles, and NK cells. The inflammatory response includes release of inflammatory mediators that attract more inflammatory cells to the site of infection, phagocytosis, and death of the inflammatory cells as well as cells of the injured tissue followed by tissue repair and scaring (Martin & Leibovich, 2005).

Pathogens or antigens that escape the innate immunity will be encountered by the adaptive immunity which is slow to start but powerful and specific to the invading pathogen (Mayer, 2006; Pancer & Cooper, 2006). The adaptive immunity is either by production of antibodies (humoral immunity) or induction of cell-mediated response accomplished by B-lymphocytes and T-lymphocytes, respectively. B-cells are responsible for attacking extracellular organisms through their specific antibodies, immunoglobulins (Ig); T-cells, on the other hand, attack intracellular organisms or kill infected host cells, using their specific T-cell receptors

(Holtmeir & Kabelitz, 2005). The binding of B-cells to the pathogen through its Ig drives the pathogen to inflammatory reaction with subsequent phagocytosis and cell death (Janeway, Travers, Walport, & Schlomchik, 2005). The binding of T-helper cells (CD+4) or cytotoxic T cells (CD+8) to the microbe or the infected host cells through their receptors will release cytotoxins that form pores in the plasma membrane of the target cells, with subsequent entry of water and toxins and cell death (Radoja, Frey, & Vukmanovic, 2006). Therefore, the outcome of the innate immunity, the inflammatory response, or the adaptive immune response can be cell death, by either necrosis or apoptosis. A brief overview on cell death including necrosis and apoptosis will be provided in the following section.

2.1.3 Cell Death (necrosis/apoptosis)

Two paradigms of cell death can be distinguished: necrosis and apoptosis (Figure 3). Necrosis is the prevailing form of cell death, resulting from a non-specific, severe and acute insult to the tissues such as sudden anoxia, ischemia, or extreme physical or chemical injury. It is characterized by the degradation of cytoplasmic structure and the release of the cellular contents into the environment, which then leads to cellular damage of the surrounding cells. Necrosis is usually accompanied by a potent inflammatory response in the surrounding tissues (Leist and Nicotera, 1998; Leist, Single, Castoldi, Kuhnle, & Nicotera, 1997).

Apoptosis, or programmed cell death, is a tightly regulated process that is essential during embryonic development and adult life to maintain a constant cell number since too much or too little apoptosis has been shown to lead to a number of diseases such as diabetes, autoimmune and neurodegenerative diseases and cancer (Leist et al., 1997). In other words,

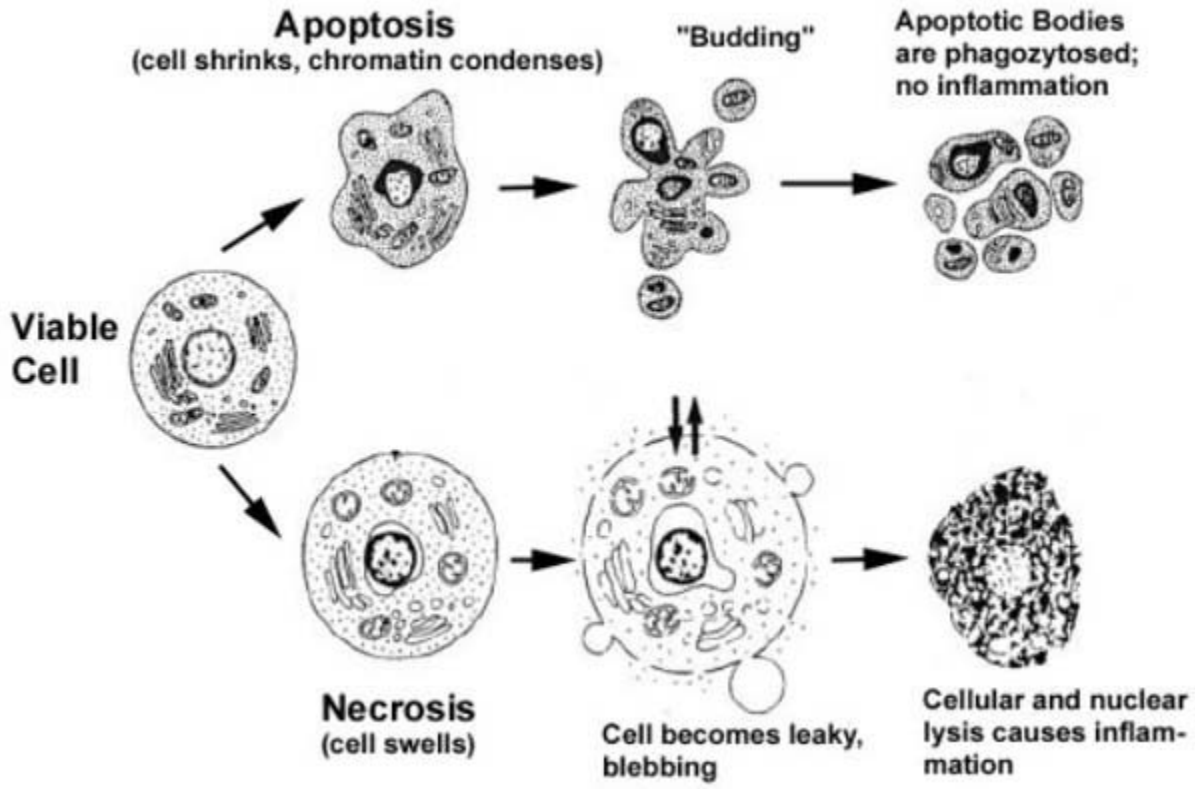

Figure 3: Apoptosis versus necrosis

Gewies (2003) from: http://www.celldeath.de/encyclo/aporev/aporev.htm

apoptosis is responsible for ordered removal of superfluous, aged, or damaged cells (Kroemer, Petit, Zamzami, Vayssière, & Mignotte, 1995; Thompson, 1995). Cells undergo apoptosis by using two major pathways: the extrinsic or death receptor (DR) pathway and the intrinsic or mitochondrial pathway. Both pathways depend on activation and involvement of caspases, which are inactive enzymes acting as death effector molecules in response to various types of apoptotic stimuli (Debatin and Krammer, 2004). Both pathways are involved in the mechanisms of IFN-induced cell death and they also can occur in the cochlea as a result of exposure to loud noise or ototoxic agents, such as aminoglycosides (Jørgensen, 1991; Li, Nevill, and Forge, 1995) and cisplatin (Devarajan, Savoca, Castaneda, Park, Esteban-Cruciani, et al., 2002).

The extrinsic pathway or death receptor pathway is activated via binding of death receptor ligands (molecules that activate the receptors) to their respective receptors. The most common cell death pathway is the Fas-Fas ligand (Fas-FasL) pathway (Figure 4). The binding of the death receptors to their corresponding ligands results in aggregation and formation of Fas activating death domain (FADD), which is followed by activation of caspase-8—upstream caspase (they are activated at the beginning of the death pathway). Activation of caspase-8 leads to activation of downstream caspases (they are activated later in the pathway), mainly caspase-3 and its release into the cytoplasm (Danial and Korsmeyer, 2004). Caspase-3 has a proteolytic effect (they break down proteins), leading to a cleavage of various intracellular proteins, including nuclear proteins, DNA, cytoskeletons, endoplasmic reticulum and cytoplasm; ending by cell death (Fraaser and Evan, 1996).

On the other hand, in the mitochondrial or the intrinsic pathway (Figure 5), the Bcl-2 family of proteins is responsible for initiating cell death. They are a family of proteins that control the permeability of the outer mitochondrial membrane. They are named such because

they are associated with the development of B-cell lymphoma type 2 (Bcl-2). Some members of this family are proapoptotic (i.e., cause apoptosis), e.g., Bax, BAD, Bak and Bok. Other members are anti-apoptotic (e.g., Bcl-xL, and Bcl-w). In response to apoptotic stimuli, Bax and Bak molecules will be activated leading to a disturbance in the mitochondrial membrane potential, which allows the release of cytochrome c from the inter-membrane mitochondrial space to the cytoplasm. Cytochrome c is an essential protein for electron transport through the mitochondrial membrane. The release of cytochrome c from the mitochondria leads to activation of procaspases-9 (Li, Nijhawan, Budihardjo, Srinivasula, Ahmad, et al., 1997), with subsequent activation of caspase-3, caspase-6, and caspase-7. Like the extrinsic pathway, caspase-3 will cause the cleavage of various intracellular proteins, including nuclear proteins, proteins of the nuclear lamina, cytoskeletons, endoplasmic reticulum and cytoplasm, leading to cell death (Fraaser and Evan, 1996). Notably, caspase-3 is a key caspase in apoptosis because it is activated in both the extrinsic and the intrinsic pathways and its activation leads to cleavage of most intracellular proteins, leading to cell death.

The inflammatory response, the immune response and apoptosis can be initiated anywhere in the body as long as there is access to the cells of the immune system. The inner ear is involved in immune reaction, inflammatory reaction as well as apoptosis as a result of viral infection, systemic autoimmune disease, e.g., systemic lopus erythrematosis (Khalidi, , Rebello, Robertson, 2008) or due to ototoxic agents. Because IFNs modulate the inflammatory and the immune responses and also initiate apoptosis, IFNs can affect the inner ear and cause hearing impairment. To illustrate this issue, the next section will shed light on IFNs, their types, their sources, their biological effects, their clinical use, their side effects, and their effect on hearing as demonstrated by previous studies.

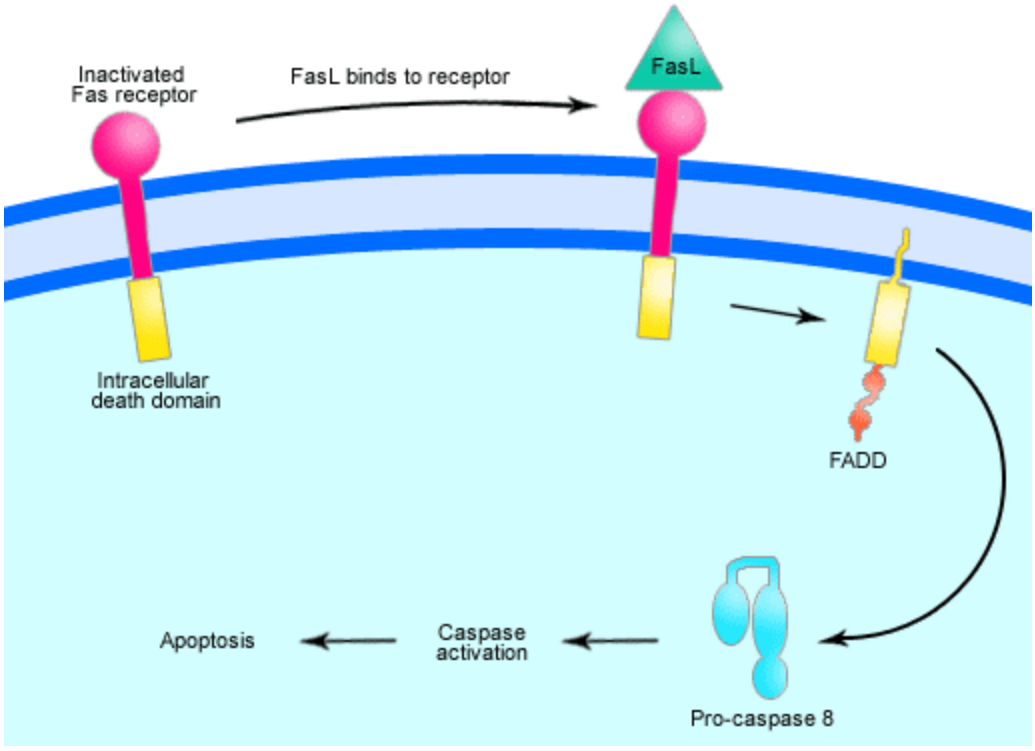

Figure 4: Extrinsic (Fas-FasL) of apoptosis

http://upload.wikimedia.org/wikipedia/commons/f/f5/Fas-signalling.png

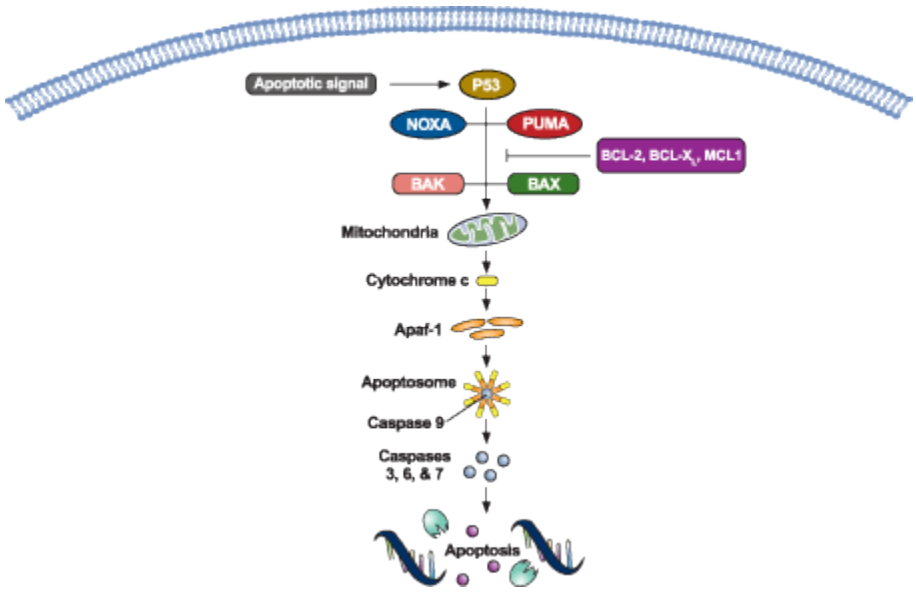

Figure 5: Intrinsic pathway of apoptosis

http://www.researchapoptosis.com/apoptosis/pathways/intrinsic/index.m

2.2 INTERFERONS

Interferons (IFNs) are categorized into three types, type I, type II (Aguet, Grobke, & Dreiding, 1984; Haque & Williams, 1989) and type III (Kotenko, Gallagher, Baurin, Lewis-Antes, Shen, et al., 2003; Sheppard, Kindsvogel, Xu, Henderson, Schlutsmeyer, Whitmore, et al., 2003; Vilcek, 2003). This classification is based on structural homology and cell-surface receptor-binding as well as functional activity. Type I IFNs include multiple members, only two of which are widely used clinically, interferon-alpha (IFN-α) and interferon-beta (IFN-β). All Type I IFNs (IFN-α & IFN-β) bind to the same receptors and are thought to elicit a common set of signaling events that ultimately regulates the expression of common biological activities (Petska, Langer, Zoon, & Samuel, 1987; Platanias, 1995). However, other studies show that IFN-α and IFN-β sometimes produces distinctive activities (Platanias, Uddin, Domanskii, & Colamonici, 1996).

On the other hand, type II- IFN is called interferon-gamma (IFN-γ) or immune IFN. It is distinctive from type I IFNs as it stimulates different types of receptors. Interferon-γ also is used clinically (Colonna, Krug, & Cella, 2002); whereas, type III (IFN-λ) has not yet been used clinically.

There is an association between IFNs and hearing impairment. Clinically, hearing impairment has been reported in patients treated with type I-IFN (IFN-α & IFN-β). Experimentally, type II-IFN (IFN-γ) is associated with autoimmune hearing loss and the induction of immune response in the inner ear. To further analyze these findings, an overview of both IFNs and their relation to hearing will be provided in the following sections.

2.2.1 Sources of IFNs in the body

Interferon alpha (IFN-α) is produced by many cell types in response to viral infection and virtually all cells are equipped to make type I-IFN. The main source, however, is the DCs, which play an important role in the immune response. Interferon-γ is secreted by the NK cells, macrophages, and T- lymphocytes, mainly CD4 Th1 cells. Since IFN-α is a prominent activator of NK cells, Th1 and macrophage, it can enhance the secretion of IFN-γ by these cells (Major, Dahari, Mihalik, Puig, Rice, et al., 2004; Tan, Derrick, Hong, Sanda, Grosse, Edenberg, 2005; Thimme, Bukh, Spangenberg, Wieland, Pemberton et al., 2002). Therefore, the effects of IFN-α on any organ or tissue can either be mediated by its own direct action or by enhancing the secretion of IFN-β or IFN- γ.

2.2.2 Signaling pathway of interferons

The signaling pathway of interferon is the sequence of events that are followed by IFNs to exert their biological activities. Three important terms will be introduced to explain the signaling pathway of IFNs; they include IFN receptors, Janus kinases, and STATs.

2.2.2.1 IFN receptors

IFN receptors are trans-membranous types of receptors because they are located on the surface of the cell and extend through the cell membrane to the cytoplasm, forming a cytoplasmic tail (Aguet et al., 1984). They are classified into two classes, class I, which is formed of α and β subunits (IFN-αR1 & IFN-αR2) and class II, which is formed of two subunits

(IFN-γR1 & IFN-γR2), corresponding to IFN-I and IFN-II respectively (Caraglia , Marra , Pelaia , Maselli , Caputi , Marsico, & Abbruzzese, 2005).

2.2.2.2 Janus Kinases

JAK1, JAK2, and Tyk2 are members of the Janus kinases family, which are enzymes that donate phosphate groups to their substrates to be activated (Ihle, Witthuhn, Quelle, Yamamoto, Thierfelder, et al., 1994).These kinases are connected to the IFNs' receptors and hence they will be activated once the receptors are activated by IFNs (Leonard, 2001).

2.2.2.3 STATs

The STATs are signal transducers and activators of transcription, a family of inactive cytoplasmic proteins that are named by the virtue of their novel dual function as they transfer signals from the receptors to the nucleus and activate transcription factors after they move to the nucleus. Transcription factors are the proteins that are involved in gene expression. There are seven known mammalian STAT proteins; only STAT1, STAT2, are involved in the pathway of IFN-α/ β and IFN-γ (Chen, Vinkemeier, Zhao, Jeruzalmi, Darnell, & Kuriyan 1998).

The most common IFN signaling pathway is the JAK-STAT pathway (Figure 6). Briefly, when IFN molecules bind to their receptors, the Janus kinases will be activated (Jak1and Tyk-2 in the case of type I-IFN and Jak1 and Jak2 in the case of type II-IFN), leading to phosphorylation and activation of STAT1 and STAT2 (Leonard and O'Shea, 1998). Both STAT1 and STAT2 quickly form homodimers (i.e., forming two identical STATs, STAT1& STAT1) in the case of type II-IFN or hetrodimers (i.e., forming two different STATs, STAT1& STAT2) in the case of type I-IFN. The activated STAT molecules dissociate from the receptors and move to the nucleus (Chatterjee-Kishore, Kishore, Hicklin, Marincola, & Ferrone, 1998),

where they bind to a transcription factor called P48 and form a complex called interferon-stimulated gene factor-3 (ISGF-3). The ISGF-3 will bind to a specific sequence on the gene called the interferon stimulated response element (ISRE) in case of type I, and gamma activating sequence (GAS) in case of type II-IFN, leading to expression of that gene. Therefore, the activation of signaling cascades mediated by IFNs will ultimately result in a new gene expression to induce certain biological changes in the target cell (Decker et al., 1991).

2.2.3 Biological effects of interferons

Interferons have many biological properties such as modulation of inflammatory response, modulation of immune response, and inhibition of cell proliferation (they can stop cell growth or lead to cell death). With regard to its effect on inflammatory reaction, interferons have a potent antiviral activity, and hence they are widely used in the treatment of many viral infections. Nowadays, IFN-α is the cornerstone therapy for chronic viral hepatitis, including hepatitis B and hepatitis C. Because of its modulating effect on the immune response, IFN-β, currently, is the most widely used therapy for multiple sclerosis (Jacobs, Cookfair, Rudick, Herndon, Richert, et al., 1996).

As an antiproliferative, many studies have shown that IFNs (IFN-α, IFN- β, and IFN- γ) can induce the regression of a number of tumors including hairy cell leukemia (Ahmed, Kanti, and Rai, 2003), chronic myelogenous leukemia, non-Hodgkin lymphoma (Ezaki, 1996), Kaposi's Sarcoma (Stadler, 1998), renal cell carcinoma (Gleave, Elhilali, Fradet, Davis, Venner,

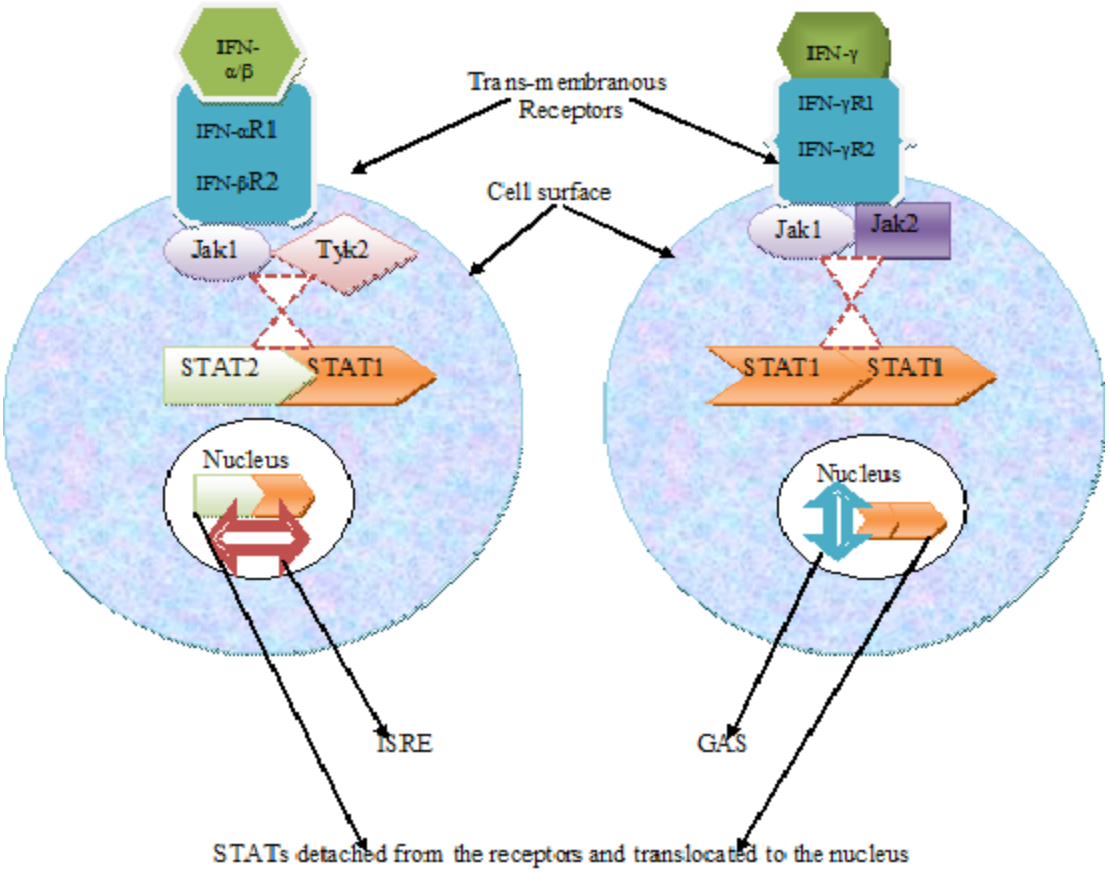

Figure 6: Interferon-mediated Jak-STAT pathway

Saad, et al., 1998), malignant melanoma (Balch, Soong, Gershenwald, et al., 2001), and breast carcinoma (Kamamura, Takahashi, Komaki, & Monden, 1998).

Since inflammatory response, immune reaction and cell death are mechanisms by which IFNs can induce their effects on any tissue, in general and inner ear in particular, the following sections will highlight these mechanisms and their contribution to hearing impairment.

2.2.3.1 Interferons as inflammatory modulators

There is increasing evidence that IFN-α/β and IFN-γ interfere with the synthesis or the release of other cytokines such as interleukin-1 (IL-1) and prostaglandin (both are primary inflammatory cytokines) causing fever and hypotension (Dinarello, 1996; Dinarello, 1984). Thus, both IFN-α and IFN-γ can be used as an anti-inflammatory; especially in treatment of disease mediated by IL-1 (Browning & Ribolini, 1987). On the other hand, both IFN-α/β and IFN- γ increase the expression of intercellular adhesion molecules (ICAM-1) (Sundelin, Roberg, Grenman, & Hakansson, 2007). ICAM-1 molecules are known to enhance the inflammatory responses by increasing migration of neutrophils from peripheral blood to the site of inflammation. Therefore, the ICAM-1 molecules can be used as a marker for the action of IFN-α and IFN- γ as inflammatory modulators. Also it has been found that IFN-α therapy promotes the production of TNF-α, which makes TNF-α a marker for the inflammatory response mediated by IFN-α (Ren, Jin, Piao, & Piao, 2007).

2.2.3.2 Interferons as immune modulators

The contributions of IFNs to the immune response involve the activation of the immune system cells as well as the increased secretion of other cytokines that can affect the immune response. For instance, IFN-α induces activation of NK cells, which are the key cells in the

innate immune response (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999). IFN-α also activates CD4+ and CD8+ T-cells and enhances their cytotoxicity, and thus promotes the adaptive immune response (York, Odom, Murphy, Ives, and Wente, 1999). Another immune cell stimulated by IFN-α is the DC, which is considered a professional APC (Cella, Facchetti, Lanzavecchia, & Colonna, 2000). Also, IFN-α and IFN-γ are potent inducers of the major histocompatibility complexes (MHC class I and class II), and thus they enhance antigen presentation to T cells (Biron, 1998). The MHC molecules are glycoprotein molecules encoded by a cluster of closely linked genes that are located on chromosome 6 in human. This region on the chromosome is called major histocompatibility complex because it causes T cells to reject tissues transplanted from unrelated donors to recipients. The MHC molecules are referred to as human leukocytic antigen (HLA) in humans. They are categorized into two classes, class-I and class-II, the expression of which is enhanced by IFN-α and IFN-γ, respectively. Hence, an increase of the expression of MHC will indicate the immune-modulating effect of IFNs. MHC-I molecules encoded by H2K1gene and present on the surface of the APCs, which function to hand off processed antigens to phagocytic cells. The APCs break down the antigens into simple peptides and use the MHC to present these peptides to phagocytic cells for phagocytosis and killing.

In addition, IFN-α stimulates the expression of other cytokines, which ultimately affects the function and activity of many immune cells. The most important cytokine that is regulated by IFN-α is interleukin-15 (IL-15), which stimulates NK cell and cytotoxic T cell proliferation, leading to enhancement of both innate and adaptive immunity (Zhang, Sun, Hwang, Tough, Sprent,1998; Durbin, Hackenmiller, Simon, & Levy, 1996). Stimulation of NK cells by IFN-α results in an increased IFN-γ level, which is essential for macrophage activation as well as T cell

stimulation (Biron, 1999). Through the activation of the immune system cells and increasing the secretion of IL-15 and IFN-γ, IFN-α will be able to initiate a global immune response and function as a key modulator of both innate and adaptive immunity.

From a clinical point of view, IFN-α production is associated with several autoimmune diseases, e.g., systemic lupus erythematosus (Farkas, Beiske, Lund-Johansen, Brandtzaeg,& Jahnsen, 2001), insulin-dependent diabetes mellitus (Devendra and Eisenbarth, 2004), rheumatoid arthritis (Lande, Giacomini, Serafini, Rosicarelli, Sebastiani, et al,. 2004), psoriasis (Nestle FO et al., 2005), and Sjögren's syndrome (Gottenberg, Cagnard, Lucchesi, Letourneur, et el., 2006). Evidence of this association is the presence of auto-antibodies in the serum of patients treated by interferon, development of an autoimmune disease, or the exacerbation of an existing one in those patients (Gota and Calabrese, 2003). IFNs contribute to the breakdown of central tolerance to the body's tissue, leading to stimulation of an immune or autoimmune response, a sequence that might occur in the inner ear.

2.2.3.3 Interferons as antiproliferative agents

The antiproliferative effect of IFNs is mainly due to their ability to induce programmed cell death or apoptosis. Apoptotic responses have been reported in response to both type I (IFN-α & IFN-β) and type II (IFN-γ) in tumor cell lines and tumor cells. IFN-induced cell death involves activation of the extrinsic pathway as well as the mitochondrial (intrinsic) pathway. In particular, IFN-α induces Fas expression (extrinsic pathway) in malignant melanoma, leukemia and basal cell carcinoma, with subsequent cell death (Fulda and Debatin, 2002; Inaba, Glibetic, Buck, Ravindranath, & Kaplan, 2004). IFN-α also can lead to the release of cytochrome c and the activation of caspase-9, with subsequent activation of downstream caspases such as caspase-3,

caspase-6, and caspase-7 (intrinsic or mitochondrial pathway of cell death) (Thyrell, Erickson, Zhivotovsky, et al., 2002). Both pathways are activated by some ototoxic agents, e.g., cicplatin (Devarajan et al., 2002) and aminoglycosides (Li, et al., 1995).

2.2.4 Side effects of interferon therapy

Although IFNs have been successful as potent antivirals, immunomodulators and anticancer agents, treatment with IFN carries significant risks (Heathcote & Main, 2005). Many side effects have been reported with IFN therapy, including flu-like symptoms, depression, and autoimmune reactions (Lisker-Melman, Di Bisciglie, Usala, et al., 1992; Marcellin, Pouteau, Benhamou, 1995; Benelhadj, Marcellin, Castelnau, et al., 1997). Other studies reported hemolytic anemia and abnormal coagulation in about 50% of patients treated with IFNs. These side effects are thought to be due to autoimmune reactions that lead to the destruction of RBCs and platelets (Poynard, Bedossa, Chevallier, et al., 1995; Becker, Winkler, Klingert, et al., 1994; Vial & Descotes, 1994).

The inner ear is a site for adverse reactions of many medications, e.g., aminoglycosides, loop diuretics, and cisplatin, which can act by the same mechanisms as IFNs. Therefore, it can be hypothesized that the inner ear may have IFN-induced side effects. A considerable number of studies have found an association between hearing impairment and IFN therapy. The following section will address this association and review the findings of these studies.

2.2.5 Effect of type I interferons (IFN-α/β) on hearing

Studies concerned with the effect of type I-IFNs on the auditory system are inconsistent. A considerable number of studies have documented the occurrence of sensorineural hearing loss (SNHL) in patients treated with IFN-α (Cadoni, et al., 1998; Elloumi, et al., 2007; Formann, et al., 2004; Johnson, et al., 2008; Kanda, et al., 1994; Le, Bader, & Fazili, 2008; Okanoue, et al., 1996; Piekarska, et al., 2007; Sachar and Polio, 2004; Salkic, et al., 2009; Wong, et al., 2005). The incidence of hearing impairment due to IFN-α was reported more frequently in patients with hepatitis C than in patients receiving IFN-α for other diseases. The deteriorating effect of IFN-α on hearing also was described in patients with chronic hepatitis B (Gorur, Kandemir, Unal et al., 2003; Tunca, Erbayrak, Aytac, Turkay, 2004). The hearing loss was described as unilateral (in most cases) (e.g., Johnson et al., 2008) or bilateral (e.g., Salkic et al, 2009), reversible (Elloumi et al, 2007; Gorur, et al., 2003; Kanda et al., 1994) or irreversible (Okanoue, et al., 1996; Sachar and Polio, 2004; Wong et al., 2005). The reported cumulative dose that was found to be associated with hearing loss due to IFN-α was around 100 million units (Cadoni et al., 1998; Gorur, et al., 2003; Kanda, et al., 1995)

On the contrary, other studies showed no toxic effect of IFN-α on hearing. For example, Kaygusuz, et al. (2004) found no effect of IFN-α on the hearing threshold of patients with chronic active hepatitis B. The study included 26 subjects treated with IFN-α.2b in a dose of 10 million units every other day for 6 months. Although the cumulative dose used in their study was higher than the cumulative dose that was found to be associated with hearing loss in other studies, no hearing loss was noticed in any of those patients. The authors attributed these findings to the small number of patients. Nonetheless, few patients complained of tinnitus, which

might suggest the presence of subtle hearing impairment. The audiological testing (conventional audiometry) used in Kaygusuz et al.'s study is not the most accurate method to detect subtle hearing loss. A more precise tool such as otoacoustic emissions might be useful in detecting subtle changes in the cochlea in such patients.

Akyol, et al. (2001) failed to find pathological changes in the organ of Corti of albino Swiss mice treated by IFN-α. The authors used a single dose of IFN, either in the form of 100,000 or 50,000 units in two groups of albino Swiss mice and compared the findings between both groups and a control group (received no IFN). The auditory brainstem response (ABR) findings showed a statistically significant shift in the hearing threshold between baseline and post-medication in the treatment group. In addition, a pairwise comparison revealed a significant difference in the hearing threshold between the groups that received IFN and the control group. Microscopic examination of the cochlea of mice treated with IFN revealed atrophy of the lateral wall as well as marked cytoplasmic vacuolation of the epithelial cells of the stria vascularis. However, no pathological changes were observed in the sensory elements of the organ of Corti (outer and inner hair cells). IFN-α may cause reversible biochemical and metabolic changes in the cochlea, rather than morphologic abnormalities. This might be consistent with the well-known reversible course of INF-induced hearing loss in many patients.

In addition, Akyol et al. (2001) used a single dose of IFN, which might not be enough to induce any hair cell damage. Most ototoxic drugs cause hair cell damage as a result of cumulative effects of the drug. In addition, if the underlying mechanism of hair cell damage is assumed to be of autoimmune origin, as has been suggested by other investigators, this response usually needs repeated doses to be elicited (Suzuki, Krug, & Cheng, 1997). Therefore, using a

single dose cannot be expected to induce significant damage to the organ of Corti, which supports the need for a study that uses repeated doses of IFN to further delineate this issue.

Nonetheless, there is evidence that IFN-α can be used for treatment of SNHL that might be of viral or autoimmune origin. For example, Kanemaru, et al. (1997) used IFN-α for the treatment of idiopathic sudden SNHL that might be due to viral infection. The authors noticed that 27 out of 42 patients (64.3%) with idiopathic SNHL showed complete recovery after IFN -α in a dose of three million units (IU) per day for 10 days. In addition, they found a significant increase in activity of 2', 5' oligoadenylate synthetase (an enzyme that indicates the antiviral effect of IFN) in 24 out of the 27 patients who showed recovery of hearing loss that was on average ≥70 dB before the treatment. The authors suggested that IFN-α can be effective as a mono-therapy for the treatment of idiopathic SNHL as such as it can replace the steroid therapy. The use of IFN-α in the treatment of idiopathic SNHL will be beneficial in patients who have limitations of using steroids, e.g., patients with diabetes mellitus. It is worth noting that IFN-α-induced SNHL was reported at doses higher than 10 million U, which is more than three times the therapeutic dose in idiopathic SNHL. This means that the effect of IFN-α on hearing could be dose-dependent, while relatively small doses were effective in treatment of SNHL, large doses, which are usually used for treatment of viral hepatitis, for example, might cause hearing loss.

Several mechanisms have been suggested as underlying causes for hearing loss in patients treated with IFN (Akyol et al., 2001; Cadoni et al., 1998; Forman et al., 2004; Fuse, Hayashi, Oota, Fukase, Asano, et al., 2003; Lorenz et al., 2002; Tunca et al., 2004). Among these mechanisms are the direct toxic effect of INF on cochlear cells (Calvet & Gresser, 1979) and immune response, including the inflammatory response (Sarraco, Touscoz, Durazzo, et al., 1990). In addition, impaired platelet function by IFN might lead to hemorrhage or vasculitis with

subsequent ischemia of the stria vascularis (hematologic effect) (Poynard, Bedossa, Chevallier, et al., 1995).

The first possible mechanism is the direct toxic effect of IFN-α on the inner ear, which might occur through the induction of apoptosis of the cellular components of the cochlea. The Fas-FasL apoptosis pathway is the most commonly described pathway in association with IFN-induced apoptosis (Choi, Park, Lee, Lim, Shin, et al., 1999; Fulda and Debin, 2002; Inbal, Cohen, Polak-Charcon, et al., 1997). There is an increase in FasL expression in the inner ear in response to induction of labyrinthitis or following stimulation with IFN-γ, which confirm the presence of FasL receptors in the inner ear (Bodmer, Brors, Pak, Keithley, Mullen et al. (2002, 2003). IFN-α enhances the secretion of IFN-γ. Therefore, it can be argued that IFN-α can lead to apoptosis of the cells of the inner ear, either directly by its own apoptotic effect or by enhancing the secretion of IFN-γ. IFN-α also can lead to the release of cytochrome c and activation of the intrinsic or mitochondrial pathway of cell death (Thyrell et al., 2002), which also occurs in the inner ear (Devarajan et al., 2002; Li et al., 1995).

The immune response (including the inflammatory reaction) is the second mechanism by which IFN-α can cause damage to the inner ear. The contribution of IFN-α to the immune system, as has been mentioned in a previous section, involves the activation of cells of the immune system and/or the increased secretion of other immunomodulatory cytokines e.g., IFN-γ, as well as the increased expression of MHC-I. Many studies have documented that the immune response contributes to the etiology of a growing number of inner ear disorders (Harris & Sharp, 1990; Arnold et al., 1987; McCabe, 1979). The evidence of autoimmunity in patients with SNHL has been supported by the presence of autoantibodies against different inner ear proteins in the sera of those patients (Suzuki, Krug, & Cheng, 1997).

In addition, increased infiltration of both CD4+ and CD8+ was observed in patients with suspected autoimmune sensorineural hearing loss (ASNHL) (Mayot, Bene, Dron, Perrin, Faure, 1993). Natural killer and Th1 cells, which are significant components of the immune system, are associated with immune-mediated inner ear diseases and Meniere's disease; the proliferation of these two cells is stimulated by IFN-α (Fuse, Hayashi, Oota, Fukase, Asano et al., 2003). This cellular infiltration usually results in an inflammatory reaction with concomitant formation of a dense extracellular matrix associated with fibrosis (Yamane, Iguchi, Konishi et al., 1995). Therefore, the outcome of the immune response could be the degeneration of the organ of Corti, stria vascularis, and spiral ganglion neurons (Berrocal & Ramirez-Camacho, 2000).

It can be speculated that IFN-α can provoke an immune response in the cochlea either directly through activation of cells of the immune system or through production of IFN-γ. This speculation is supported by many studies (Cadoni, Agostino, Manna, Santis, Fetoni, et al., 2003; Cadoni et al., 1998; Ottaviani, Cadoni, Marinelli, Fetoni, De Santis, et al., 1999). For example, Ottaviani, et al. (1999) found anti-endothelial cell antibodies in 15 out of 32 (47%) patients who had sudden hearing loss and in two out of 14 of the controls (14%). This suggested an association between sudden hearing loss and immune-mediated microvascular damage. These anti-endothelial cell antibodies react against surface antigens on the endothelial cells of the inner ear blood vessels, leading to their damage via a complement-mediated or antibody-dependent cellular cytotoxic mechanism. The same effect has been detected in retinal vascular lesions during interferon therapy (Guyer, Tiedeman, Yannuzzi, et al., 1993). Therefore, it can be argued that interferon therapy can provoke an immune response in the inner ear, resulting in hearing impairment.

The third possible mechanism of IFN-induced hearing impairment is hemorrhage or vasculitis with subsequent ischemia of the stria vascularis. Anemia and thrombocytopenia (decreased platelet counts) are among the side effects of IFNs, where they occur in about 50% of patients receiving IFN-α (Poynard, et al., 1995). Ogawa & Kanzaki (1994) observed a sudden sensorineural hearing loss in some patients who were receiving IFN-α and had aplastic anemia and thrombocytopenia. Those patients experienced total unilateral hearing loss accompanied by tinnitus, severe vertigo, and nausea. Ischemic disorders in the stria vascularis, which plays an important role in the nutrition of the organ of Corti, might result in SNHL.

In conclusion, IFN-α can affect the cochlear structures either directly by the virtue of its immune, inflammatory, and apoptotic effects or indirectly by enhancing the secretion of IFN-γ. To date, no clinical data have suggested that IFN-γ has any effect on hearing. However, many experimental studies have shown that IFN-γ can affect the cochlear structures. Therefore, the following section will review the findings presented in these studies.

2.2.6 Effect of type II interferon (IFN-γ) on hearing

Although according to the available literature, hearing loss was not reported with the exogenous (therapeutic) use of IFN-γ in the clinical arena, IFN-γ has been studied at the molecular level in the inner ear in experimental animals and tissue culture. Indeed, exogenous IFN-γ was found to be associated with an increase in ICAM-1 molecules (Pawankar, Tomiyama, Jinnouchi, Ikezono, et al., 1998), MHC class II molecules (Gloddek, Bodmer, Brors, Keithley, and Ryan, 2002), and Fas-FasL molecules (Bodmer et al., 2003) which reflect inflammatory, immune and apoptotic responses, respectively in the inner ear.

2.2.6.1. Interferon-γ as an inflammatory modulator in the inner ear

IFN-γ can be expressed in various parts of the inner ear as a result of induction of an antigen with subsequent expression of inflammatory molecules—ICAM-1. For example, Pawankar, Tomiyama, Ikezono, Nonaka, Jinnouchi, et al. (2004) found increased expression of IFN-γ in different parts of the inner ear of systemically pre-sensitized rats after keyhole limpet hemocyanin (KLH) antigen challenge into the endolymphatic sac. The amount and the timing of IFN-γ expression were correlated with the expression pattern of the ICAM-1 molecules, with subsequent recruitment of inflammatory cells into the inner ear. These adhesion molecules play an important role in the cellular interaction during the immune or the inflammatory response (Dustin, Singer, Tuck, & springer, 1988). ICAM-1 is strongly expressed in many sites of the cochlea after KLH antigen challenge into the endolymphatic sac (Pawankar, et al., 1998). These observations demonstrate that antigen challenge into the endolymphatic sac induces IFN-γ expression, which can then upregulate (enhance) ICAM-1 expression and induce cell infiltration, suggesting that IFN-γ may play a crucial role in inflammatory/immune-mediated inner ear diseases.

2.2.6.2 Interferon-γ as an immune modulator in the inner ear

IFN-γ is a potent inducer of major histocompatibility complex class II (MHC-II), which has a critical role in initiation of immune responses, where they present the processed antigens to lymphocytes for phagocytosis (Cresswell, 1994). For example, Takahashi and Harris (1988) indirectly indicated the presence of MHC class II by showing the invasion of the cochlea by macrophages, which produce MHC class-II following immune response. The presence of MHC-II was directly demonstrated in the normal human endolymphatic sac (removed at autopsy) by

using immunohistochemical study with anti- MHC-II antibodies (Altermatt, Gebbers, Muller, Arnold, & Laissue, 1990). Since MHC molecules are located on the APCs, including macrophages and dendritic DCs, this indicates the presence of these cells in the cochlea (Tomiyama & Harris, 1987). Dendritic cells (DCs) were found to produce between 200-1000 times more IFN-α than any other white blood cells, which might indicate the possibility of production of IFN-α in the cochlea.

In addition, Gloddek et al. (2002) used fluoresce-activated cell sorter (FACS) to study the effect of induction of sterile labyrinthitis and repeated doses of IFN-γ on the inner ear structures. They found significant increase in MHC-II molecules in the cochlear explants of the lateral wall, organ of Corti, modiolus, and spiral ganglia versus the untreated explants. They concluded that MHC-II molecules play an important role during inflammatory and immune responses of the cochlea, which might lead to irreversible damage of cochlear structures. Gloddek et al. (2002), in light of their findings, attributed the hearing loss associated with IFNs administration to their robust immunological effects on many cochlear structures.

In addition, Lorenz, Solares, William, Sikora, Pelfrey et al. (2002) demonstrated the presence of IFN-γ producing T-cells in the peripheral blood of patients with autoimmune SNHL versus control subjects whose peripheral blood showed no evidence of IFN-γ-producing T-cells. Moreover, by using the enzyme-linked immunospot (ELISPOT), Solares and Tuohy (2005) observed an increase of peripheral blood IFN-γ-producing T-lymphocytes in patients with autoimmune SNHL. Therefore, they suggested that IFN-γ is involved in the pathogenesis of autoimmune SNHL.

2.2.6.3. Interferon-γ as an inducer of apoptosis in the inner ear

Interferon-γ increases the expression of the Fas-FasL complex in the inner ear. For example, Bodmer, et al. (2002) used FACS to demonstrate the effect of repeated doses of IFN-γ and the induction of immune-mediated labyrinthitis in the inner ear cells. They found an increase of Fas-FasL from 3-8% in untreated inner ear cells to 35-53% in those treated with IFN-γ, with maximum expression of Fas-FasL in the lateral wall, organ of Corti, and modiolus after 24 hours, and in the spiral ganglion after 48 hours. There was also a moderate FasL immunostaining in marginal cells of stria vascularis and spiral ganglia, whereas, there was a weak FasL reactivity in the supporting cells of the organ of Corti.

In another study, Bodmer et al. (2003) analyzed the cloned DNA (cDNA) generated from unstimulated and IFN-γ-stimulated organ of Corti by using real time Polymerase Chain Reaction (PCR). They found no changes in transcription of the gene encoding Fas; however, confocal microscopy showed dramatic changes in the distribution of Fas in the cellular components of the organ of Corti as a result of IFN-γ-stimulation. The distribution of Fas following IFN-γ-stimulation was consistent with the recruitment of preformed Fas molecules from intracellular compartments, e.g., endoplasmic reticulum into the cytoplasm and the plasma membrane rather than its biosynthesis as a consequence of genetic expression. The authors suggested that the presence of preformed Fas makes its distribution on the cell membrane more rapid than if it resulted from genetic expression. Furthermore, they concluded that expression of Fas on the plasma membrane might indicate the involvement of Fas in the protection of the inner ear from stress by autoimmune or inflammatory responses. Supporting cells were the cells that have this protective role because they showed marked distribution of Fas, in contrast to the hair cells that showed no labeling with Fas antibodies. This protective role of Fas also was found in the anterior

chamber of the eye (Griffith, Yu, Herndon, Green, & Ferguson, 1996). In addition, mice with deficient Fas genes show progressive hearing loss. This hearing loss may be due to autoimmunity because it was associated with progressive accumulation of autoreactive T-lymphocytes, degeneration of stria vascularis cells, and progressive endolymphatic hydrops (Trune, Craven, Morton & Mitchell, 1989; Ruckenstein, Keithley, Bennett, Powell, Baird & Harris, 1999). This reaction means that absence of Fas makes the inner ear more vulnerable to the immune response. However, the increased expression of Fas has been observed in cochlear cell culture exposed to cisplatin, which is known for its ototoxicity (Van de Water, Nguyen, Shoemaker, Schipor, et al., 2001). Therefore, the Fas-FasL complex may participate in the protection of cochlear hair cells or it may play an important role in inner ear pathology (Bodmer et al., 2003). IFN-γ, which increases Fas expression, can be in turn protective or pathologic to the cochlea. Further investigations are needed to delineate this suggestion. Table 1 summarizes the studies concerned with the effect of IFN-α/β and IFN-γ on hearing.

Table 1: Summary of the studies concerned with IFN and hearing

Reference	Type of IFNs	Type of study	Findings/comments
Kanda & Shigeno,(1994)	IFN-α or IFN-β	Prospective (Hepatitis C&B)	HL was more with IFN-β than IFN- α..
Kanda et al. (1995)	IFN-α or IFN-β	Prospective (Hepatitis C&B)	No difference between the effect of IFN-α or IFN-β on hearing.
Cadoni et al(1998)	IFN-α	Case study	Anti-endothelial cells antibodies detected in the patient's serum.
Okanoue, et al. (1996)	IFN-α	Retrospective (Hepatitis C)	Irreversible SNHL in one patient out of 677
Kanemaru, et al. (1997)	IFN-α	Prospective	IFN-α was used in the treatment of idiopathic SNHL.
Akyol, et al. (2001)	IFN-α	Experimental on mouse cochlea	There was a significant rise in hearing thresholds in the IFN-treated groups by ABR. Histologically, there were a decreased number of fibroblasts in the spiral limbus, with cytoplasmic vacuolation No loss of hair cells.
Görür, et al. (2003)	IFN-α	Prospective on Hepatitis B patients	IFN-α in patients with hepatitis B may cause mild reversible SNHL.
Formann et al.(2004)	IFN-α	Retrospective of 4 patients	Irreversible SNHL.

Reference	Type of IFNs	Type of study	Findings/comments
Sachar and Polio (2004)	IFN-α	Case study	Reversible SNHL.
Wong et al. (2005)	IFN-α	Case study	Irreversible unilateral severe SNHL.
Elloumi et al.(2007)	IFN-α	Case study	Reversible SNHL.
Piekarska et al. (2007)	IFN-α	Case report	Irreversible bilateral SNHL in a 27-year-old patient with Turner syndrome .
Johnson et al. (2008)	IFN-α	Case report	Irreversible severe SNHL, tinnitus and Vertigo.
Atug, et al. (2009)	IFN-α	Case report in patient with chronic hepatitis C	Unilateral reversible SNHL.
Le, Bader, Fazili. (2009)	IFN-α	Case report patient with chronic hepatitis C	Unilateral SNHL, Prednisone while maintaining IFN-α.
Salkic et al. (2009)	IFN-α	Case report in patient with chronic hepatitis C	Reversible SNHL.
Bodmer, et al. (2002)	IFN-γ	Experimental animals IFN-γ and the induction of sterile labyrinthitis	Expression of Fas-FasL in the lateral wall, organ of Corti, and modiolus, and the spiral ganglia.

Reference	Type of IFNs	Type of study	Findings/comments
Gloddek et al. (2002)	IFN-γ	Experimental animals Induction of sterile labyrinthitis and repeated doses of IFN-γ	Increase in MHC- II molecules in the cochlear explants of the lateral wall, organ of Corti, modiolus, and spiral ganglia.
Pawankar et al. (2004)	IFN-γ	Experimental animals systemically pre-sensitized rats after KLH antigen into the endolymphatic sac.	IFN-γ in different parts of the inner ear was associated with the expression of ICAM1.

2.3 SUMMARY OF THE LITERATURE REVIEW

In summary, data concerning the effect of IFNs is inconsistent with a wide range of variability; whereas some studies documented their pathological effects on the inner ear, others documented their therapeutic usefulness in management of SNHL. Different mechanisms were suggested for the effect of both IFN-α/β and IFN-γ. The first mechanism is the direct toxic effect on the cellular components of the cochlea that lead to cell death as has been reported with different ototoxic agents, such as aminoglycosides and cicplatin. The immune response—including the inflammatory reaction—is the second mechanism by which both IFNs can affect the inner ear. The third mechanism is the hematological effect with the resultant hemorrhage and vasculitis of the stria vascularis (Stria vascularis will not be included in the cochlear cell line used in the current study). Ultimately, the outcome of these different mechanisms (i.e., inflammatory, immunologic, or hematologic) can be assumed to be programmed cell death of the cochlear structure. Therefore, IFN-α can lead to apoptosis of the cochlear cells either directly or through provoking an immune and/or inflammatory response. This conclusion can be supported by the following evidence:

- Various reports have shown that an autoimmune response, either cell-mediated (produced by T-lymphocytes) or antibody-mediated (induced by B-lymphocytes), results in enhancement of the Fas expression with subsequent cell death of the target cells (Ju, Cui, Panka, Ettinger, and Marshak-Rothstein, 1994). These findings mean that if IFNs initiate

an immune response in the inner ear, apoptosis of the inner ear cells can be assumed to be the result of this immune response.

- The ability of Fas itself to induce cell death and its involvement in the IFN-induced apoptosis suggests that IFNs can cause Fas-FasL-induced apoptosis of the cochlear structures through the extrinsic pathway.
- Moreover, activation of the STAT1, or interferon regulatory factor 3 (IRF-3) (both are included in the IFN signaling pathway), is associated with nitric oxide (NO) and reactive oxygen species (ROS) production, with subsequent apoptosis. The production of ROS is the underlying mechanism of aminoglycosides (Priuska and Schacht, 1995) and cisplatin-induced apoptosis (Kopke, Liu, Gabaizadeh, Jacono, Feghali, Spray, et al., 1997). Therefore, activation of STAT1 by IFNs might lead to increased production of NO and ROS, with subsequent apoptosis of the cochlear cells by the intrinsic pathway of cell death.
- Furthermore, as it has been mentioned in a previous section of the current review, the inflammatory and/or the immune response will result in cell death, either by necrosis or apoptosis. Mixed necrotic and apoptotic cell death can be found as the pathogenesis of the same disease. Some evidence supports that different types of cell death can share common mechanisms (Clarke, 1990; Demarchi, Bertoli, Copetti, Tanida, Brancolini, Eskelinen, et al., 2006; Yasuhara, Asai, Sahani, Martyn, 2007).

Figure 2.7 summarizes the effects of both IFN-α (directly or indirectly) through increasing of IFN- β and/or IFN- γ on the inner ear structures and shows a theoretical outcome for these effects, which is cell death, apoptosis/ necrosis.

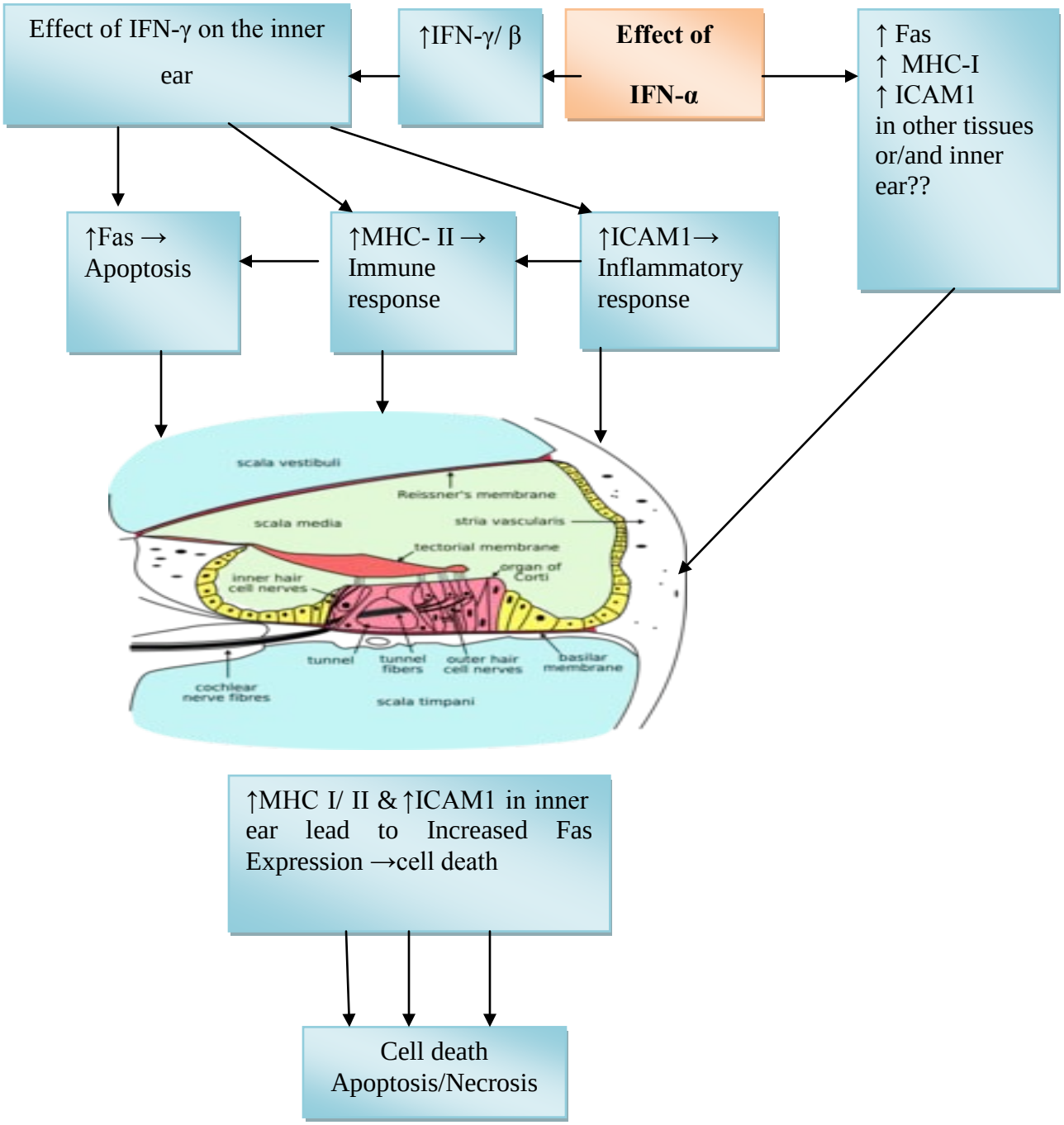

Figure 7: Schematic diagram showing the predicted effect of IFN on the cochlea

MHCI/II= major histocompatibility class I/II.

ICAM1= Intercellular adhesive molecules. ↑ = increase level. → = result in

2.4 SIGNIFICANCE AND GOALS OF THE PRESENT STUDY

The current study is designed to address the following unresolved issues with regard to the effect of IFN-α on the auditory system.

First, clinical findings have suggested an association between hearing loss and IFN-α therapy; yet, the underlying mechanism of this association is still unclear. Despite increasing evidence for the association of hearing loss with IFN-α, the only experimental study exploring this association, to date is that of Akyol, et al. (2001). The authors investigated the effect of IFN-α, using audiological and histological approaches which might not be the most accurate approaches to precisely rule out the influence of IFN-α on the inner ear—an effect that can be determined more precisely at the molecular level. Therefore, there is a need for a study that can explore the effect of IFN-α on the inner ear at a molecular or genetic level.

Second, from a clinical point of view, because IFNs are widely used today in the clinical arena, it is important to ensure the safety of such medications to reduce any subsequent handicap (in this case, hearing impairment). Regular monitoring of the hearing function might be required in those patients, who are receiving IFNs, but the underlying mechanism first must be explored; otherwise the particular monitoring used might be misguided. Most of the clinical studies concerned with the effect IFN-α has on hearing are retrospective studies; no prior hearing evaluation was obtained before IFN-α therapy.

Third, and in general, understanding the molecular and cellular signals that regulate and control the effect of IFNs on the cochlear structures will help in understanding the origin and mechanism of many inner ear diseases, especially that of autoimmune origin. This knowledge might lead to developing effective methods for preventing unwanted side effects on the inner ear

and opening an avenue for new therapeutic agents to manage many inner ear disorders. In addition, these findings help in identifying novel diagnostic markers for susceptibility, severity, and outcomes of any cochlear pathology.

To possibly delineate these issues, the current study was designed to accomplish two main goals. The first goal was to investigate if the cochlea has receptors for IFN-α and if IFN-α would exert its effect directly through stimulation of these receptors or indirectly through the enhanceing of the expression of other members of type I-IFN (e.g. IFN-β) or type II-IFN (IFN-γ). The second goal was to determine the mechanism by which IFN-α can affect the cochlear structures, and if these mechanisms can affect the viability of the cochlear cells. The first goal was achieved by examining the expression of IFN-α1, IFN-β, and IFN-γ. The second goal was achieved by examining the expression of those genes that might be involved in the induction of inflammatory response (ICAM1 and TNF-α), immune response (MHC-1), and apoptosis (Fas gene, Caspase-3, and Bax genes). In particular, the following research questions will be addressed:

1- Does the cochlea have receptors for IFN-α?
2- Does IFN-α act directly on the cochlear cells or by induction of the expression of IFN-γ and/or IFN-β in the cochlear cell line?
3- Does IFN-α induce expression of inflammatory markers, markers for immune response and/or markers for apoptosis?
4- Does IFN-α affect cell viabiliy (cell count) in the cochlear cell culture?
5- Are the effects of IFN-α dose and/ or time dependent?

3.0 CHAPTER III: METHODOLOGY

3.1 EXPERIMENT-1

3.1.1 Experimental Design

To accomplish the stated goals, a cochlear hair cell line called HEI-OC1 (House Ear Institute, Los Angeles, CA) was used as an in-vitro system to examine the molecular events associated with treatment of the organ of Corti cells with IFN-α. The cells obtained from the culture were treated with IFN-α in three different doses (20, 40, and 80 units/ml of culture media) to test if the effect of IFN- α is dose dependent. The dose was calculated according to the antiviral assay of IFN-α (Familletti, Rubinstein, and Pestka, 1981). In this assay, a dose of one unit/ml IFN-α was found to produce a cytopathic effect of 50% as antiviral. Because in viral challenge, the cells are more vulnerable to the effect of IFN than unaffected cells as the case in the current experiment, 20U was used as an initial dose.

Because different mechanisms might be involved in the effect of IFN-α on the inner ear (the inflammatory, the immune reaction, and apoptosis) and these mechanisms can occur simultaneously or at different periods of treatment with IFN-α, five time points were used to assess cell counts and gene expression. These time points included 3, 6, 12, 24, and 48 hours. Therefore, the cells resulting from the culture were seeded in the density of $5x10^4$ per well

containing 2 ml of complete medium, using 5 flat-bottomed 12-well plates, with a total of 60 wells. The 60 wells were divided into five groups according to the number of hours. They corresponded to 3-hour, 6-hour, 12-hour, 24-hour, and 48-hour treatment periods, respectively. Each of the five groups was divided into three subgroups according to the dose of IFN-α, namely, IFN20 for a relatively small dose (20U/ml), IFN40 (40U/ml), and IFN80 for a relatively large dose of IFN-α (80U/ml). In addition, a fourth subgroup represented a control group (IFN0) that was treated with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) in a dose of 80μL/ml. The PBS was used to dilute IFN-α to obtain a concentration of 10U/μL. This means that the control group received the same buffer solution, but without IFN-α. Each of these subgroups was included in three wells (triplicate) to increase the number of the experimental units and the power of the statistical test. Summary of the experimental design is shown in Table 2. After treatment with IFN-α or PBS (control), the cells from each well were collected and counted. Then, the cells were subjected to RNA extraction. Afterward, reverse transcription and real time polymerase chain reaction (RT-rt-PCR) were used to examine the expression of three sets of genes.

3.1.1.1 Sets of the genes that were examined in the current study

The three sets of genes included genes that are specifically expressed by HEI-OC1; the second set included those genes that might be triggered by IFN-α (IFN-α-induced genes); and the third set included a reference gene.

Specific genes to the HEI-OC1: These included the following:

- Myosin 7a is a marker for outer hair cells (Hasson & Mooseker, 1996).
- Nestin is a marker for stem-like cells (Lendahl, Zimmerman, McKay, 1990).

- Connexin26 is a marker for supporting cells (Lautermann, ten Cate, Altenhoff, et al., 1998).
- Calbindin2 is a marker for sensory cells (Slepecky & Ulfendahl, 1993).

Table 2: **Summary of the experimental design (Experiment-1)**

Hours / Dose	***3 hours***	***6 hours***	***12 hours***	***24 hours***	***48 hours***	Total # of wells
20U/ml	IFN20/3	IFN20/6	IFN20/12	IFN20/24	IFN20/48	
# of wells	3	3	3	3	3	15 wells
40U/ml	IFN40/3	IFN40/6	IFN40/12	IFN40/24	IFN40/48	
# of wells	3	3	3	3	3	15 wells
80U/ml	IFN80/3	IFN80/6	IFN80/12	IFN80/24	IFN80/48	
# of wells	3	3	3	3	3	15 wells
Control	IFN0/3	IFN0/6	IFN0/12	IFN0/24	IFN0/48	
# of wells	3	3	3	3	3	15 wells
Total	12	12	12	12	12	60 wells

Genes or markers for IFN-α treatment: These include the following genes:

i. IFN-α gene was examined to verify the presence of IFN-α receptors in the cochlea.
ii. IFN-β gene was examined because it is expected to be expressed by IFN-α.
iii. IFN-γ gene was examined because its secretion is expected to be increased by IFN- α.
iv. ICAM-1 was examined as an inflammatory marker of IFN-α in the cochlear cells.
v. TNF-α gene was examined as an inflammatory marker for IFN-α.
vi. H2K1 gene that encodes MHC-1 molecules was examined as a marker for the immune response of IFN-α.
vii. Fas gene was examined as a marker for the extrinsic pathway of apoptosis.
viii. Caspase-3 was examined as evidence of apoptosis.
ix. Bax gene was examined as a marker for the intrinsic pathway of apoptosis.

Reference gene: The 18S rRNA gene was used as a reference to measure the expression of IFN-α-induced genes. The 18S is a housekeeping gene that is present in all nucleated cells and it is necessary for basic cell functions. The mRNA of the housekeeping gene is stable and would not be changed by experimental treatments (Foss, Baarsch, & Murtaugh, 1998; Marten, Burke, Hayden, & Straus, 1994; Thellin, Zorzi, Lakaye, De Borman, Coumans, Hennen,et al., 1999).

Since the current experiment involved the cochlear cell line as an in-vitro tool to study the effect of IFN-α on the cochlear cells, using rt-PCR, the following section provides an overview for both of them.

3.1.1.2. The cochlear cell lines

For the present study, a HEI-OC1 cell line was used. This cell line was obtained as a gift from Dr. Federico Kalince (House Ear Institute, Los Angeles, CA) and was derived from the

organ of Corti of 7 days postnatal transgenic mice (Immortomouse H-2Kb-tsA58, Charles Rivers Laboratories, Willmington, MA, USA). Because the organ of Corti cells do not proliferate, Immortomouse was used to facilitate the establishment of the cell lines. Most cells in this animal harbor a temperature sensitive oncogene (tsA58), a mutant of the SV40 T antigen (Tag) gene (Jat and Sharp, 1989). This oncogene is regulated by the MHC-I promoter that can be induced by IFN-γ. Under permissive conditions (33°C, and IFN-γ), this gene will be expressed and the cells will proliferate; whereas, under non-permissive conditions (39°C and no IFN-γ), proliferation will stop and the cells start to differentiate.

This cell line was obtained as follows (Kalince, Webster, Lim, & Kalince, 2003; Devarajan, Savoca, Castaneda, Park, Esteban-Cruciani, 2002; Rivolta & Holley, 2002; Bertolaso, Martinin, Bindini, Lanzoni, Parmeggiani et al., 2001; Kalince, Kalince, Boukhvalova, & Kachar, 1999). Cochlear half turns from the Immortomice were cultured on uncoated plastic culture dishes under permissive conditions (33°C, 50 U/ml IFN-γ, and 10% CO2). The Culture contains a high glucose Dulbecco's Eagle medium (DMEM) (Gibco BRL, Gaitherburgh, MD., USA) and 10% fetal bovine serum (FBS; Gibco BRL). Afterward, the cultures were moved to non-permissive conditions (39°C and no IFN-γ, and 5% CO2), where cellular proliferation stopped and cellular differentiation began. The culture remained under these conditions for up to 180 days and was monitored by phase contrast and video microscopy. No antibodies were added to the culture. Between the outer sulcus and the inner sulcus, where the cells of the organ of Corti were expected to grow, clusters of newly generated, epithelial-like rounded cells appeared and then coalesced together to form a belt at the boundary with the original explant (Figure 4). Afterward, the culture was incubated for 2-5 minutes with trypsin-EDTA (0.25% trypsin, 1 mM EDTA; Gibco BRL) to detach the cells from the culture media. Later, the cells were isolated by

lifting them using a micropipette and seeded in new culture dishes. Different cell lines were established and cloned including, OC-K1, OC-K2, OC-K3, and OC-K4. They were collected and cloned using the limiting dilution method.

The use of hair cell lines will help to overcome the limitations imposed by using human or animal subjects. First, using human subjects to study the effect of IFNs will be limited by the frequent use of adjuvant agents with IFN-α, e.g., ribavirin in treatment of hepatitis C and fluorouracil in hepatocellular carcinoma (Heathcote & Main, 2005). Their effects on hearing should be excluded (Urabe, Kaneko, Matsushita, Unoura, Kobayashi, 1998). A second limitation of using human subjects to study the effect of IFN on the auditory system is the comorbidity of other diseases that might themselves have a detrimental effect on the auditory system. A third limitation (probably, the most important one) is studying the biological effect of IFN-α at a molecular and genetic levels will be difficult in human subjects. Although these previous limitation imposed by human subjects can be avoided by using an animal model, the high expense of animal care, the small amount of tissue available from the animal, as well as the pharmacokinetics of the drug make the use of animal models difficult. Moreover, and most importantly, using cell lines enables in-depth molecular studies for signaling pathways to be performed with no limitations on sample size (Bertolaso et. al., 2001). In particular, the HEI-OC1 line, derived from these cells expresses several markers that are characteristic of the sensory cells of the organ of Corti, such as, calbindin, brain-derived neurotrophic factor, calmodulin, connexin 26, organ of Corti protein 2, Math1 and myosin VIIa (OHC) (Rivolta, Grix, Lawlor, Ashmore, Jagger, & Holley, 1998). In addition, many studies indicated that HEI-OC1 cells are five-fold more vulnerable to apoptosis resulting from aminoglycosides than cells originating from fibroblasts (Kalince, Lim, & Kalince, 2002).

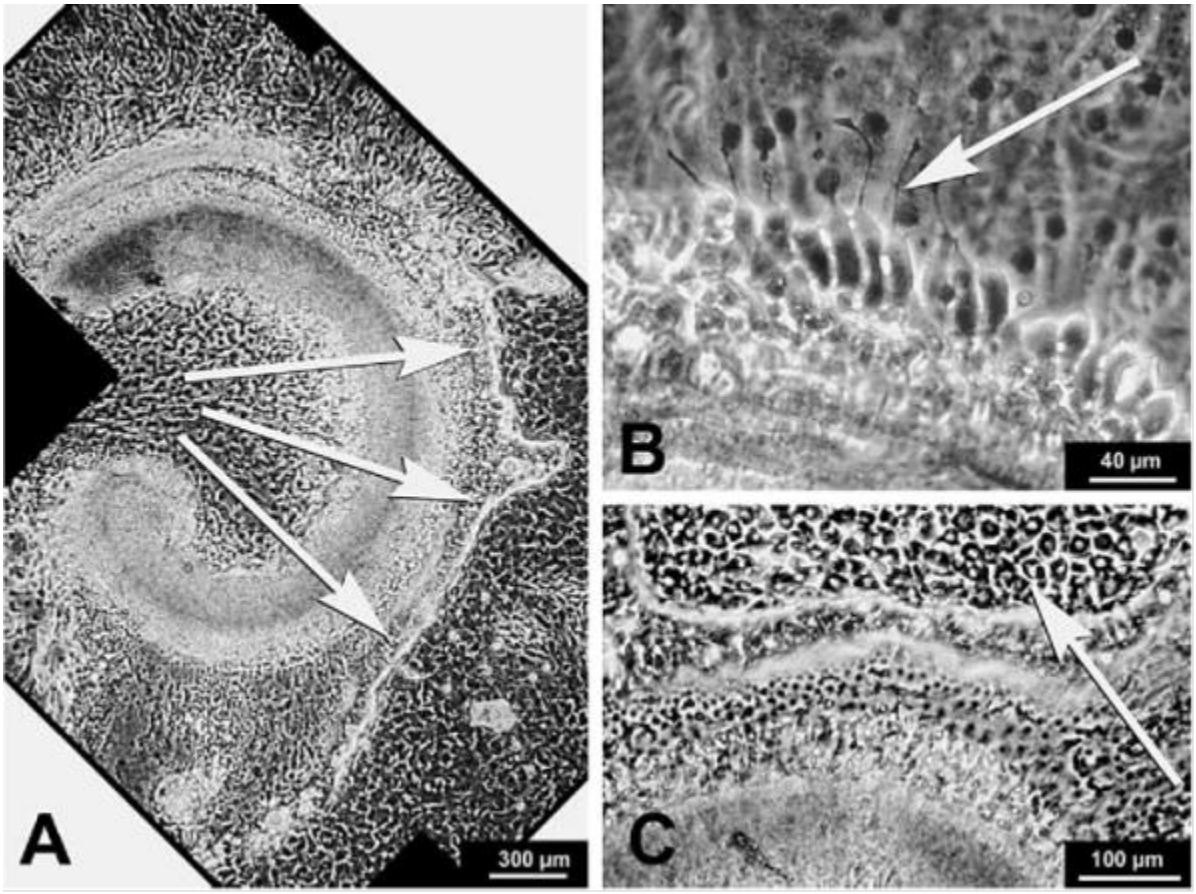

Figure 8: Preparation of the cochlear cell line

A) Morphology of a cochlear explant cultured for 28 days. Note the ‘ridge’ between the multilayered region of the original explant and the monolayer of cells growing in the periphery (arrows). B) Higher magnification of the ‘ridge’ area with the focal plane on the top of the multilayered cell region (note that the monolayer of cells is out of focus). Cylindrical cells with projections resembling Deiters' phalangeal processes (arrow) start to develop after 40 days under nonpermissive conditions (39°C). C) The cells in the monolayer, at the boundary with the original explant, display a very homogeneous phenotype (arrow). (From Kalinec, et al., 2003)

Therefore, HEI-OC1 might be the most suitable cell line to study the effects of IFNs on the organ of Corti cells in the present study.

3.1.1.3. Polymerase chain reaction

Polymerase chain reaction (PCR) is an in-vitro technique of nucleic acid formation, by which a particular segment of the DNA molecule can be replicated and amplified. The technique includes amplification of two oligonucleotide primers that flank the DNA molecules in question by repeated cycles of heat denaturation to separate double-strand DNA (dsDNA) into single strands (ssDNA), annealing the primers to their complementary sequences, and extending the annealed primers with a heat-stable DNA polymerase. Afterward, PCR data are automatically collected and analyzed by computer software to calculate relative gene expression between several samples based on a standard curve. This technique is very useful to amplify DNA; however, to study gene expression, messenger RNA (mRNA) which is carrying the genetic information transcript from the gene is the one that needs to be amplified. The messenger RNA is formed as a Xerox for the DNA that carries the gene in question, but it is different from DNA in being a single helix, rather than double helix. Because PCR is designed to amplify DNA, the mRNA must first be reversely transcribed to DNA by an enzyme known as a reverse transcriptase, using a technique called reverse transcription (RT).

Many studies suggest that real time PCR (rt-PCR) can be used for analysis of limited quantities of tissue and may show promise for further qualitative and quantitative studies on the molecular mechanisms of hair cell transduction and regeneration (Lee and Cotanche, 1995). Therefore, rt-PCR is considered the method of choice for simultaneous detection, amplification and quantifying changes in gene expression in a minute number of cells (for example, cochlear

tissue) over time (Kubista, Andrade, Bengtsson, Forootan, Jonák, et al., 2006). This method provides a very powerful inexpensive and sensitive approach to reveal differentially expressed genes.

In the current study, rt-PCR was used to reveal time and dose-dependent changes of the target genes in the hair cell culture challenged by IFN-α. In particular rt-PCR was used to measure the magnitude of expression, if any, of some genes that might be stimulated by IFN-α.

Analysis of PCR data

The PCR has three phases, exponential phase, linear phase and plateau phase. The exponential phase is the earliest phase and during which, the PCR product increases exponentially since the reagents are available in large amounts. The linear phase is characterized by a linear increase of PCR product because the PCR reagents start to be limited. In the plateau phase, the PCR product reaches plateau because some of the reagents start to be depleted. During the exponential phase, the PCR product will double during each cycle if the amplification efficiency is close to 100%, which can be achieved by optimizing the PCR conditions, primer characteristics, template purity and amplicon length (the length of the product molecule).

The rt-PCR system is based on the detection and quantitation of a fluorescent reporter, (fluorescent dye that binds specifically to the minor groove in ds-DNA), the signal of which increases in direct proportion to the amount of PCR product in a reaction (the amplified DNA). By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template (amplicon). The cycle at which this significant increase in the target amplicon occurs is called cycle threshold (Ct). The higher the starting copy number of the target cDNA, the sooner a significant increase in fluorescence is

observed, and the lower the Ct number (i.e., Ct is inversely correlated to the logarithm of the initial copy number of cDNA). The level of gene expression is revealed by the ΔCt, where the ΔCt represents the Ct of the target gene normalized to the 18S rRNA gene ($\Delta Ct = Ct_{18SrRNA} - Ct_{target}$). Therefore, ΔCt is the statistic that determines the gene expression, and hence it is the statistic of interest in the current study.

In most experiments, rt-PCR data are quantified relatively by comparing the expression of a target gene to the expression of a reference gene (18S rRNA gene was used in the present study) and the expression of the same gene (gene in question or target gene) in treatment samples and control samples (Pfaffl, 2001). The experiments include treatment samples and control samples; for each sample, target genes and a reference gene are included. The goal is to statistically test for differences between target and reference genes in treatment samples versus control samples. Therefore, the null hypothesis is: the Ct differences between target and reference genes will be the same in treatment samples versus control samples. Yuan, Reed, Chen, and Stewart (2006) suggested four methods for statistical analysis of PCR data: multiple regression model, analysis of covariance (ANCOVA), T-test and Wilcoxon two-group test (non-parametric test). The authors found that the four methods led to similar results. Therefore, first, ΔCt is calculated ($=Ct_{target} - Ct_{18SrRNA}$); and then, one of these statistical tests should be performed on the ΔCt of various treatment conditions (e.g., doses or hours).

In addition, relative quantification of the mRNA expression levels of target genes is calculated by fold change, which reflects how many folds the amplicon has been changed. This fold change can be calculated by using two mathematical models, namely, the efficiency calibrated method (Pfaffl, 2001) and the ΔΔCt model (Livak & Schmittgen, 2001); both methods are similar. In the current study, the ΔΔCt model was used by calculating ΔCt for the target gene

in the control sample and the ΔCt for the target gene in the IFN-treated sample, followed by calculation of ΔΔCt:

$\Delta\Delta Ct = (Ct_{18SrRNA} - Ct_{target})_A - (Ct_{18SrRNA} - Ct_{target})_B$,

where A and B are the treatment and control samples respectively (Schmittgen and Zakrajsek, 2002).

Fold change= $2^{\Delta\Delta Ct}$

Therefore, in the current study, two variables from the PCR data were obtained; first was the ΔCt, on which the statistical testing was done; the second was the fold change, by which the amount of the gene expression was quantified. More than two-fold change in the PCR product was assumed to reflect a reliable expression of the gene in question (Proudnikov, Yuferov, LaForge, Ann Ho and Kreek, 2003).

The following sections present the materials that were used and the procedures that were followed in this experiment.

3.1.2 Materials

The following materials were used in the current experiment:

3.1.2.1 Cell culture

HEI-OC1 cell was obtained as a gift from Dr. Federico Kalince (House Ear Institute, Los Angeles, CA). The cell line was cultured on uncoated plastic culture dishes, using Dulbecco's modified Eagle medium (DMEM).

3.1.2.2. Drugs and reagents

The following drugs and reagents were used to run the current experiment:

- Recombinant mouse interferon-α was used in the present study because IFNs are species specific with regard to their biological effects (Uze, Lutfalla, & Gresse, 1990).
- Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) was used for cell culture.
- Trypsin-EDTA was used to detach the adherent cells from the bottom of tissue culture plate.
- Trypan blue stain was used to count the cells and to estimate cell viability.
- RNeasy Mini Kit for RNA extraction.
- TaqMan Reverse transcription reagents for reverse transcription.

3.1.2.3 Real time PCR kit

For the present study, SYBR Green was used for RT- PCR. The kit contains sufficient reagents to perform 200 50-µL reactions. The reaction contains SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs, Passive Reference, and optimized buffer components. During the reaction SYBR Green dye binds to double strands DNA (dsDNA), and upon excitation emits light. Therefore, as the amount of PCR products increases, fluorescence increases.

3.1.2.4 Primers

Gene-specific primers were designed using OLIGO Primer Analysis Software (v6.3, Molecular Biology Insights, Inc., Cascade, CO). The following primers were designed (Appendix B):

Primers for hair cell markers*:* these included the following:

i. Primer for Myosin-VIIa gene.

ii. Primer for Nestin gene.

iii. Primer for connexin26 gene Forward r

iv. Primer for Calbindin2 gener

Primers for IFN-α-induced genes

i. Primer for IFN-α1 gene.

ii. Primer for IFN-β gene.

iii. Primer for IFN-γ genes.

iv. Primer for ICAM-1 gene.

v. Primer for TNF- α gene.

vi. Primer for H2K1 gene that encodes MHC-1.

vii. Primer for Fas gene.

viii. Primer for Caspase-3gene.

ix. Primer for Bax gene.

Primer for 18S rRNA gene: The 18S rRNA was used to detect 18S rRNA expression as a house keeping gene that would not be changed by any treatment.

3.1.3 Procedures

The following procedures were followed to pursue the goals of the current study.

3.1.3.1 Cell culture

The HEI-OC1 cell line was cultured on uncoated plastic culture dishes, using DMEM media in the presence of 10% CO2, 50 U/ml of recombinant IFN-γ, and 10% fetal bovine serum under two conditions, permissive conditions and non permissive conditions. The permissive conditions

were in the form of 33°C, 10 % CO2, high-glucose DMEM media, 10% FBS, and 50µ IFN-γ; whereas, the non-permissive conditions included 39°C, 5% CO2, high-glucose DMEM, and 10%, FBS. The culture was monitored daily for any change in the color or clarity of the medium and for the confluence. The change in color or clarity of the media might reflect depletion of the supplies or infection; once detected, the media should be changed or the culture is discarded. None of these changes were noticed in the current experiment. Otherwise, the medium of the culture was changed twice a week. The cells were split when the cells reach near 90-95% confluence. The splitting was accomplished as follows:

- Removal of the old media.
- Washing the culture twice using PBS.
- Adding 5ml trypsin-EDTA to dislodge the cells from the bottom of the culture plate.
- Incubating the mixture for 5 minutes.
- Adding equal amount of the media and placing the mixture in a centrifuge tube.
- Place 500 micro liter of the mixture for cell count.
- Spinning the remaining of the mixture at around 700 rpm for 10 minutes.
- Removing the supernatant and re-suspending the cells in X amount of media according to the number of cells and according to the required concentration of the cells in the new plates.
- Moving the culture into one or more new culture flasks.
- Incubating the new culture under the same conditions.
- Splitting and passing the cells was repeated according to the rate of cell growth and the number of cells needed for the experiment.

3.1.3.2 Cell viability assay:

Cell viability assay was performed to monitor the growth of the culture and to determine the effect of IFN-α on cell growth and cell replications. Cell viability was measured by Trypan blue exclusion. This dye does not penetrate healthy cell membrane; however it passes through the cell membrane of dead cells. Therefore, viable cells will be unstained, whereas dead cells will be stained blue. Briefly, 50 µL of cell suspension was mixed gently with an equal volume of 0.4% Trypan blue in a test tube for five minutes at room temperature. Then 10 µl of the mixture was placed in a hemocytometer to count the number of viable (unstained) and dead (stained) cells. The average number of unstained cells were calculated in each quadrant of the hemocytometer, and multiplied by 2×10^5 to find cells/ml. The cell count was averaged across the three wells (triplicate) for each subgroup. Cell viability assay was performed during passing the cells to new flasks, during seeding the cells into plates and after treatment of the cells with different doses of IFN or 0.1% PBS at different time points.

3.1.3.3. Treatment of the cell culture with IFN-α / PBS

When the cells in the culture flasks reached approximately 85% confluence, the cells were seeded into 12-well culture plates ($5x10^4$cells/well); each containing 2ml culture media. Five plates were used—each was labeled for the corresponding time points, namely, 3, 6, 12, 24, and 48 hours (forming a total of 60 wells). The 12 wells in each plate were labeled corresponding to the four treatment groups, namely 20U, 40U/ and 80U/ml of IFN-α, and a control group (sham) treated with 80U/ml of PBS. Each treatment group as well as the sham was included in 3 wells. After treatment with IFN-α, the plates were incubated in 39°C, 5% CO2 for the

corresponding period of time. After each particular time, the cells were collected from each well and stored in -80C for RNA extraction.

3.1.3.4 RNA extraction

Total RNA was extracted from each group of the cells collected from the previous steps, using RNeasy Mini Kit (Qiagen). According to manufacture protocol: cells were mixed with 350µL RLT buffer (for lysis of cells before RNA isolation) and 5mm metal bead and homogenized by TissueLyzer (Qiagen, Valencia, CA) to dissolve the sample from other cell components, while keeping the integrity of RNA. Then cell lysate was centrifuged for one minute and supernatant was transferred to a new tube, leaving the cell pellet in the old tube. The supernatant was mixed with 350 µL of ethanol 70% and then centrifuged in RNeasy mini 2mL collecting tube. Afterward, the sample was washed with 350 µL RWI solution and centrifuged to discard the flow through reuse collecting tube. The sample was treated with DNaseI solution to purify the sample from any genomic DNA, followed by washing with RWI. The sample also was washed by RPE solution. At this point the sample contained RNA only that was eluted in RNase-free water to ensure protection of the RNA from any contamination. RNA quality and quantity were evaluated by gel electrophoresis on a 1.5% agarose/Tris-Borate-EDTA (TBE) gel using ethidium bromide staining and 260/280 absorbance (ABS) ratio in a Beckman DU 600 Spectrophotometer (Beckman, Fullerton, CA, USA). Optical density-OD quantification method was run to measure the total amount of RNA extracted from each sample.

3.1.3.5 Reverse transcription

Reverse transcription (RT) of RNA was performed to convert RNA resulting from the previous procedure into recombinant DNA (cDNA), using TaqMan Reverse transcription reagents. These reactions included 10.0 µl of 10X PCR TaqMan Gold buffer II (Applied Biosystems), 30 µl of 25 mM MgCl2, 4 µl of 25 mM of each dNTP, 5 µl of 100 µM of random primers (Gibco BRL), 2 µl of RNasin (40 units; Applied Biosystems), 1.25 µl of Super-Script II (250 units; Applied Biosystems) and 5 µl (250 ng) of DNA-free total RNA in a final volume of 100 µl. The reaction was incubated at 25^{o}C for 10 min, 50^{o}C for 50 min and 85^{o}C for 5 min in a 2700 Thermocycler (Applied Biosystems Inc., Foster City, CA).

3.1.3.6 Real time Polymerase chain reaction (rt-PCR)

Quantitative comparisons of gene expression among different groups with regard to different doses of treatment and different time points of treatment were measured by means of rt-PCR (Schmittgen, Zakrajsek, Mills, Gorn, Singer, & Reed, 2000), using SYBR Green Master Mix Kit (Applied Biosystems). The kit contained sufficient reagents to perform 200 50-µL reactions. The reaction contained SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs, Passive Reference, and optimized buffer components. A PCR reaction of 50 µl included 5 µl of 10× SYBR PCR buffer, 6 µl of 25 mM MgCl2, 4 µl of each dNTP (a mixture of 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP (dUTP was used instead of dTTP to help in minimizing the carried over PCR products), 2.5 µl (5 µM) of each gene-specific primer (forward and reverse for all target genes), 0.5 µl of AmpErase UNG (0.5 units), 0.25 µl of AmpliTaq Gold (1.25 units) and 5 µl of cDNA (250 ng) in a final volume of 50 µl. All the reagents were kept on ice during the set up for the experiment. This mix was placed in a 96-well plate. The plate was

sealed, mixed, and spun to remove any air bubbles in the wells. The reaction was submitted to the following thermal cycling parameter.

Incubation in 50 C° for 2 minutes, in 95C° for 12 minutes for Taq Activation, then, 35 cycles at 95 C° for 15 seconds for DNA denaturation and in 60C° for 1 minutes for annealing and extension in ABI PRISM® 7300 Sequence Detection System (SDS) (Applied Biosystems).

7300 Sequence Detection software (Applied Biosystems) was used for instrument control, automated data collection and data analysis. Relative quantification (fold difference) of the expression levels of each transcript was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Zakrajsek, 2002) with modifications (Li-Korotky et al., 2009). The ΔCt represents the Ct of the target gene normalized to 18S rRNA ($\Delta Ct = Ct_{Target} - Ct_{18S\ rRNA}$). Relative quantification (fold change) of the expression level of the target gene was calculated using a modified $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{18S\ rRNA})_{Target} - (Ct_{Target} - Ct_{18S\ rRNA})_{Control}$.

3.1.4 Data analysis

Data were collected and included the following:

- Fold change for each gene.
- ΔCt for each gene.
- Cell count for each treatment group.

First, the presence or absence of each gene was determined by measuring its fold change. More than two-fold increase is considered a reliable indication of change in the expression of the gene relative to the control group (sham), which was normalized to the 18S rRNA gene. Because calculation of fold changes is considered a quick way to determine the presence or absence of a

particular gene, an initial screening was done for some genes to examine if the dose regimen (20, 40, & 80U/ml) was sufficient to induce expression of any of these genes that were questioned in the current study. These genes included ICAM1 and TNF-α as inflammatory genes and caspase-3 and Bax genes as apoptotic genes. The results of this screening procedure showed that none of those genes were expressed in a significant amount (none of them exceeded two- fold increase). The results of experiment-1 are depicted in the results section (Chapter IV). Because none of these genes were expressed, no further statistical tests were performed on their ΔCt.

Therefore, another experiment (experiment-2) was run, using relatively higher doses, namely 200 and 2000U/ml media. The dose of 200U/ml, which is almost two times the maximum dose (80U/ml) used in experiment-1, was chosen to represent a relative increase in the previous dose regimen. Most studies on interferons used a wide range of doses starting with one unit to 100,000U/ml media. The strategy when the effect of cytokines (receptors ligand) needs to be tested on different cell types is to increase the dose by 10 folds, 1, 10, 100, 1000,….100,000. According to the available literature, one unit of IFN-a was found to produce 50% cytopathic changes in antiviral assay (Familletti,et al., 1981), and based on that, a dose of 20U was chosen as an initial dose in the first experiment. Therefore, in the new experiment 200U (10 times of the initial dose) and 2000U (10 times of 200U) were used. In addition, only four time points, including 6hrs, 12hrs, 24hrs, and 48hrs were used in the second experiment because there was not much change in gene expression at 3hrs in Experiment 1. Eliminating this step saved laboratory supplies from being wasted by running unnecessary steps.

The following section presents the experimental design, the materials used as well as the procedures followed in experiment-2.

3.2 EXPERIMENT- 2

3.2.1 Experimental design for experiment-2

The cells resulting from the culture were seeded in the density of $5x10^4$ per well containing 2 ml of complete medium, using 4 flat-bottomed 9-well plates, with a total of 36 wells. The 36 wells were divided into four groups according to the number of hours. They were labeled as: 6hrs, 12hrs, 24hrs, and 48hrs.

Each of the four groups was divided into three subgroups according to the dose of IFN-α (table 3), which included:

- IFN0 acted as a control (sham) group that was treated by 0.1% PBS in a dose of 2000μL/ml, with no IFN-α.
- IFN200, which was treated by IFN-α in a dose of 200U/ml media.
- IFN2000, which was treated by IFN-α in a dose of 2000U/ml.

All the other elements of the design were the same as in the first experiment.

3.2.2 Materials and procedures for experiment-2

All the materials that were included in experiment-1 also were used in experiment-2. Also, all the procedures followed in the experiment-1 were followed in experiment-2 except for the treatment procedure, in which the number of wells as well as the number of time points was changed (see Table 3 for details).

Table 3: Summary of experimental design for experiment-2

Hours / Dose	***6 hours***	***12 hours***	***24 hours***	***48 hours***	Total # of wells
IFN0	IFN0/6	IFN0/12	IFN0/24	IFN0/48	
# of wells	3	3	3	3	12wells
IFN200	IFN2000/6	IFN200/12	IFN40/24	IFN200/48	
# of wells	3	3	3	3	12 wells
IFN2000	IFN2000/6	IFN2000/12	IFN2000/24	IFN2000/48	
# of wells	3	3	3	3	12 wells
Total	9	9	9	9	36 wells

When the cells in the culture flasks reached 85% confluence, the cells were seeded into 9-well culture plates ($5x10^4$cells/well); each well contained 2ml culture media. Four plates were used; each plate was labeled for the corresponding time points, namely, 6, 12, 24, and 48hrs. The nine wells in each plate were divided into three groups—3 wells each corresponding to the three different doses of IFN-α, namely (IFN0, IFN200, and IFN2000). After treatment with IFN, the cultures were incubated in 39°C, 5% CO2 for the corresponding period of time. After each particular time, the cells were collected from each well and stored in -80C for RNA extraction. The IFN0 group was treated with 2000U/ml of PBS forming the control group.

3.2.3 Data analysis for experiment-2

Data were collected and included the following:

- Fold change for each gene.
- ΔCt for each gene.
- Cell count for each treatment group.

First, the presence or absence of each gene was determined by measuring its fold change, including the genes that are specific to HEI-OC1 and the IFN-α-induced genes.

The second part of the analysis was to quantitatively determine those genes that were expressed in the cochlear cells as a result of IFN-α treatment with respect to dose and time of IFN-α treatment, using the ΔCt as the statistic of interest. The ΔCt for each gene was collected and then compared across different doses and across different time points. This part of the analysis was not applied for the genes that are specific to HEI-OC1, the presence of which would validate the presence of viable hair cells in the growing culture used in the current experiment.

Also, the cell count from the cell viability assay procedure was averaged across the wells included in each subgroup and then compared across different doses and across different time points.

Therefore, there were two independent variables (dose and time) with three and four levels respectively and two dependent variables (ΔCt and cell counts), which created a 3X4 factorial design for the ΔCt and for the cell count.

3.2.4 Statistical analysis for experiment-2

The goal of the first level of data analysis was achieved by calculating the fold-increase for each gene; the gene was considered reliably expressed if there was more than two-fold increase in the PCR products relative to the control group as well as to the 18S rRNA. The calculation was done for those genes that are specific to the cochlear cells as well as for each of IFN-α-induced gene to initially determine if these genes were expressed or not.

The goal of the second level of the analysis was to statistically test for the presence of significant effects of both the dose and the time (two independent variables) on the ΔCt of each IFN-α-induced gene and on the cell count (two dependent variables). Livake and Schmittgen (2001) documented that ΔCt calculation satisfies assumption for the use of parametric statistics such as t-test and ANOVA. Since t-test compares two means—each should be with one level only, the alternative to t-test in the case of multiple means with multiple levels would be the analysis of variance "ANOVA". Therefore, for this experiment, two-way ANOVA was performed (four levels of time X three levels of the treatment). In particular, a separate two-way ANOVA for each gene and one for cell count (a total of 9 two-way ANOVAs) were performed.

Differences will be considered significant when P-value < 0.05 (i.e. alpha=0.05). ANOVA testing was applied only for those genes that showed a reliable fold increase (more than two folds).

A post hoc analysis, using the least difference test (LSD) was done to examine for significant differences across the three levels of the dose (IFN0, IFN200, and IFN2000); likewise, for significant differences among the four time points (6hrs, 12hrs, 24hrs, and 48hrs). LSD does not correct for alpha, which is beneficial for the current study as the least difference would be sufficient to justify significance for this kind of data.

4.0 CHAPTER IV: RESULTS

4.1 RESULTS OF EXPERIMENT-1

Fold change was used to determine if the gene was expressed or not. The gene is considered expressed if there was two-fold increase in its level relative to the control, which was normalized to the 18S rRNA gene.

Figures 9, 10, 11, 12 and 13 showed the relative fold changes for IFN-γ, TNF-, ICAM1, Caspase-3, and Bax genes, respectively. None of these genes showed more than two-fold increase, which means that none of them was expressed by such small doses of IFN-α used in experiment-1 (20, 40, & 80 U/mL). However, the patterns of the fold change look similar along the three doses. Because none of these genes were expressed in a significant amount, there was no need to run two-way ANOVA or any further statistical test for any of them.

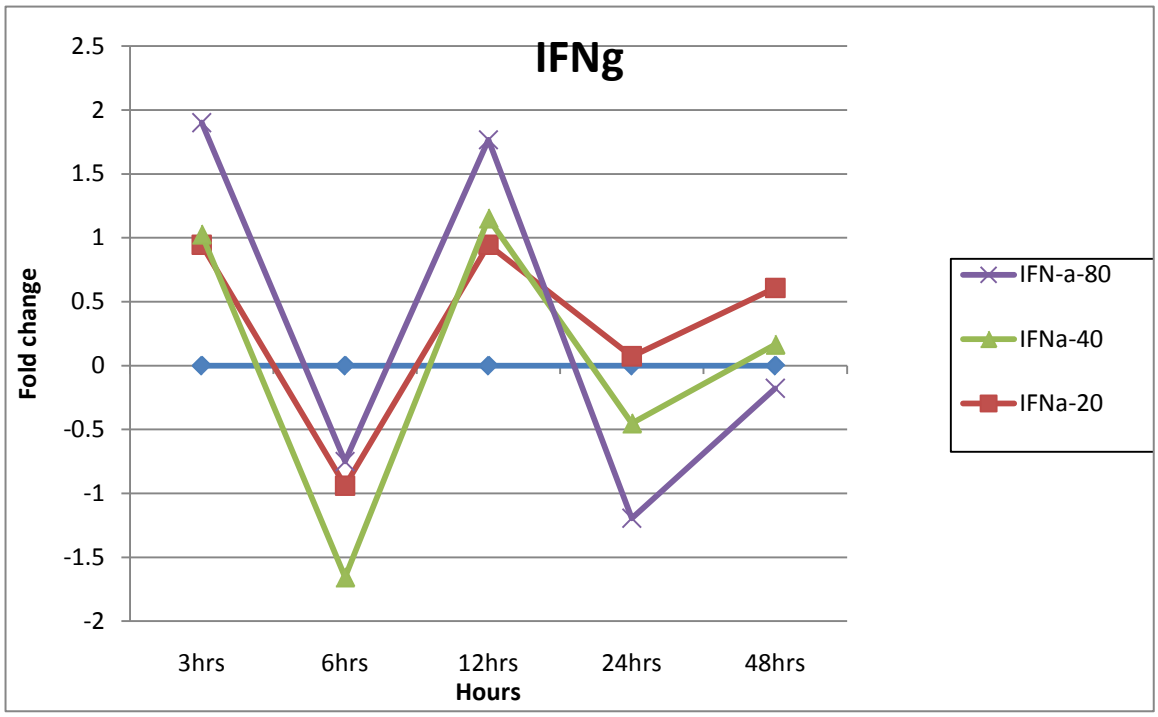

Figure 9: Relative fold changes in IFN-γ gene expression- Experiment-1

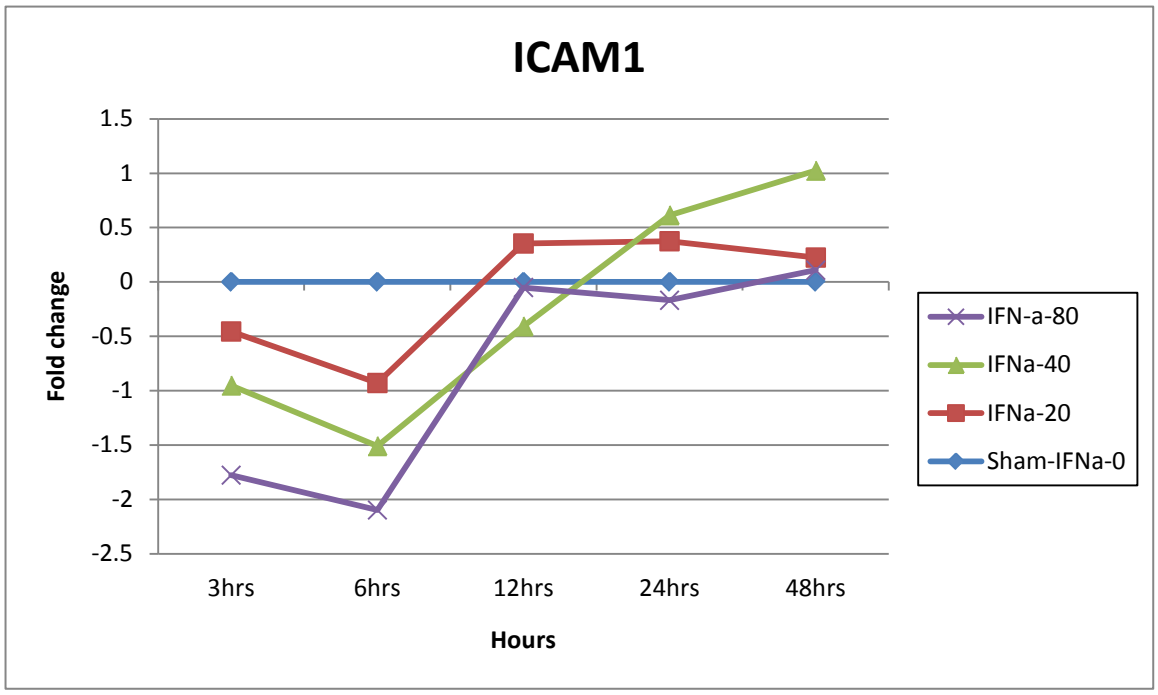

Figure 10: Relative fold changes in ICAM1 gene expression- experiment-1

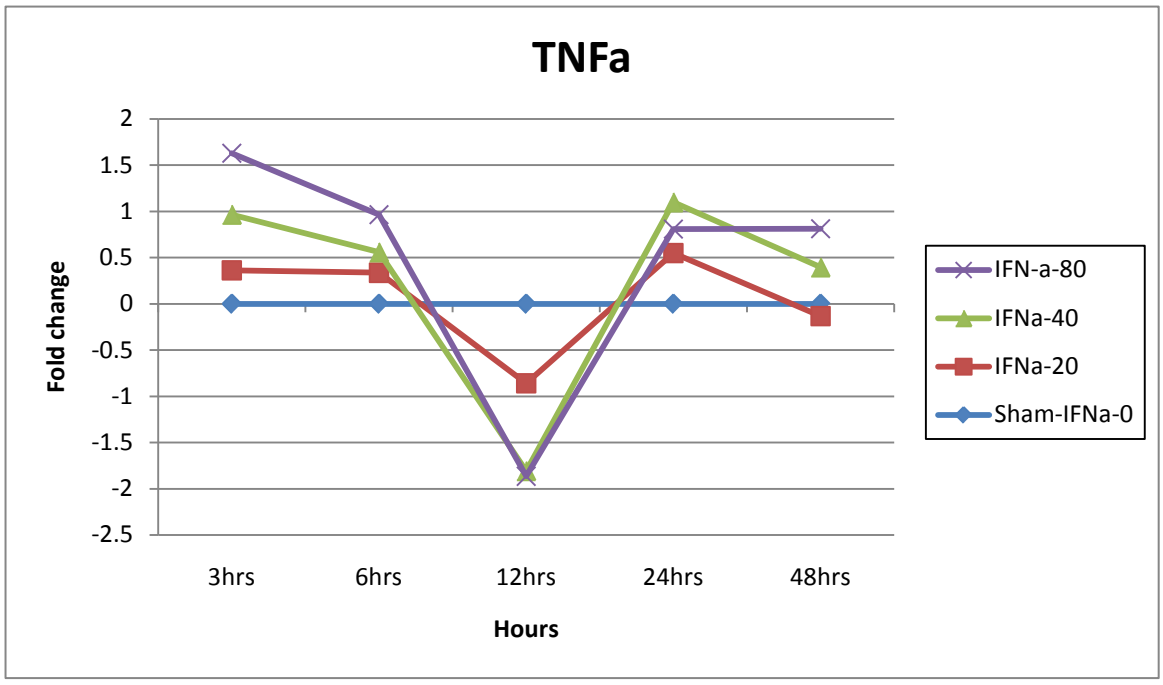

Figure 11: Relative fold changes in TNF-α gene expression in experiment-1

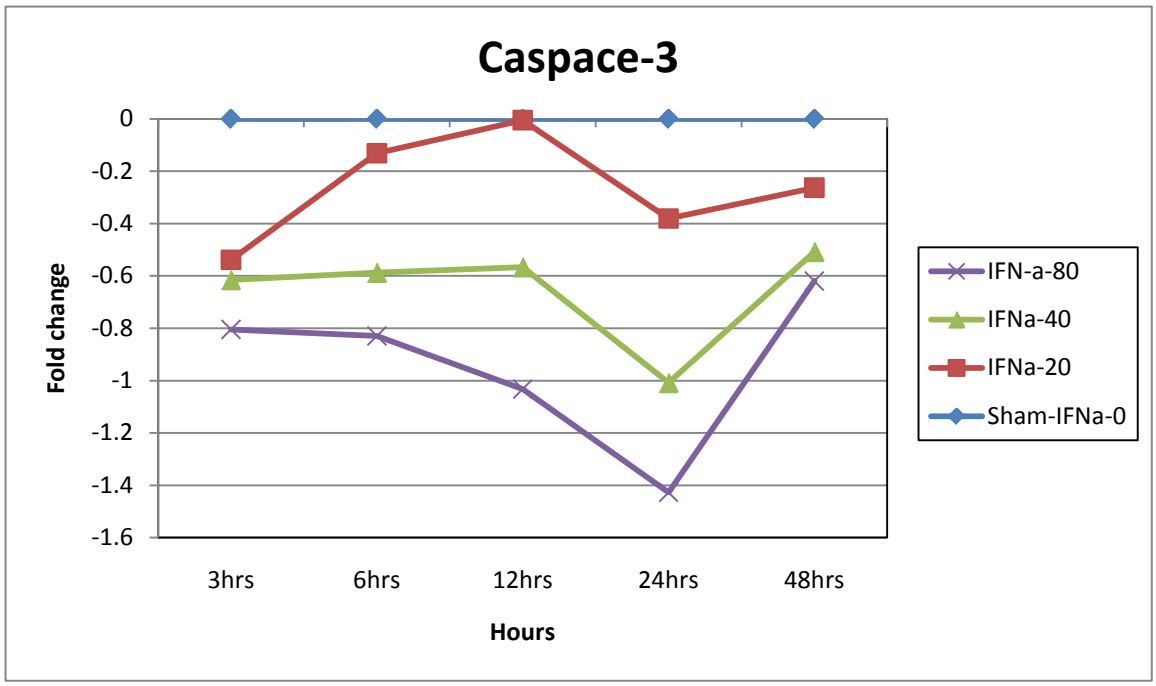

Figure 12: Relative fold changes in caspase-3 gene expression in experiment-1

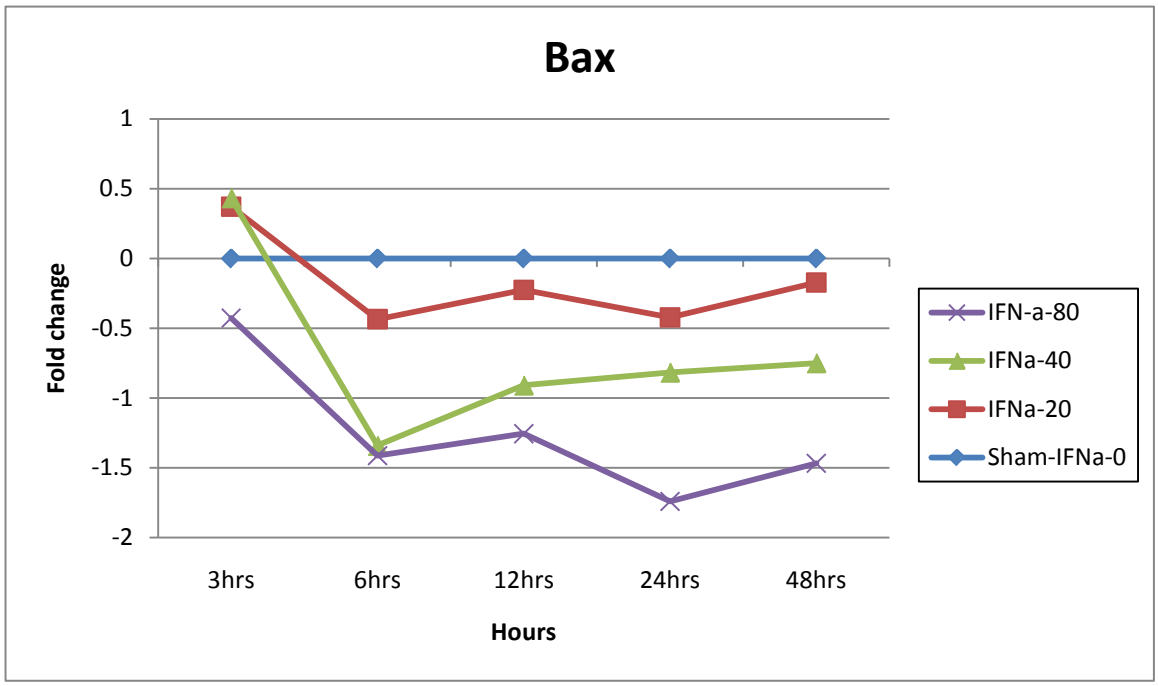

Figure 13: Relative fold change in BAX gene expression in experiment-1

4.2 RESULTS OF EXPERIMENT-2

First, the fold change was used to determine if the gene was reliably expressed or not. For the genes that are specific for the hair cells, only fold change calculation was done to determine the presence or absence of these genes. For the IFN-α-induced genes, the results include those from the fold change calculation for each gene and the results of the two-way ANOVA followed by the results of post hoc analysis, using LSD. The following section will illustrate the details of these results.

4.2.1 Expression of genes specific for hair cells

- Myosin VIIa: Figure 14 shows the expression of myosin-VIIa gene. There was an increase in the fold up to 10 folds, which indicates the presence of myosin-VIIa gene.
- Connexin26: Figure 15 shows the expression level of connexin gene, which did not exceed 2 fold changes, i.e., the hair cells in the cochlear cell line used in the current experiment did not express connexin 26 gene.
- Nestin gene: There was an increase in the expression level of nestin gene that ranged from 2-6 folds (Figure 16).
- Calbinidin2 gene: Figure 17 shows the fold changes in the calabindin2 expression level that ranged from 6-13 folds; however, the gene could not be detected at 48 hours.

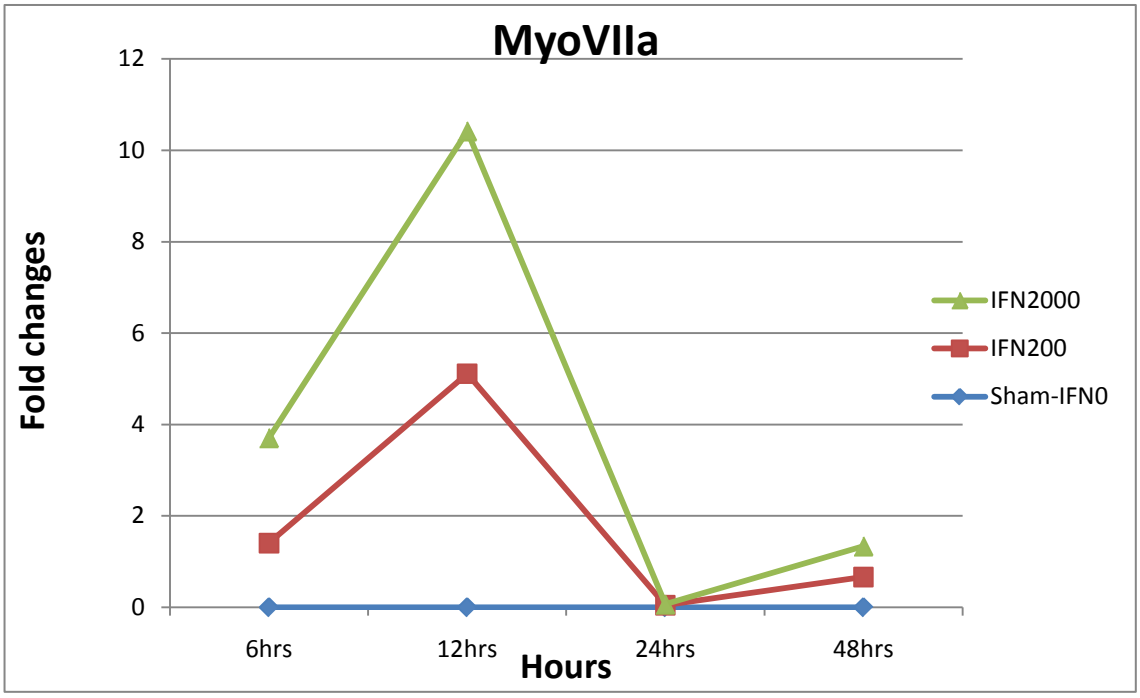

Figure 14: Relative fold changes in Myosin-VIIa gene expression

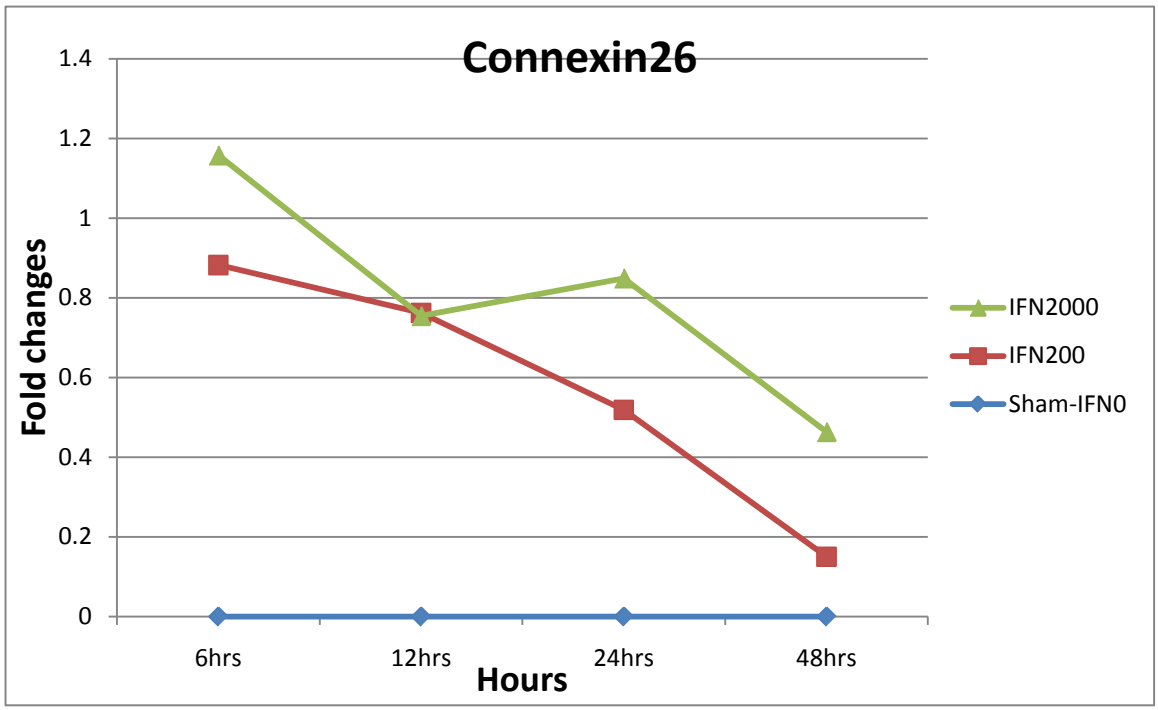

Figure 15: Relative fold changes in Connexin26 gene expression

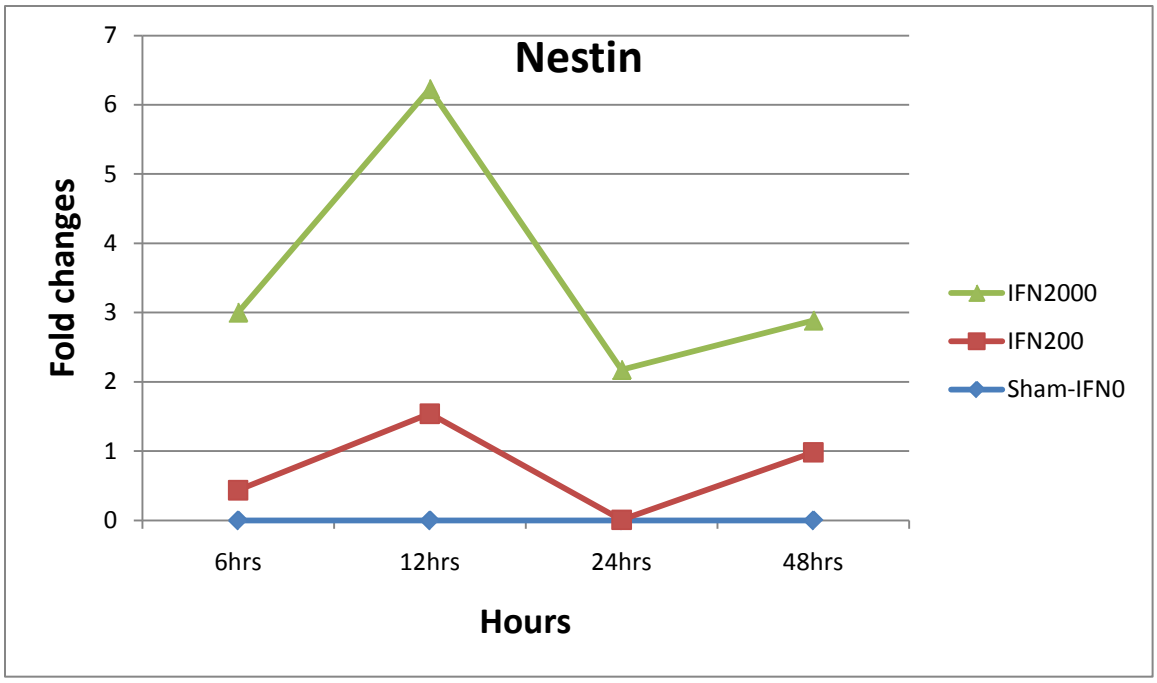

Figure 16: Relative fold changes in Nestin gene expression

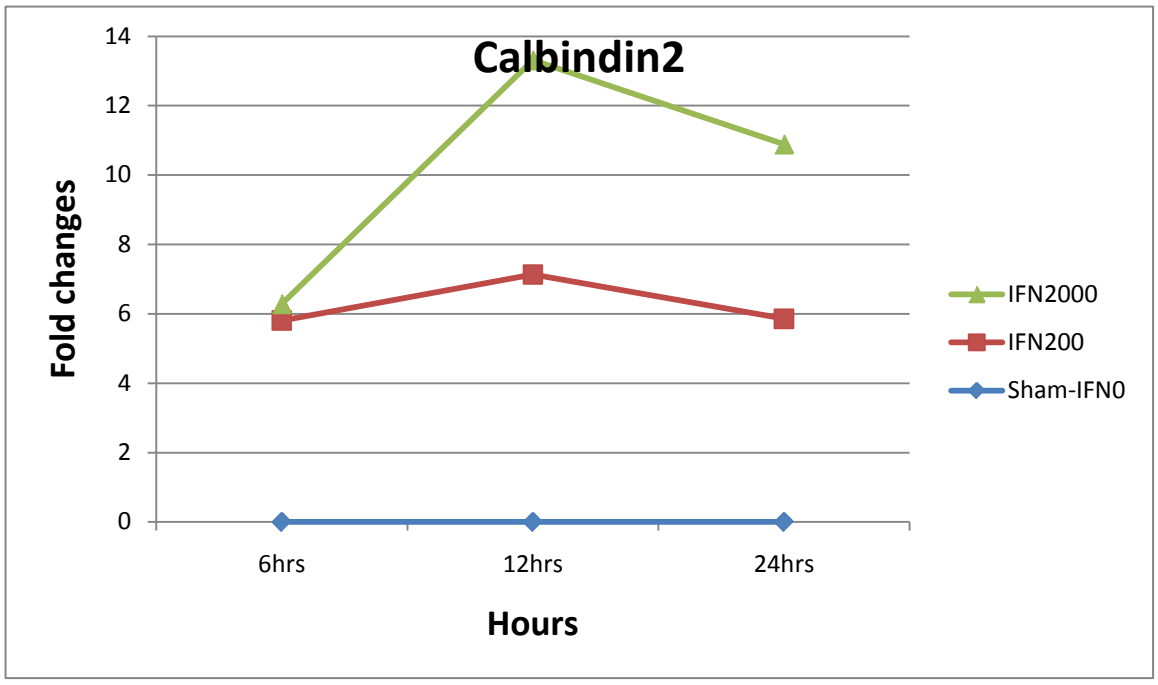

Figure 17: Relative fold changes in Calbidin2 gene expression

4.2.2 Expression of IFN-α -induced genes

Table 4 shows the means and standards deviation for ΔCt of all the IFN-α-induced genes. This will be followed by a detailed description of the findings for each gene.

Table 4: Descriptive Statistics for ΔCt of all IFN-α-induced genes

Gene	N	Minimum	Maximum	Mean	STDV
ΔCT-IFN- α1	36	23.41	29.94	26.1142	1.86281
ΔCT-IFN-β	36	21.36	28.93	24.7644	2.17972
ΔCT-TNF-α	36	20.33	32.23	25.7675	1.88526
ΔCT-ICAM1	36	16.85	20.42	18.8992	0.93173
ΔCT-MHC1	36	14.66	17.84	16.0106	0.99651
ΔCT-Fas	36	10.51	14.08	12.6733	0.62402
ΔCT-Casp-3	36	10.20	12.67	11.4900	0.66508
ΔCT-Bax	36	8.91	11.84	10.3467	0.65771

4.2.2.1 Expression of other types of interferons

The results showed expression of type I interferon (IFN-α1 and IFN-β); however there was no reliable expression of type II interferon (IFN-γ).

4.2.2.1.1 Expression of IFN-α1gene

The first research question of the current study was to determine if IFN-α can be expressed in the cochlear cells. Figure 18 shows that there was about 2-4 folds increase of IFN-α1 in the case of 200 U/ml and about 3.5-6 folds increase in the case of 2000U/ml.

Table 4 shows the output of two-way ANOVA, which revealed a significant effect of the dose on the expression of the IFN-α1 ($P<.05$, $P=.000$). However, the effect of time in hours was not significant ($P=0.342>0.05$). The interaction between the dose and the time was not significant ($P=0.916$), which means that the effect of dose was independent of the effect of the time of treatment.

Since the dose effect was significant, a post-hoc analysis was done to examine the significant difference between all possible pairs of the dose levels, using LSD test (table 5). The analysis reflected a significant difference between IFN0 & IFN200, IFN0 & IFN2000, and IFN200 & IFN2000 ($P=0.000$, 0.000, & 0.004 respectively).

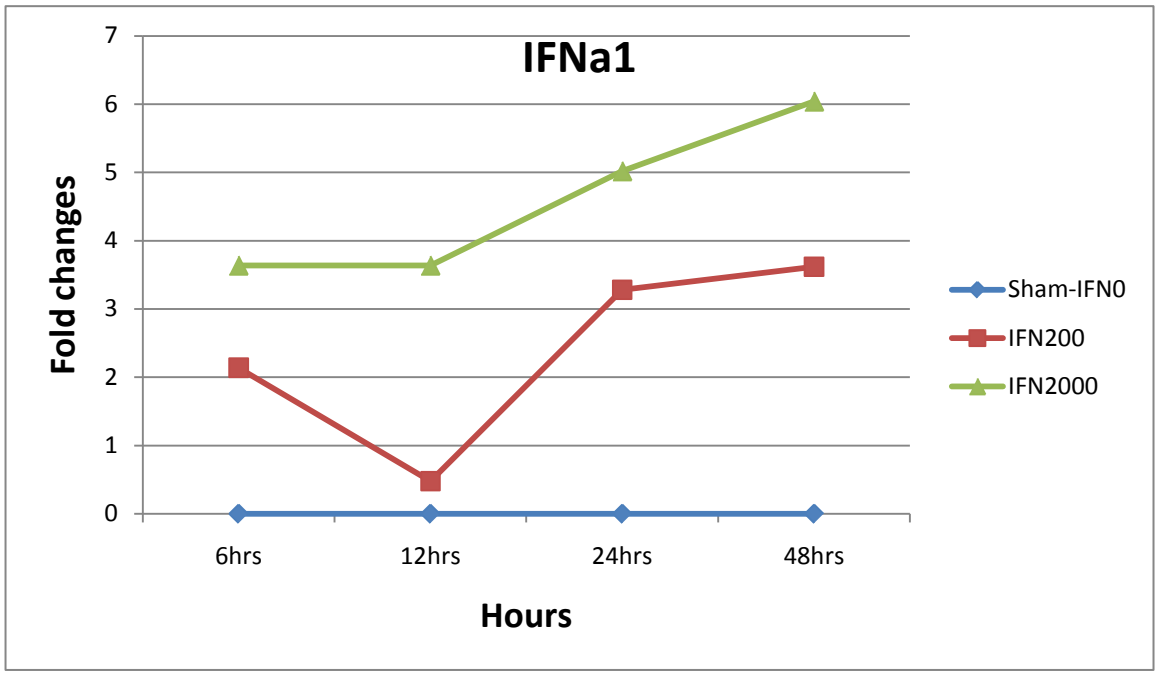

Figure 18: Relative fold changes in IFN-α1 gene expression

Table 5: Output of two-way ANOVA for ΔCt of IFN-α1 gene

Variable	df	Mean Square	F-value	Significance
Dose*	2	50.085	69.373	0.000
Time	3	0.845	1.170	0.342
Dose X time	6	0.237	0.328	0.916

*: The mean difference is significant at the 0.05 level

Table 6: Post-hoc analysis for the effect of dose on ΔCt for IFN-α gene

Levels of dose	Std Error	Significance
IFN0-IFN200*	0.34688	0.000
IFN0-IFN2000*	0.34688	0.000
IFN200-IFN2000*	0.34688	0.004

*: The mean difference is significant at the 0.05 level

4.2.2.1.2 Expression of IFN-β gene

Figure 19 shows that IFN-β was expressed in the cochlear cells. The expression level was higher in the case of 2000U/ml (6-11folds) than in the case of 200U/ml of IFN-α (2-5 folds). However, the pattern of change across different time points was almost the same for both doses, with the highest level of expression was during the 48-hrs-time point.

Table 7 shows the output of the two-way ANOVA, which revealed a significant effect for both the dose and the time on the expression of the β gene (P=0.000 & 0.005 respectively). However, the interaction between the dose and the time was not significant (P= 0.855).

Post-hoc analyses were completed to examine the significant difference of all possible pairs between different levels of the dose and different levels of the time, using LSD test (tables 8 & 9, respectively). The analysis reflected significant difference between all levels of the dose (P=0.000, 0.000, & 0.004); however, the hour levels were significant at 6-24 hrs, 12-24 hrs, and 24-48hrs only (P=0.000, 0.006 & 0.009).

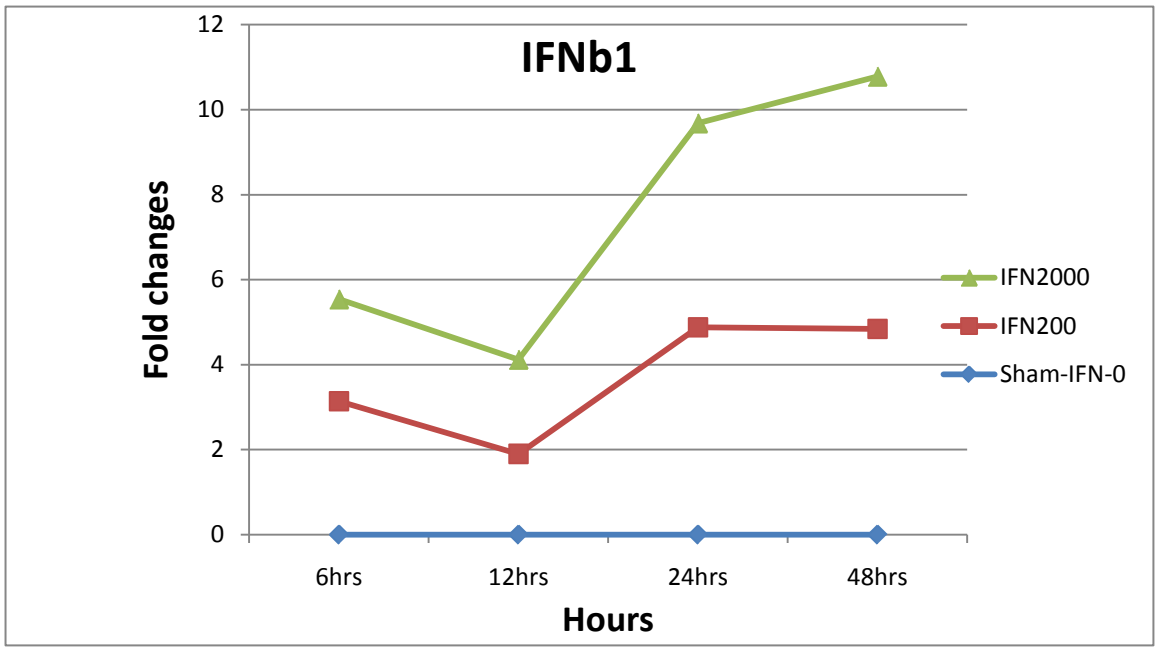

Figure 19: Relative fold changes in IFN-β gene expression

Table 7: Output of two-way ANOVA for ΔCt of the IFN-β gene

Variable	df	Mean Square	F-value	Significance
Dose*	2	52.786	42.552744	0.000
Time*	3	7.835	6.3160129	0.005
Dose X time	6	0.595	0.425	0.855

*: The mean difference is significant at the 0.05 level

Table 8: Post-hoc analysis for the effect of dose on ΔCt of IFN-β gene

pairwise	Std Error	Significance
IFN0-IFN200*	0.34688	0.000
IFN0-IFN2000*	0.34688	0.000
IFN200-IFN2000*	0.34688	0.004

*: The mean difference is significant at the 0.05 level

Table 9: Post-hoc analysis for the effect of time on ΔCt IFN-β gene

Levels of time	Std Error	Significance
6-12 hrs	0.52504	0.210
6-24 hrs*	0.52504	0.000
6-48 hrs	0.52504	0.169
12-24 hrs	0.52504	0.006
12-48 hrs*	0.52504	0.900
24-48 hrs*	0.52504	0.009

*: The mean difference is significant at the 0.05 level

4.2.2.1.3 Expression of IFN-γ

Another gene that might be upregulated by IFN-α is IFN-γ gene, which is a macrophage marker. The level of the gene expression could not be detected by fold change (Figure 20), which indicates that IFN-γ gene was not expressed by IFN-α treatment even in small amounts at the highest Ct cycle number used for PCR. Therefore, there was no need to run two-way ANOVA in this case.

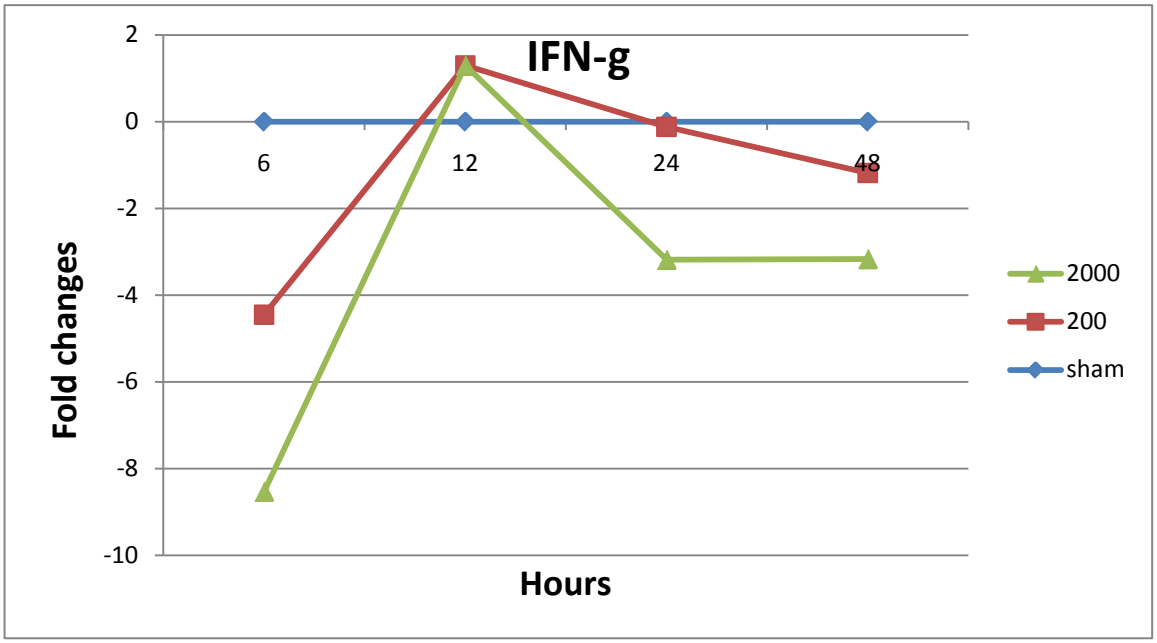

Figure 20: Relative fold changes in IFN-γ gene expression in experiment-2

4.2.2.2. Expression of inflammatory markers

The first part of the third research question was to detect if IFN-α induced inflammatory markers in the cochlear cell culture. Two inflammatory genes were examined in the present study including, ICAM1 gene and the TNF-α gene.

4.2.2.3.1. Expression of ICAM1 gene

Figure 21 shows the expression level of ICAM1 gene. There was an increase in the expression level of about 2-5 folds for 2000U dose, with less than two fold-change in the case of 200U dose. However, the expression level declined during the later hours, reaching its minimum level at 24 and 48 hours in both cases (both 200& 2000U of IFN-α showed similar pattern of change in ICAM1 gene expression).

In addition, two- way ANOVA was performed to determine if changing the dose and time of IFN-α treatment had an effect on ICAM1gene expression. Table 10 shows that changing the dose did not have a significant effect on ICAM1 gene expression (P=0.230). On the other hand, changing the time had a significant effect on ICAM1gene expression (P=0.000). The interaction did not show any significant effect (P=0.386).

Post-hoc analysis, using LSD (table 11) reflected significant differences of different levels of hours, including 6 &12 hrs, 6 & 24hrs, 12 & 24hrs, and 12 & 48 hr (P=0.015, 0.000, 0.001, & 0.005, respectively). There was no significant difference in the period between 6 hr and 48 hr- time points (P=0.428).

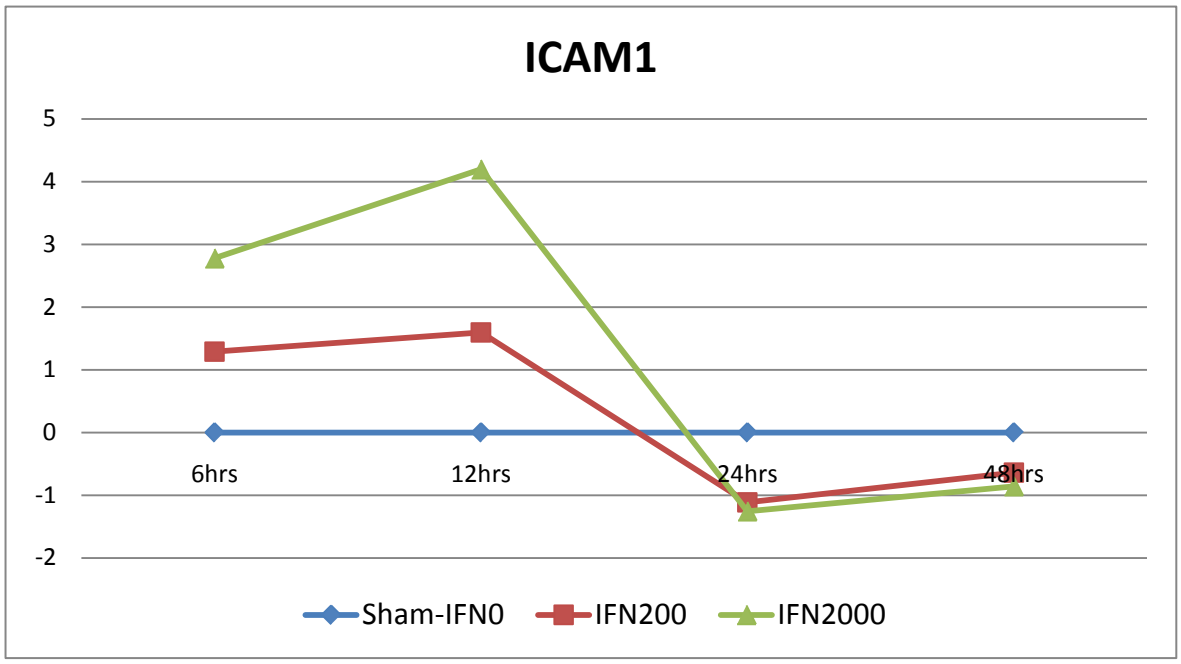

Figure 21: Relative fold changes in ICAM1 gene expression in experiment-2

Table 10: Output of Two-way ANOVA for ΔCt of ICAM1gene

Variable	df	Mean Square	F-value	Significance
Dose	2	0.680	1.563	0.230
Time *	3	5.232	12.033	0.000
Dose X time	6	0.482	1.108	0.386

*: The mean difference is significant at the 0.05 level

Table 11: Post-hoc analysis for the effect of time on ΔCt of ICAM1 gene

Levels of time	Std Error	Significance
6-12 hrs*	.20369	0.015
6-24 hrs*	.20369	0.000
6-48 hrs	.20369	0.173
12-24 hrs*	.20369	0.001
12-48 hrs*	.20369	0.005
24-48 hrs	.20369	0.232

*: The mean difference is significant at the 0.05 level

4.2.2.3.2 Expression of TNF-α gene

TNF-α showed about 2-7-fold increase in the case of 2000U and 2-4 folds increase in the case of 200U. The peak increase was at 12 hours; however the gene expression declined afterward to reach its minimum at 48hrs in both doses (Figure 22).

Two-way ANOVA (Table 12) indicated no significant difference for the effect of dose (P=0.722) or the interaction (P=0.316) on the ΔCt of TNF-α. However, the effect of time on ΔCt of TNF-α gene was significant (P=0.003).

A post-hoc analysis (Table 13) reflected significant difference for the effect of IFN-α at 6-12 hrs, and 6 - 24 hrs (P=0.000 & 0.012 respectively) as well as between 12 hrs and 48 hrs (P=0.013).

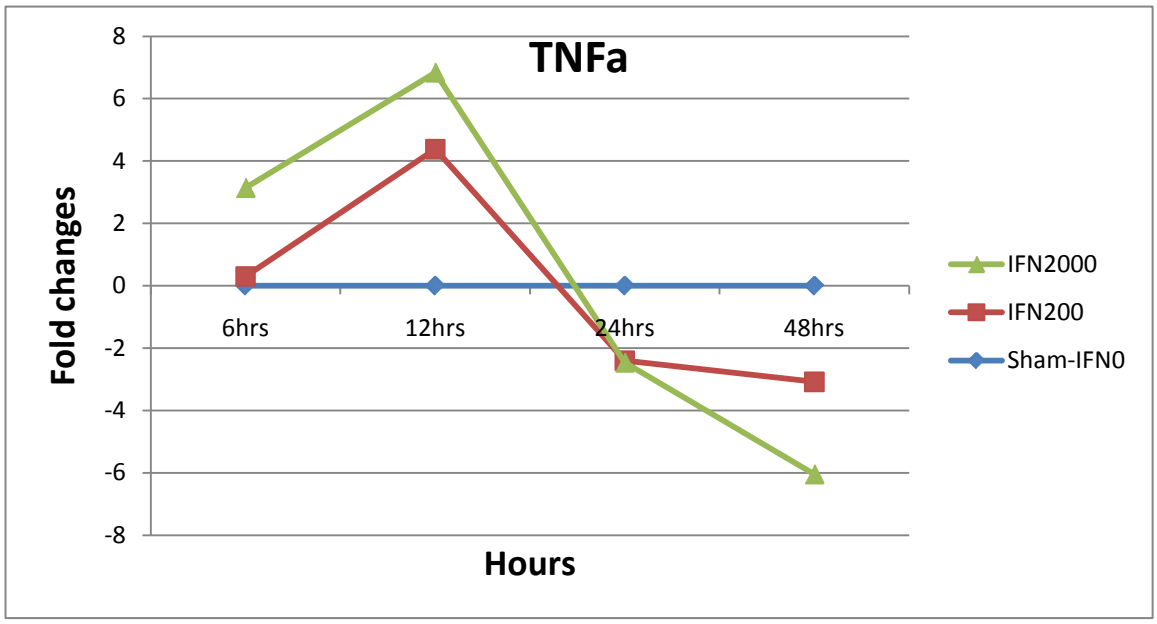

Figure 22: Relative fold changes in TNF-α gene expression in experiment-2

Table 12: Output of two-way ANOVA for ΔCt of TNF-α gene

Variable	df	Mean Square	F-value	Significance
Dose	2	0.816	0.333	0.722
time*	3	15.191	6.200	0.003
Dose X time	6	3.065	1.251	0.316

*: The mean difference is significant at the 0.05 level

Table 13: Post-hoc for the effect of time on ΔCt of TNF-α1 gene

Levels of time	Std Error	Significance
6-12 hrs*	0.740	0.000
6-24 hrs*	0.740	0.013
6-48 hrs	0.740	0.168
12-24 hrs	0.740	0.175
12-48 hrs*	0.740	0.013
24-48 hrs	0.740	0.211

*: The mean difference is significant at the 0.05 level

4.2.2.3 Expression of markers for the immune response

The second part of the third research question was to determine if IFN-α can induce genes that might be specific for an immune response; the H2K1 gene that encodes MHC1 was examined for this purpose. The result of experiment-2 showed an increase in the MHC1 gene expression by about 3 folds in the case of 200U of IFN-α versus 6-8 fold-increases in case of 2000U of IFN-α. The pattern of the fold change was similar for both cases (Figure 23).

In addition, two-way ANOVA indicated significant effects for both dose and time on the ΔCt of MHC-I gene, with P= 0.000 and 0.001, respectively (Table 14).

The results of LSD test indicated significant differences between IFN0 & IFN200, IFN0 & IFN2000, and IFN200 & IFN2000 (P=0.000, 0.000, and 0.000, respectively) (Table 15). On the other hand, Table 16 shows the output of the LSD test indicating significant differences between 6 & 24 hrs, 12 & 24 hrs, 24 & 48 hrs time points (P=0.001, 0.029, and 0.000, respectively).

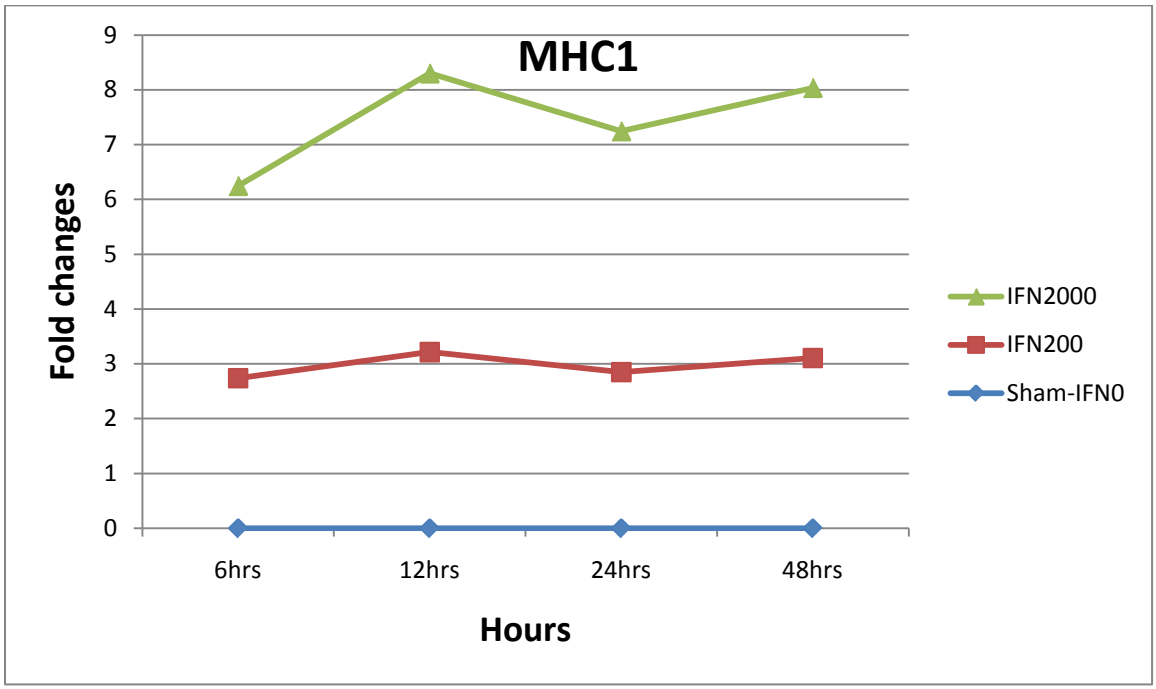

Figure 23: Relative fold changes of MHC1 gene expression in experiment-2

Table 14: Output of two-way ANOVA for ΔCt of MHC-I gene

Variable	df	Mean Square	F-value	Significance
Dose*	2	15.618	246.631	0.000
Time*	3	0.464	7.320	0.001
Dose X hours	6	0.102	1.604	0.189

*: The mean difference is significant at the 0.05 level

Table 15: Post-hoc analysis for the effect of dose on ΔCt of MHC-I gene

Levels of dose	Std Error	Significance
FN0-IFN200*	0.10273	0.000
IFN0-IFN2000*	0.10273	0.000
IFN200-IFN2000*	0.10273	0.000

*: The mean difference is significant at the 0.05 level

Table 16: Post-hoc analysis for the effect of time on ΔCt of MHC-I gene

Levels of time	Std Error	Significance
6-12 hrs	0.11863	0.116
6-24 hrs*	0.11863	0.001
6-48 hrs	0.11863	0.868
12-24 hrs*	0.11863	0.029
12-48 hrs	0.11863	0.085
24-48 hrs*	0.11863	0.000

*: The mean difference is significant at the 0.05 level

4.2.2.4 Expression of markers for apoptosis

The third part of the third research question was to determine if IFN-α can induce expression of markers for apoptosis, which included, Fas gene, Caspase3 gene, and Bax gene.

4.2.2.4.1. Expression of Fas gene

Figure 24 shows that the expression level of Fas gene exceeded three-fold increase only at 12 hrs in the case of IFN2000; whereas the expression level showed less than two-fold increase at any time point in the case of IFN200. However, the pattern of fold-change was almost similar in both cases (peak at 12 hrs and minimum change at 48hrs).

In addition, two- way ANOVA (Table 17) indicated no significant effect of the dose (P=0.127) or the interaction (P=0.592); however, there was significant effect of changing the time on Fas gene expression (P=0.002).

Post-hoc analysis using LSD test (Table 18) indicated significant differences between 6 & 48 hrs, 12 & 48 hrs, 24 & 48 hrs time points (P=0.001, 0.001, and 0.001, respectively).

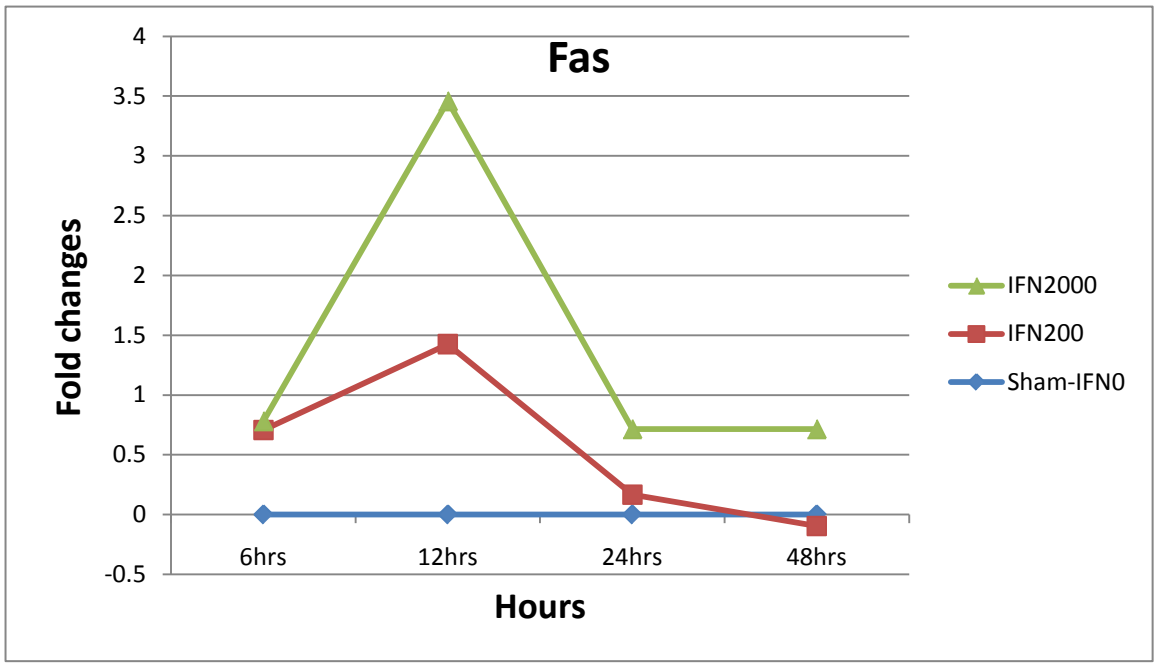

Figure 24: Relative fold changes in Fas gene expression in experiment-2

Table 17: Output of two-way ANOVA for ΔCt of Fas gene

Variable	df	Mean Square	F-value	Significance
Dose	2	0.575	2.250	0.127
Time*	3	1.715	6.711	0.002
Dose X Hours	6	0.200	0.782	0.592

*: The mean difference is significant at the 0.05 level

Table 18: Post-hoc analysis for the effect of time on ΔCt of Fas gene

Levels of time	Std Error	Significance
6-12 hrs	0.23832	0.649
6-24 hrs	0.23832	0.737
6-48 hrs*	0.23832	0.003
12-24 hrs	0.23832	0.905
12-48 hrs*	0.23832	0.001
24-48 hrs*	0.23832	0.001

*: The mean difference is significant at the 0.05 level

4.2.2.4.2 Expression of caspase-3 gene

Figure 25 shows the gene expression level of Caspase3. Although the expression level exceeded up to three folds in the case of 2000U of IFN-α, there was less than a two-fold increase in the case of 200U of IFN-α. The pattern of gene expression was similar in both cases, showing peaks around 12 hrs and maximum decline at 48hrs.

In addition, two- way ANOVA reflected significant effect of the dose and time on caspase-3 gene expression (P=0.048 & 0.007, respectively); whereas no significant effect of the interaction (P=0.555) (Table 19).

Post hoc analysis revealed significant differences between IFN0 & IFN2000 (P=0.017) only, no significant differences between IFN0 & IFN200, or IFN200 & IFN2000 (P=0.085, and 0.450 respectively) (Table 20). Analysis also revealed significant difference between 6-48hrs, 12-48hrs, and 24-48hrs (P=0.027, 0.001, & 0.005, respectively) only (Table 21).

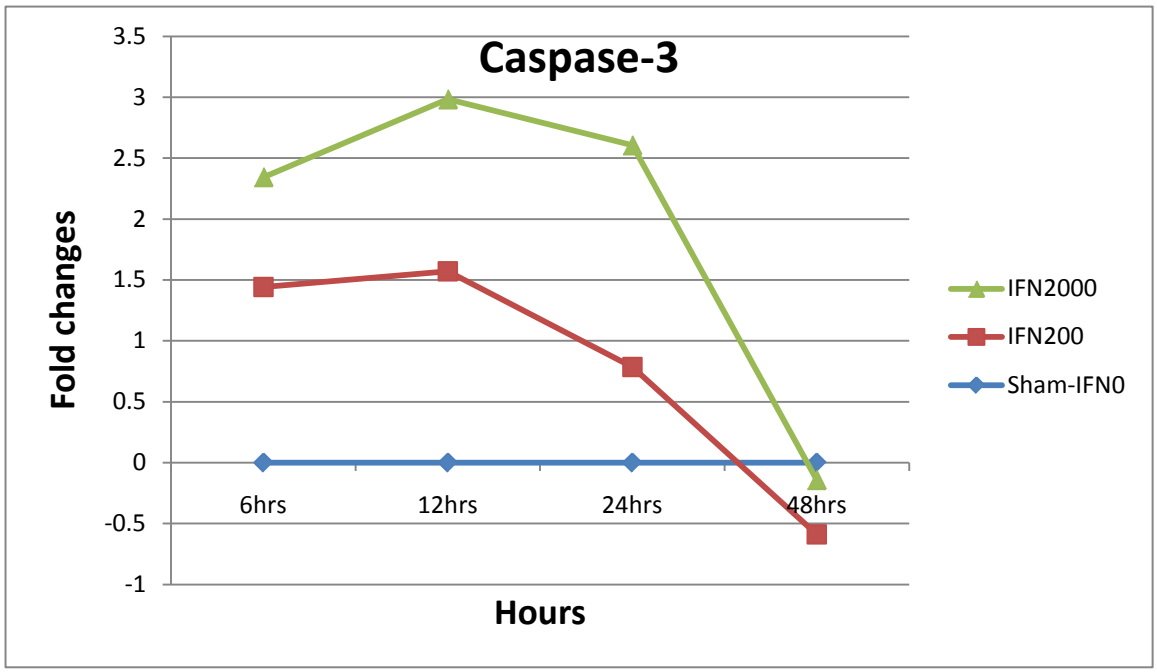

Figure 25: Relative fold changes in Caspase-3 gene expression - experiment-2

Table 19: Output of two-way ANOVA for ΔCt of Caspase-3 gene

Variable	df	Mean Square	F-value	Significance
Dose*	2	1.039	3.458	0.048
Time*	3	1.562	5.199	0.007
Dose * Time	6	0.251	0.836	0.555

*: The mean difference is significant at the 0.05 level

Table 20: Post-hoc analysis for the effect of dose on ΔCt of Caspase-3 gene

Levels of dose	Std Error	Significance
IFN0-IFN200*	0.22378	0.085
IFN0-IFN2000*	0.22378	0.017
IFN200-IFN2000*	0.22378	0.450

*: The mean difference is significant at the 0.05 level

Table 21: Post-hoc analysis for the effect of time on ΔCt of Caspase-3 gene

Levels of time	Std Error	Significance
6-12 hrs	0.25839	0.199
6-24 hrs	0.25839	0.482
6-48 hrs*	0.25839	0.027
12-24 hrs	0.25839	0.550
12-48 hrs*	0.25839	0.001
24-48 hrs*	0.25839	0.005

*: The mean difference is significant at the 0.05 level

4.2.2.4.3 Expression of Bax gene

Figure 26 shows less than two-fold increase in the expression of the Bax gene for either dose of IFN-α. However, there was a peak, reaching around two folds at 24hrs in the case of 2000U of IFN-α. The two- way ANOVA results (Table 22) indicated significant effect of time on ΔCt of Bax gene (P=0.000); whereas, the effects of the dose or the interaction between the dose and hours were not significant (P=0.138 & 0.172, respectively).

In addition, LSD test revealed significant differences between 6-24, 6-48hr, 12- 24hr, 12-48hr, and 24- 48hr time points (P= 0.001, 0.000,0.028, 0.000, & 0.000) (Table 23).

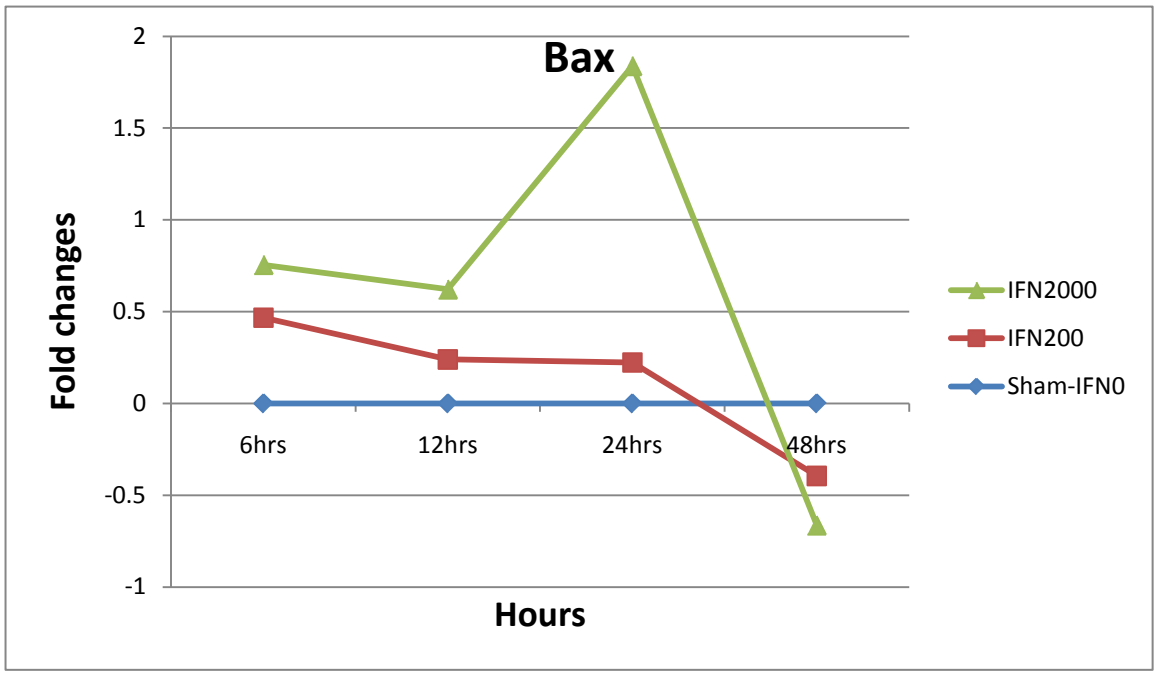

Figure 26: Relative fold changes in BAX gene expression - experiment-2

Table 22: Output of two-way ANOVA for ΔCt of Bax gene

Variable	df	Mean Square	F-value	Significance
Dose	2	0.202	2.151	0.138
Time*	3	3.848	40.986	0.000
Dose X Time	6	0.157	1.668	0.172

*: The mean difference is significant at the 0.05 level

Table 23: Post-hoc analysis for the effect of time on ΔCt of Bax gene

Levels of time	Std Error	Significance
6-12 hrs	0.14444	0.147
6-24 hrs*	0.14444	0.001
6-48 hrs*	0.14444	0.000
12-24 hrs*	0.14444	0.028
12-48 hrs*	0.14444	0.000
24-48 hrs*	0.14444	0.000

*: The mean difference is significant at the 0.05 level

Table 24 summarizes the findings for IFN-α-induced genes examined in the current study

Table 24: Summary of the findings for IFN-α-induced genes

Gene	**Fold change**		**ANOVA**		**Indication for**
	IFN-200	***IFN-2000***	***Dose***	***Time***	
IFN-α	2-4folds	3-6folds	S[a]	N-S[b]	IFN-α expression
IFN-β	2-5folds	4-11folds	S[a]	S[a]	other types of IFN
IFN-γ	ND[c]	ND[c]	NA [d]	NA[d]	No IFN-γ expression
TNF-α	2-4folds	2-6folds	N-S[b]	S[a]	Inflammatory response
ICAM1	N-S[b]	3-4folds	N-S[b]	S[a]	Inflammatory response
MHC-I	3folds	6-8folds	S[a]	S[a]	Immune response
Fas	N-S[b]	3.5folds	S[a]	N-S[b]	Apoptosis
Caspase-3	N-S[b]	2-3 folds	S[a]	S[a]	Apoptosis
Bax	N-S[b]	2folds	N-S[b]	S[a]	Apoptosis

[a]: S= significant [b]: N-S= not significant

[b]: ND= not detected [d]: NA=not-applicable

4.2.3 Effect of IFN-α on cell count of the cell culture

The fourth research question examined if IFN-α treatment would affect the cell count of the cochlear cells. Figure 27 shows an initial increase in the cell count at 6hr in the sham-treated and 200U/ml-treated groups. However, at the same time point, there was apparent reduction of cell count after treatment with 2000U/ml IFN-α, which continued to reach a maximum reduction at 48hrs. After 12 hrs, the trend in cell count reduction was almost the same for both doses of IFN-α.

Table 25 shows the output for two-way ANOVA which revealed significant differences for the effect of both dose and time on the cochlear cell count (P= 0.000 in both cases). However, the interaction showed no significant effect (P=0.228), which means that the dose and time affect the cochlear cell count independently.

Post hoc testing (Table26) shows that there was significant differences between sham and both 200 and 2000U/ml of IFN- α; however there was no significant difference between 200 & 2000U/ml (P=0.00, 0.00, and 0.169 respectively). On the other hand, levels of time showed significant differences between 6 & 24hrs and 6 & 48hrs only (P=0.019 & 0.002, respectively) (Table 27)

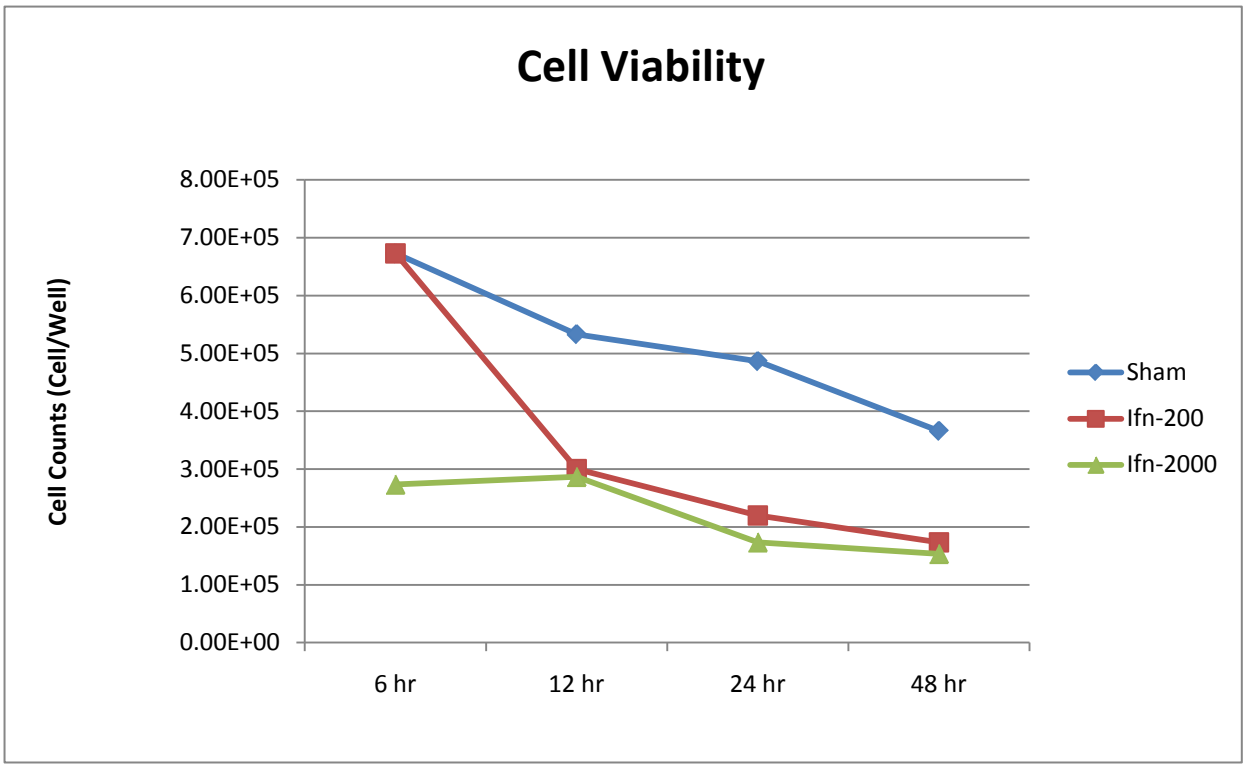

Figure 27: Cell count change across time points for different doses of IFN- α

Table 25: Output of two-way ANOVA for cell count

Variable	df	Mean Square	F-value	Significance
Dose	2	2.837E+11	58.301	0.000
Time	3	9.524E+10	19.570	0.000
Dose X time	6	7185185185	1.476	0.228

*: The mean difference is significant at the 0.05 level

Table 26: Post hoc analysis for the effect of dose on cell count

Levels of dose	**Std Error**	**Significance**
Sham-IFN200*	47441.26264	0.000
Sham-IFN2000*	47441.26264	0.000
IFN200-IFN2000	47441.26264	0.169

*: The mean difference is significant at the 0.05 level

Table 27: Post hoc analysis for the effect of time on cell count

Levels of time	Std Error	Significance
6-12hrs	71072.03788	0.188
6-24hrs*	71072.03788	0.019
6-48hrs*	71072.03788	0.002
12-24hrs	71072.03788	0.269
12-48hrs	71072.03788	0.054
24-48hrs	71072.03788	0.388

*: The mean difference is significant at the 0.05 level

5.0 CHAPTER V: DISCUSSION

The study was designed to investigate the signaling pathway of IFN-α in the cochlear cell line and, in particular, the study was aimed at: first, determining if the cochlea has receptors for IFN-α that might lead to the expression of IFN genes including IFN-α, IFN-β, and IFN-γ and if IFN-α acts directly or indirectly through IFN-β and IFN-γ. Second, the study aimed to detect those genes or markers that can be stimulated by the effect of IFN-α and if the cochlear cell count would be changed by the effect of IFN-α. In addition, a subsidiary goal was to confirm the presence of specific markers for the cochlear cell line.

As expected, the fold-change calculation reflected more than two-fold increases in the expression of most of the hair cell markers which indicates that viable hair cells grew in the cell culture used in the current experiment.

The results of experiment-1, using small doses of IFN-α (20, 40, 80U/ml) showed less than two-fold increase in a subset of the IFN-α-induced genes (TNF-α, ICAM, Caspase-3 or Bax). This indicates that IFN-α in small doses did not induce expression of some of the IFN-α-induced genes such as inflammatory genes and apoptotic genes.

However, the results of experiment-2 using higher doses of IFN-α (200 & 2000 U/ml) reflected more than two-fold increases in the inflammatory markers (ICAM1& TNF-α), immune response marker (MHC-I) as well as some apoptotic markers (Fas & caspase-3 genes). The

results also reflected significant fold-increase of IFN-α1 gene, IFN-β gene with no significant increase of type II-IFN (IFN-γ). This might suggest that IFN-α acted directly on the cochlear cell line, not through IFN-γ; however its action through IFN-β genes is more likely. These results suggest that high doses of IFN-α stimulated the IFN-α receptors either directly or through IFN-β, led to induction of inflammatory response, immune response and apoptosis of the cochlear cells, reflected in the reduction of the cell number.

The results also showed significant effects of both the dose and the time of IFN-α treatment on the pattern and amount of expression of some of the IFN-α-induced genes.

The following section will discuss the results of experiment-2 in terms of each research question and compare them to the results of experiment-1.

5.1 Dose the cochlear cells have receptors for IFN-α?

One subtype of IFN-α was tested to detect the presence of IFN-α receptors in the cochlear cell line, i.e., the IFN-α1 gene. The results showed more than two-fold increases in the expression of the IFN-α1 gene (Figure 18). This increase was more apparent in the case of the IFN2000 group than in the IFN200 group. These findings suggest that the dose of IFN-α affects the level of expression IFN-α1 and also indicate that the cochlear cells have receptors for IFN-α.

However, not only the dose can cause differences in the effects of IFN-α, it can be the type of IFN used or the associated disease that induces different biological action. For example, Kaygusuz, et al. (2004) found no effect of IFN-α on the hearing threshold of patients with chronic active hepatitis B treated with IFN-α2b/day (the cumulative dose was about 720 million units over 6 months). On the other hand, Johnson, et al. (2008) reported a case of bilateral SNHL

and vestibular dysfunction in a patient receiving the same type of IFN-α with similar cumulative dose for the treatment of hepatitis C. This suggests that the associated disease, hepatitis C versus hepatitis B, could be the determinant factor in causing hearing impairment, not the dose of the IFN-α.

In the current study, the IFN-α1 gene was the type of IFNs that were expressed, not the IFN-α2b, which might have another distinctive pathway. The differential expression of individual IFN-α subtypes was due to distinct nucleotide substitutions in the promoter of IFN genes (Au, Yeow, & Pitha, 2001; Au, Su, Raj, & Pitha, 1993; Civas, Genin, Morin, Lin, & Hiscott, 2006; Lopez, Reeves, Island, Bander, Christelt, et al., 1997) as well as the presence of negative regulatory sequences located in the upstream (previous location) promoter regions of some IFN-α subtypes (Lopez, Island, Drouin, Bandu, Christeff, et al., 2000) (details about this issue are outside of the scope of the current study). However, the possibility that those patients with hepatitis C are more vulnerable to develop SNHL by IFN therapy cannot be excluded; further investigations are needed to delineate this vulnerability.

In addition, the results of the current study appear to contradict with the results from Akyol, et al. (2001) who failed to find pathological changes in the organs of Corti of albino Swiss mice treated by IFN-α. The authors investigated the effect of IFN-α, using audiological and histological approaches, but these methods might not be the most accurate approaches to precisely rule out the influence of IFN-α on hearing—an effect that can be determined more precisely at the molecular level. However, the audiological and the histopathological changes observed in their study might be the outcome of the expression of some IFN-stimulated genes that led to a shift in the APR threshold after IFN-α treatment. Otoacoustic emissions would be a more precise tool to detect early changes if any in the cochlea.

In conclusion, the IFN-α1 gene was detected in the cochlear cell line, which suggests that the cochlea has receptors for IFN-α. The expression of this gene in the cochlear cell line was dose and time dependent which might lead to subsequent expression of other genes with diverse reactions at different time points.

5.2 Did IFN-α act directly on the cochlear cell line?

Two genes were tested to answer this question, including IFN-β and IFN-γ genes.

5.2.1. Expression of IFN-β gene

The results revealed more than two-fold increases in the expression of IFN-β gene (about 5 folds by 48 hrs in the case of 200U/ml & 11 folds in the case of 2000U/ml dose) (Figure 19). It is interesting that the pattern of gene expression was not only identical for both doses, but also very similar to the pattern of expression in the case of the IFN-α1 gene (Figure 18). This might be due to the fact that all type I interferons (IFN-α, IFN-β, and IFN-ω) bind to a common receptor called IFNR, which is formed of two subunits (α subunit and β subunit) and elicit a common set of signaling events that ultimately regulate the expression of common biological activities (Novick, Cohen, & Rubinstein, 1994; Stark, Kerr, Williams, Silverman, & Schreiber, 1998; Uze, Lutfalla, & Gresser, 1990). However, this is not always the case; there is accumulating evidence that significant differences among the effects of distinct Type I IFNs in vitro and in vivo exist (Platanias, 1995). In particular, IFN-β induces a selective association of a tyrosine-

phosphorylated protein (p100) with the α-subunit of the receptor, which might result in a distinctive pathway for IFN-β (Platanias, Uddin, & Colamonici, 1994).

The expression of IFN-β as a result of IFN-α treatment can be explained in terms of two levels: First at the cellular level: IFN-β and IFN-α are secreted from the same type of cells, which are the DCs, in particular the plasmacytoid cell subtype of dendritic cells (pDCs). If the cochlear cell line has this type of cells; upon stimulation by IFN-α, the DCs will secrete IFN-β. The secretion of IFN-β may have a negative feedback on the production of IFN-α1, decreasing its level, which might explain the relatively low expression of IFN-α gene in the same time window (the fold-increase of IFN-β was more than that of IFN-α1). However, according to the available literature to date, no evidence confirms that DCs are inhabitants of the inner ear. The results of the current experiment as it will be mentioned later support the presence of the DC cells in the cochlea as reflected by the pronounced expression of the MHC-I gene, which is a marker for the immune response. MHC-I are known to be present on the surface of the APCs, among which are the DC cells.

Second at the molecular level: the expression of the IFN-α and IFN-β genes is regulated at transcriptional (during copying the mRNA from DNA) and post-transcriptional (after formation of mRNA) levels. The family of interferon regulatory factors (IRFs) is the most important transcriptional factors that regulate the expression of type-I IFN, in particular, IRF1, IRF3, and IRF7. The expression of IFN-α gene is associated with activation of IRF3, which in turn will homo-or heterodimerize with IRF-7 and translocate to the nucleus, where they stimulate transcription of IFN-β (Grandvaux, Servant, tenOever, Sen, & Balachandran, 2002; Lin, Heylbroeck, Pitha, & Hiscott, 1998; Lin, Mamane, & Hiscott, 1999; Peters, Smith, Stark, & Sen,

2002; Weaver, Kumar, & Reich, 1998; Yoneyama, Suhara, Fukuhara, Fukuda, Nishida, & Fujita, 1998).

The expression of IFN-β in larger amounts than IFN-α1 might be in agreement with what has been reported by Kanda et al. (1994) who found that the incidence of hearing impairment was more associated with IFN-β than with IFN-α. However, in another study, Kanda et al. (1995) found no difference in the incidence of hearing loss between patients treated with IFN- α and those who were treated with IFN-β. This similarity might be consistent with the similar pattern of expression between IFN-α and IFN- β in the current study.

In conclusion, IFN-β gene expression in the cochlear cell line can be induced by IFN-α treatment either by stimulation of DC cells or through IRF3 signaling pathway. The apparent low expression level of IFN-α in comparison to that of IFN- β might suggest that the IFN-β took over the action of IFN-α1, i.e., the action of IFN-α on the cochlear hair cell line can be mediated by IFN-β. This means that IFN-α can act directly on its receptors or by enhancing the secretion of IFN-β. Gene-gene interaction study might be helpful in delineating this relation between both genes. The effects of dose and time were significant on the IFN-β gene expression, which means that the IFN-β gene expression was dose and time dependent.

5.2.2. Expression of IFN-γ

Although it is well known that type I-IFN enhances the secretion of IFN-γ by mediating the activation of NK and T cell responses (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999; Orange & Biron, 1996), the results of the current study revealed less than two-fold increases in the IFN-γ gene expression (Figure 20). This means that the growing cells from the cochlear cell

line used in the current experiment did not produce IFN-γ. IFN-γ can be produced in the inner ear by induction of labyrinthitis in experimental animals (e.g., Gloddek et al., 2002; Pawankar, et al., 2004) and human subjects (Lorenz, et al., 2002; Solares & Tuohy, 2005) as a result of antigen challenge in the inner ear or associated with autoimmune SNH, respectively. Moreover, the upregulation of IFN-γ in the inner ear in these studies was associated with upregulation of ICAM1 (Pawankar, et al., 2004) and MHC-I (Gloddek et al., 2002), which means that IFN-γ was associated with induction of inflammatory and immune responses in the inner ear. These findings provide evidence for the production of IFN-γ in the inner ear in association with inner ear injury or pathology, which is not the case in the current study. The events in the current study were not based on ongoing antigen challenges or stimulation of the immune system cells (the cochlear cell line used in the current study was not exposed to any kind of antigen or cell injury), rather they reflected molecular or genetic signaling events and this may explain the difference in findings.

At the molecular level, the results of the current study might be in agreement with the study of Nguyen, Cousens, Doughty, Pien, Durbin & Biron (2000) who found that IFN-α and IFN-β did not enhance the expression of IFN-γ. They attributed this negative regulation between type I-IFN (IFN-α & IFN-β) and type II-IFN to STAT1, which is involved in the signaling pathway of type I-IFN because when these two cytokines had an alternative signaling pathway that did not involve STAT1, they promoted the expression of IFN-γ. Therefore, it can be argued that IFN-α and IFN-β used the Jak-STAT signaling pathway with activation of STAT1 in the cochlear cell culture, and hence, they were not able to promote the expression of IFN-γ. This pathway needs to be tested in order to elucidate this issue.

Treatment of the cochlear cell line with IFN-α did not induce expression of the IFN-γ gene, which suggests that IFN-γ did not mediate the action of IFN-α. Instead, IFN-α might have acted directly or through IFN-β as was mentioned in the previous section.

5.3 Did IFN-α induce expression of inflammatory markers?

To answer this question, the expression of two inflammatory genes was examined, including the ICAM1 gene and the TNF-α gene.

5.1.1 Expression of ICAM1 gene

The results of experiment-2 revealed more than two-fold increases in the expression of the ICAM1 gene as a result of IFN-α treatment of 2000U/ml; whereas, a dose of 200U/ml showed less than two fold-change (Figure 21). In both cases, the maximum fold-increase was at12hrs, a point at which both IFN-α and IFN-β showed maximum decline in their levels (Figures 18 & 19). The level of ICAM1 expression started to increase at the beginning (for instance 6hrs) then reached maximum at 12hrs and declined to its minimum level at 48hrs, a pattern that is similar to the pattern of changes in the ICAM1 concentration in other studies. For example, Suzuki and Harris (1995) found that the concentration of ICAM1 in the inner ear following antigen challenge was weak at 6hrs and reached its maximum on the second day, and then it faded away. Also, Pawankar et al. (1998 & 2004) observed the same pattern of expression of ICAM-1 molecules in different parts of the inner ear following an antigen challenge and IFN-γ treatment.

These previous studies found that the expression of ICAM1 was concomitant with the expression of IFN-γ. The authors suggested that IFN-γ plays an important role in the expression of the ICAM1 gene. The IFN-γ gene was not detected in the current experiment; however the observation that ICAM1 reached its maximum level at 12hrs, where both IFN-α and IFN-β expression reached their minimum levels suggests the possibility of a relationship between ICAM1 and both IFNs. Notably, the levels of ICAM1 maximally declined at 24 & 48hrs, whereas both IFNs reached their highest levels. It seems that once the level of both IFNs declined, ICAM1 molecules increased and the reverse is correct. This might be explained by the presence of a negative feedback between the expression ICAM1 and the expression of both IFNs. It is known that the production of cytokines is usually transient, and never can be sustained to initiate a certain action or to stimulate another cells to induce different cytokines (Guttermann, 1995), which might be the case with ICAM1 and both IFNs in the current study. The scenario of the events could be as follow: the expression of both IFNs enhanced the expression of ICAM1, the expression of which might have led to a transient decline in the IFNs' levels (for example at 12hrs). The function of ICAM1, as inflammatory molecules, is mainly the recruitment of inflammatory cells and inflammatory mediators to the scene of inflammation; once these mediators are formed, ICAM1 molecules have no further role. This might explain the transient increase in their levels in the current experiment and other studies.

In addition, the association between ICAM1 and IFNs was not limited to that between IFN-γ and inner ear pathology, but there is also an association between them in retinal pathology. For instance, Lee, Hooper, Kump, Hayashi, Nussenblatt et al. (2006) found that there was a concomitant expression of both IFN-β and ICAM1 in the sera of patients with retinal vasculitis. There was also an association between IFN-α and ICAM1 in the islet endothelial cells of the

pancreas in type I diabetes mellitus (Chakrabarti, Huang, Beck, Henrich, McFarland, et al., 1996; Foulis, Farquharson, & Meager, 1987; Somoza, Vargas, Roura-Mir, Vives-Pi, Fernandez-Figueras, et al., 1994). Therefore, IFN-α and IFN-β might have enhanced the expression of the ICAM1 molecules, which in turn led to the reduction of these two cytokine levels by a negative feedback mechanism.

5.3.2. Expression of TNF-α gene

The pattern of expression of the TNF-α gene was very similar to the ICAM1 gene expression (Figure 22). Therefore, it can be argued that both TNF-α and ICAM1 as inflammatory genes were expressed in the same time and they had a similar relationship (negative feedback) with IFN-α and IFN-β. Also, TNF-α expression was time-dependent and dose-independent. IFN-α has an inhibitory effect on the TNF-α gene expression in vivo and in vitro, producing an anti-inflammatory effect (Abu-Khabar, Armstrong, & Ho, 1992; Larrea, Garcia, Qjan, Civiera, & Prieto, 1996). The findings of these authors' studies appear to contradict the conclusion that was drawn from the current study about the presence of a feedback between TNF-α and IFN-α & IFN-β as in case of ICAM1. However, the pattern of changes observed in the expression of all four genes is more consistent with a feedback mechanism than with an inhibitory mechanism. If the case was an inhibitory mechanism, the level of IFN-α and IFN-β would not decline when the levels of ICAM1 & TNF-α were high (as was observed at 12hrs); instead, the levels of both IFNs should remain high all the time, as long as the levels of both ICAM1 & TNF-α are low (in the case of an inhibitory effect).

In agreement with the results of experiment-2 is the study of Ren, Jin, Piao, and Piao (2007) who found that IFN-α therapy significantly increased the level of TNF-α and IFN-γ in patients with hepatitis B. This means that IFN-α promotes the production of TNF-α to accomplish its antiviral effect on the hepatitis B virus, which might support the feedback relationship between TNF-α and both IFNs.

Also, other studies showed that both IFN-α and TNF-α share some activities. For instance, a series of studies reported that combined treatment with IFN-α and TNF-α exhibited much stronger antitumor effects (Fiers,1991; Muro, Naomoto, & Orita, 1991; Naomoto, Tanaka, Fuchimoto, & Orita,1987; Orita, Ando, & Kurimoto, 1987; Yasuoka, Naomoto, Yamatsuji, Takaoka, Kimura, et al., 2001). The authors of these studies found that the two cytokines had the same signaling pathway to induce their apoptotic or antitumor effect. This reflects that both of these cytokines have synergetic or additive effect and not inhibitory effects as it was reported by Abu-Khabar, et al. (1992) and Larrea, et al. (1996).

Therefore, it can be concluded that IFN-α have induced the expression of the TNF-α gene in cultured cochlear cells. It appeared that a negative feedback relationship exists between them. However the nature of their action together, either inflammatory, anti-inflammatory or apoptotic cannot be determined within the scope of the current study. Further signaling pathways need to be tested to resolve this issue. Conclusively, the results and the supportive evidence from previous studies suggest that IFN-α treatment resulted in expression of both ICAM1 & TNF-α genes (inflammatory markers) in the cultured cochlear cells of this experiment and these genes had a negative feedback on IFN-α1 and IFN-β expression.

5.4. Did IFN-α induce expression of immune response markers?

To address this question, the expression of MHC-I gene was tested. There was much more than two-fold increases in the expression of the MHC-1 encoded by H2K1 gene in the cochlear cells treated by IFN-α. The expression was relatively high in both doses of IFN-α and over all time points, which might suggest that MHC-I was expressed constantly in the treated cochlear cell culture (Figure 23). The function of the MHC-I molecules is to present processed antigen to cytotoxic T cells and bind to CD8 on the surface of T-cells. IFN-α induces activation of CD8+ T-cells and thus promotes their cytotoxicity, with subsequent expression of the MHC-I molecules (York, Odom, Murphy, Ives, and Wente, 1999). In addition, IFN-α stimulates the DCs, which are considered professional APCs (Cella, Facchetti, Lanzavecchia, & Colonna, 2000). Therefore, IFN-α can enhance antigen presentation to T-cells with a subsequent expression of MHC-I and phagocytosis of a foreign antigen (Biron, 1998).

At the molecular level, the promoter (i.e., a part of the gene with which a stimulating molecule will attach to induce expression of that gene) of the MHC-I genes contains an interferon response sequence (IRS), which leads to expression of MHC-I once IFN-α is introduced (Ten, Blank, Le, Kourilsky, & Israël, 1993). Therefore, treatment of the cochlear cell culture with IFN-α most probably led to expression of MHC-I gene.

The results of the current study may be in agreement with what has been documented by many studies that MHC-I is a “hallmark” of type I-IFN, including IFN-α (Brucet, Marqués, Sebastián, Lloberas, & Celada, 2004). In addition, IFN-α treatment has been found to be important for clonal expansion and survival of the CD8+ T-cell population (MHC-I binds to the surface of these cells) in an antigen-independent manner, a mechanism called “bystander effect”

(Parronchi, Mohapatra, Sampognaro, Giannarini, Wahn, et al.,1996; Tough, Borrow, Sprent, 1996). Although this bystander effect is not as powerful as a response during viral infection, it still represents a unique mechanism of controlling an adaptive immune response (Tough et al., 1996). Moreover, IFN-α has been found to cause alteration of the MHC with increased expression of class I molecules on the tumor cells (Oberg, 1992). All these findings support the notion that IFN-α is a major enhancer of T-cell activation and hence, it is a potent inducer of MHC. Furthermore, in agreement with the current study, other studies on both animal and human have indicated that IFN-γ and IFN-α may upregulate MHC-I molecules on various types of normal cells (Jarosinski and Massa, 2002; Keir et al, 2002; Ljunggren and Anderson, 1998) and tumor cells (Abril., Mendez, Garcia, et al., 1996; Anderson, Elder, Brown et al., 2002; Atta, Irving,Powell, et al., 1995).

Notably, lymphocytes (which are the cells of the adaptive immune response) are not normally present in the cochlea; however, the cochlea's innate immunity may be responsible for initiating the adaptive immune response with subsequent recruitment of lymphocytes. Therefore treatment with IFN-α, which is well known by its priming action (initiates the innate immune response) and its bystander effect (needs no antigen) will promote the development of adaptive immune response with subsequent lymphocytic activation, leading to MHC-I production in the cochlear cell culture regardless of the absence of antigen exposure. This conclusion might explain the constant expression of MHC-I over all the time points and in both doses (200 & 2000U/ml).

Many studies have shown the involvement of lymphocytes, including CD+4 & CD+8 T-cells with the production of IFN-γ and ICAM1 in the pathogenesis of immune-mediated inner ear disorders in human subjects as well as in experimental animals (Arnold, Pfaltz, & Altermatt,

1987; Gloddek et al., 1999; Lorenz et al., 2002; McCabe & McCormic, 1984; Yamanobe & Harris, 1992). This association between hearing loss and the immune response suggests that IFN-α can cause hearing impairment by promoting the innate as well as the adaptive immune responses in the cochlea. Therefore, IFN-α can induce an immune response in the cochlea.

5.5 Did IFN-α induce expression of apoptotic markers?

Three markers were examined to address this question, including Fas, caspase-3 and Bax genes.

5.5.1 Expression of Fas gene

The results of the current study showed more than two-fold increase in the expression of Fas gene only at the 12hrs time point in the case of IFN2000, and less than two-fold increase with IFN200 or other time points in either case (Figure 24). This transient pattern of expression is more or less similar to the pattern of expression of both ICAM1 & TNF-α. Notably, there were many events occurring at the 12hrs time point: IFN-α1 and IFN-β reached their minimal level of expression, ICAM1, TNF-α and Fas genes reached their maximum levels of expression. Therefore, it can be argued that Fas gene expression has the same negative feedback relationship with both IFN-α1 and IFN-β. The scenario of these events can be as follows: treatment of the cochlear cell culture with IFN-α led to the expression of IFN-α1 and IFN-β, which stimulated the expression of inflammatory genes (ICAM1 & TNF-α). By their roles as inflammatory genes, an inflammatory reaction started that drove the cells into apoptosis by concomitant expression of

the Fas gene. It is well-known that Fas-FasL system is a common extrinsic pathway of cell death, by which IFN-α induces apoptosis (Fulda & Debatin, 2002; Inaba, et al, 2004). Also, the Fas-FasL system occurs in the cochlea due to aminoglycoside and cisplatin ototoxicity (Van de Water, Nguyen, Shoemaker, Schipor, et al., 2001).

Moreover, there is evidence about the presence of Fas in the inner ear following repeated doses of IFN-γ to inner ear cell culture in vitro and the induction of immune-mediated labyrinthitis in vivo (Bodmer, et al., 2002; Bodmer, et al., 2003). The authors of these studies noticed dramatic changes in the distribution of Fas in the cellular components of the organ of Corti but no changes in the transcription of the gene encoding Fas (by using PCR). The distribution of Fas following IFN-γ-stimulation was consistent with the recruitment of preformed Fas from intracellular compartments, e.g., endoplasmic reticulum into the cytoplasm and the plasma membrane rather than its biosynthesis as a consequence of genetic expression. Bodmer et al. (2003) concluded that expression of Fas on the plasma membrane might indicate the involvement of Fas in the protection of the inner ear from stress by autoimmune or inflammatory responses. This protective role of Fas also was suggested to be exerted in the anterior chamber of the eye (Griffith, Yu, Herndon, Green, & Ferguson, 1996). A supporting finding for these suggestions is that mice with deficient Fas-FasL system show immune reactions with progressive hearing loss (Trune, Craven, Morton & Mitchell, 1989; Ruckenstein, Keithley, Bennett, Powell, Baird & Harris, 1999). This means that the absence of Fas makes the inner ear more vulnerable to immune reactions.

This protective role of Fas looks to be unlikely in light of the current results as the expression of Fas occurred simultaneously with the expression of the inflammatory genes, which are indicators of an inflammatory response, the end result of which is usually cell death. Also,

the reduced cell viability reflected by reduced cell count (discussed in a later section) should have resulted from cell death that most likely was initiated by Fas as a cell death orchestrator, not as a protective agent. The expression of the Fas gene was time-dependent but not dose-dependent.

5.5.2. Expression of caspase-3 gene

Caspase-3 is an executioner caspase that cleaves cellular substrates following either the extrinsic or the intrinsic pathway (Budihardjo, Oliver, & Lutter, 1999; Vaux & Strasser, 1996); therefore its expression does not specify which pathway of cell death has taken place. The results of experiment-2 revealed more than two-fold increase in the gene expression of caspase-3 that started at 6hrs, reaching its maximum at 12-24hrs and declined to reach its minimal level at 48hrs (Figure 25). This pattern of expression of caspase-3 gene suggests a sequence of events that started earlier with the increased expression of IFN-α1 and IFN-β genes, followed by an increase of inflammatory markers and the expression of death molecules (Fas) that orchestrated the extrinsic pathway of apoptosis. This extrinsic pathway stimulated a series of downstream (later in the sequence) caspases, among which was the executioner caspase-3. The caspase-3 gene causes cleavage of proteins necessary for cell survival, resulting in cell death.

The results of this experiment are in agreement with most of the studies that investigated the extrinsic pathway of apoptosis (Budihardjo, Oliver, Lutter et al., 1999; Cao, Luo, Nagayama, et al., 2002; Lesuisse & Martin, 2002). Caspase-3 activation has been detected also in hair cells damaged by aminoglycosides (Cunningham, Cheng, & Rubel, 2002; Lee, Nakagawa, Kim, et al., 2004; Mangiardi, McLaughlin-Williamson, May, et al., 2004), cisplatin (Zhang, Liu, Ding, &

Salvi, 2003), and acoustic trauma (Hu, Henderson, & Nicotera, 2002; Nicotera, Hu, & Henderson, 2003).

In conclusion, the results suggest that IFN-α induced expression of caspase-3 gene, which might be through a sequence of events that started by the induction of inflammatory markers and engagement of the Fas gene, which orchestrated an extrinsic pathway of cell death that involved caspase-3 as an executioner caspase.

5.5.3. Expression of Bax gene

The results showed less than two-fold increase in the expression of Bax gene (Figure 26). Bax is a pro-apoptotic Bcl-2 family member that can trigger the intrinsic pathway of apoptosis. Because Bax is activated only during the intrinsic pathway, it can be assumed that the cells of the cochlear cell line in the current experiment did not undergo apoptosis through the intrinsic pathway. However, this cannot be totally ruled out for two reasons. First, there was an apparent peak in the expression of Bax gene at 24hrs, which might indicate an initiation of the intrinsic pathway of cell death, especially because this peak was timed with the expression of caspase-3 gene that is involved in both pathways of cell death. Second, the expression of other caspases, such as caspase-9 or other proapoptotic genes of Bcl2 family such as Bak or cytochrome-c, needs to be examined to confirm or to rule out the occurrence of the intrinsic pathway of cell death.

Therefore, the results of experiment-2 suggest that treatment of the cochlear cell culture with IFN-α led to expression of the Fas gene, which orchestrated an extrinsic pathway of apoptosis. Fas in turn, activated downstream caspases included caspase-3, which is an

executioner caspase that cleaved cellular structures leading to cell death. This suggests that IFN-α was able to induce apoptotic markers in the cochlear cell culture.

5.6. Did IFN-α reduce the cell count of the cochlear cells?

The results of experiment-2 also reflected a significant reduction of the cell count as a result of IFN-α treatment; however the difference in the cell count between 200 & 2000U/ml was not significant. The results also showed evidence for the occurrence of cell death, which was most likely initiated by Fas-induced apoptosis, an extrinsic pathway of cell death. On the other hand, the Bax gene, which is a marker for the intrinsic pathway of apoptosis, was not expressed in this experiment. However, the reduced cell count extended over all time points, especially in the case of IFN2000, which might indicate the presence of an alternative pathway of cell death that continued over all time points. The persistence of caspace-3 (which is a common caspase in both pathways of cell death) over longer time points support the occurrence of apoptosis over an extended time period. Notably, the expression of both Fas and caspase genes were not dose dependent, which might explain why the reduced cell count did not show significant difference between the two doses IFN-α.

In addition, it is possible that cell death resulted from the immune response mediated by IFN-α. The expression of MHC-I occurred across all time points, which indicates that an immune reaction was going on along with the reduced cell count. Moreover, the inflammatory response might be another cause behind the reduction in the cell viability. In either case (immune response or inflammatory response), cell death might have occurred due either to apoptosis or

necrosis. Other inflammatory markers as well as markers for necrosis need to be examined to confirm this possibility. Likewise, other genes of the intrinsic pathway, such as Bak and caspase-9, need to be examined to confirm this pathway.

The reduced cell count noticed over time in case of the control (sham) was most probably due to exhaustion of the nutrients in the media; yet there were still significant differences between the cell count in case of the sham and either doses of IFN-α.

5.7. Were the effects of IFN-α dose and/or time dependent?

Overall, the effects of IFN-α on the expression of IFN-α-induced genes were dose and time dependent in most cases. Also, the pattern of changes in the gene expression across hours look to be identical for both doses (200 & 2000U/ml). Notably, both doses induced upregulation of most of the IFN-α-induced genes even if the fold increase did not reach the cut off (2 folds). These results, when viewed together with the results of experiment-1 show similar, yet opposite effects. That is, in experiment 2, using higher does, there was an increase in the fold (i.e., increase expression or upregulation of the genes); yet with experiment-1, with very low doses, a decrease or downregulation of these genes were noticed.

These findings may be consistent with the dose-dependent response typically seen with cytokines treatment. For instance, low-dose treatment has a stimulating effect, whereas, high-dose produces tolerating or suppressant energies (Biron, 2001; Havell & Spitalny, 1983a; 1983b; Taylor, & Grossberg, 1998). This observation did not point to an absolute stimulation or an absolute depression; rather it meant that low and high doses have distinctive outcomes. For

example, Yoshino (1996) found that a low dose of IFN-α caused downregulation of delayed hypersensitivity and cellular infiltration in lymph nodes. On the other hand, high doses were found to be clinically effective against neoplasm and against viral infection, but they are poorly tolerated by the patients (Tovey, Meritet, Guymarho, & Maury, 1999). Also, it has been documented by some studies that systemic administration of low doses of IFN-α during anti-neolplastic or antiviral therapy will act as a priming cytokines for the host immune system, resulting in protection from viral challenge and increase of the tolerance of the patient to the effect of IFN-α (Beilharz, McDonald, Waston, Heng, McCeachie, & Lawson, 1997; Ferbas, Toso, Logar, Navratil, & Rinaldo, 1994).

Theoretically, low-doses of IFN-α mimic the early endogenous priming effect by innate immunity versus high-dose treatment that resembles the systemic adaptive immune response, which is usually vigorous and more generalized (Tompkins, 1999). The innate and the adaptive immunity have distinctive cytokines production and distinctive cellular activation, which might make the outcome of low-dose treatment distinctive from the high-dose treatment.

This assumption might provide an explanation for some clinical findings associated with IFN-α treatment. For example, Kanemaru et al. (1997) used IFN-α to treat patients with severe idiopathic SNHL due to viral infection. A significant number of those patients showed complete recovery after IFN-α therapy of a total cumulative dose of 30 million units. The recovery of the hearing loss in their study was most probably due, first, to enhancement of the antiviral effect that in turn overcame the viral assault in the inner ear. This antiviral effect was indicated by the concomitant increase in 2,5 oligoadenylate synthetase (2,5 A-S) activity. Second, the recovery of the hearing loss might be attributed to a different pathway of IFN-α in the inner ear when it is used in low doses. This different pathway might have led to induction of other genes such as

anti-inflammatory or anti-apoptotic genes with subsequent recovery of the hearing loss. There is evidence from other studies that IFN-α has an antiapoptotic effect by enhancing the expression of some anti-apoptotic genes of the Bcl-2 family (Imam, Gobl, Eriksson, & Oberg,, 1997).

Furthermore, the results of experiment-2, in particular the expression of the inflammatory genes, the immune-response genes as well as the apoptotic genes by high doses, may be consistent with a considerable number of studies that found an association between SNHL and IFN-α therapy, especially in patients with hepatitis C viral infection (Elloumi, et al., 2007; Piekarska, et al., 2007; Wong, et al., 2005; Formann, et al., 2004; Okanoue, et al., 1996; Cadoni, et al., 1998; Kanda, et al.,1994). The authors of these studies attributed this hearing loss to the inflammatory response, the immune response or the direct toxic effect of IFN-α on the hair cells that might have led to apoptosis. The reported cumulative dose that was found to be associated with the occurrence of SNHL was more than 100 million units (i.e. about three times the cumulative dose used in the treatment of SNHL in the study of Kanemaru et al. (1997). Therefore, it can be concluded that IFN-α, when used in small doses, can produce different effects on the cochlear cells from when it was used in large doses. This suggests that IFN-α has dual actions; protective in small doses and harmful in large doses.

The dose and the administration schedule of IFN-α treatment may play an important role on its biological and therapeutic effects by shaping the innate and the adaptive immune responses. In particular, low-dose of IFN-α can be used to protect against viral infection or immune-mediated disorders. On the other hand careful observation and follow ups might be necessary in case of long-term, high-dose therapy of IFN-α; two measures that can be applied to protect against hearing impairment.

6.0 CHAPTER VI: SUMMARY AND CONCLUSIONS

6.1 SUMMARY AND IMPACT OF THE CURRENT STUDY

In summary, previous studies have demonstrated that IFN-α can cause hearing impairment in patients receiving IFN-α therapy. The association between IFN-α therapy and hearing impairment was mainly in patients with hepatitis C. The results of the previous studies were diverse and inconsistent, while some demonstrated that IFN-α caused hearing impairment, others showed its therapeutic usefulness in the treatment of idiopathic SNHL. Different mechanisms have been suggested to explain the association between IFN-α and hearing impairment. These mechanisms included direct toxic effect of IFN-α on the cochlear structures that led to apoptosis of the cochlear cells, inflammatory reaction and/or immune response.

The current study investigated the signaling pathway of IFN-α in the cochlear cell line to shed light on these various mechanisms. The cell culture derived from the HEI-OC1 cochlear cell line was treated by different doses of IFN-α and at different time points. Using relatively small doses (20, 40, & 80U/ml) at five time points (3, 6, 12, 24, & 48hrs) in experiment-1 showed less than two-fold increase of the tested genes (IFN-γ, ICAM, TNF-α, caspase-3 or Bax). This can be explained by the inability of small doses of IFN-α to cause enough stimulation of IFN-α receptors to induce subsequent expression of other IFN-α-induced genes.

However, the results of experiment-2, using large doses resulted in more than two-fold increase in other types of IFN genes as well as IFN-α-stimulated genes, including the inflammatory genes (ICAM & TNF-α), immune response gene (MHC-I), and apoptotic genes (Fas & caspase-3). These outcomes suggest that the cochlear cells have receptors to IFN-α, which were stimulated by relatively higher doses of IFN-α. Stimulation of these receptors led to the expression of IFN-α1 and IFN-β genes. These genes led to initiation of an inflammatory response as well as an immune response that might have led to cell death of the cochlear cells as revealed by the reduced cell count.

The inflammatory response, the immune response and apoptosis are the underlying mechanisms involved in the hearing impairment noticed in patients under IFN-α therapy. The immune response was the most pronounced response as it has been reflected by the expression of the MHC-I gene which showed the highest expression level among the other genes. The inflammatory and the apoptotic responses looked to be transient as it has been revealed by the expression of ICAM-1, TNF-α, Fas and caspase-3, which showed peak expression at the same time point (12hrs). Particularly at this time point, both IFN-α1 and IFN-β genes showed maximum decline in their expression level, which might indicate the presence of a negative feedback between the occurrence of the inflammatory and the immune responses and the level of both IFNs. It can be suggested that this feedback relationship functions to limit both responses and protect against further insult. This conclusion is supported by the notion that the production of cytokines is usually transient, and never can be sustained, to initiate a certain action or to stimulate other cells to produce different cytokines (Guttermann, 1995). Also, this pattern of responses that occurred in the same time might reflect that of IFN-α induced inflammatory, immune as well as apoptotic responses simultaneously not as a sequence of events.

All these responses might have led to cell death that was reflected in the reduction of cell count noticed in all time points and occurred with both doses of IFN-α. Although there was significant increase in the markers of the extrinsic pathway of apoptosis, the occurrence of the intrinsic pathway cannot be ruled out because the reduction of cell count was continuous over all time points (i.e., beyond the time points at which extrinsic pathway markers were expressed), especially with high dose. Likewise, the inflammatory response, as well as the immune response, can drive the cells to cell death, which most likely was through necrosis. Further markers for the intrinsic pathway of cell death as well as markers for necrosis need to be examined to rule out or to prove the occurrence of either intrinsic pathway and/or necrosis.

The effect of IFN-α treatment on the expression of most of the genes included in the current study seemed to be dose and time dependent; the dose of IFN-α has been found to determine the nature of its effect.

Therefore, the current study implies that IFN-α can affect hearing by different mechanisms including inflammatory reaction, immune response, and apoptosis. These mechanisms might occur as a sequence of events or occur simultaneously. The dose and the time of IFN-α treatment influence the outcome of its effect, which implies that IFN-α in small doses can be used in the treatment of some immune-mediated disorders as well as viral infection of the inner ear. It also implies that pre-treatment hearing evaluation as well as close monitoring of hearing function in patients undergoing long-term, high-dose IFN-α therapy might be necessary to avoid or to minimize its harmful effect on hearing. The decision whether to continue or to stop IFN therapy needs to be made carefully by the physician according to the patient medical condition. If the treatment needs to continue, physicians should be aware of the possible ototoxic effects of IFN-α requiring appropriate surveillance.

6.2 DIRECTIONS FOR FUTURE RESEARCH

- Better design can be used to investigate the signaling pathway of IFN-α in the cochlear cell line, including more time points, multiple dose regimens that include a wide range of doses; most of the IFN-α studies included a wide range of doses that extended from one to 100,000U/ml. This will help in determining different biological characteristics of IFN-α that are largely dependent on its dose. Also, repeated doses might be helpful to mimic the actual therapeutic course of IFN-α and to help in magnifying an immune response, if any.

- Different methods of cell count can be used such as flow cytometry.

- To investigate if the inflammatory/y response, immune response, and the apoptosis occur in sequence or simultaneously, specific antibodies can be used to suppress one or both of the two first responses (inflammatory or immune or both) and determine if evidence of apoptosis is still there. This can help in inventing new therapeutic agents that might cut off this sequence of events. Also it will help in better understanding the underlying mechanisms involved in IFN-α therapy.

- Likewise, markers for other apoptotic pathways such as the intrinsic pathway, as well as markers for necrosis need to be examined.
- Immunohistochemical studies might be helpful in determining the functional effect of these genes that affected by IFN-α treatment by detecting their corresponding proteins.
- Animal models can be used to mimic the biological events that can occur in vivo as a result of IFN-α treatment. In addition, animal model for hepatitis C would be the ideal

model to simulate the real scenario occurring in patients with hepatitis C undergoing IFN-α therapy.

- Prospective studies that include subjects receiving IFN-α therapy for different diseases (such as hepatitis C, hepatitis B, malignancies.etc) need to be run. These patients should have their hearing tested before starting the therapy and every three or six months during the therapy. The hearing evaluation should include basic audiological testing as well as otoacoustic emissions to detect early changes that might occur in the cochlea.

APPENDEX A

LIST OF ABBREVIATIONS

Abbreviation	Meaning
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
U	Unit
IU	International unit
B-lymphocytes	Bone-marrow lymphocytes
T-lymphocytes	Thymus lymphocytes
Th- cells	Thymus helper cells
DCc	Dendritic cells
NK	Natural killer cells
CD	Clusters of differentiation
HPSC	Hematopoietic stem cells
BM	Bone marrow

Ig	Immunoglobulins
RBCs	Red blood cells
Fas	folic acid synthesis
DR	Death receptors
Caspases	cysteine-aspartic acid proteases
FasL	Fas-ligand
Bcl	B-cell leukemia
JAK	Janus kinases
STAT	Signal transduction and activators of transcription
Tyk	Tyrosine kinases
ISGF-3	Interferon stimulated gene factor-3
P-48	Protein-48
GAS	Gamma activating sequence
IL	Interleukin
ICAM	Intercellular adhesion molecules
APCs	Antigen presenting cells
MHC-I	Major histocompatibility class I
MHC-II	Major histocompatibility class II
ABR	Auditory brainstem response
ASNHL	Autoimmune SNHL
KLH	Keyhole limpet hemocyanin
ELISPOT	Enzyme-linked immunospot

FACS	fluoresce-activated cell sorter
PCR	Polymerase chain reaction
DNA	Deoxy- ribonucleic acid
cDNA	Cloned/complementary DNA
RNA	Ribonucleic acid
IFR-3	Interferon regulatory factor-3
ROS	Reactive oxygen species
NO	Nitric oxide
TNF-α	Tumor necrosis factor-α
HEI-OC	House Ear Institute- Organ of Corti
Hrs	Hours
ml	milliliters
µL	micro litter
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
rt-PCR	Real time PCR
RT	Reverse transcription
ds DNA	Double strand DNA
mRNA	Messenger RNA
CO2	Carbon dioxide
BSA	Bovine serum albumin
Ct	Cycle threshold

ANOVA	Analysis of variance
ANCOVA	Analysis of the covariance
DMEM	Dulbecco's modified Eagle medium
rRNA	Ribosomal RNA
dGTP	Deoxy-guanine triphosphate
dNTPs	Deoxy- nucleotide triphosphates
dUTP	Deoxy- undine triphosphate
MgCl	Magnesium chloride
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytosine triphosphate
IRS	Interferon response sequence
SDS	Signal detection system
LSD	Least significant difference
STDV	Standard deviation

APENDEX B

LIST OF PRIMERS

Primers are used as complementary sequences to the target sequences in the PCR reaction. Therefore, each primer has a complementary sequence to each gene in question.

A.1 PRIMERS FOR HIAR CELL MARKERS

These include the followings:

1. Primer for Myosin-VIIa gene
    - Forward: 5'- CATCCGCAACGACAACTCT -3'
    - Reverse: 5'- TCGATGGCACCACGCTTGTTA -3'.
2. Primer for Nestin gene:
    - Forward: 5'- TCAGCTGAGCCTATAGTTCAA -3'
    - Reverse: 5'- GCCAGAGCAGTCTCGT -3'.
3. Primer for connexin26 gene Forward r:
    - Forward: 5'-CTGATGGTCCTGGCGGTGCT-3'

- Reverse: 5’-CCTTCTCCTGCGGGAATCCAA-3’.

4. Primer for Calbindin2 gener:
    - Forward: 5’- TGTGCCTGCGTTGGGTCA -3’
    - Reverse: 5’- CCTAAATCATACAGCGAAGGA -3’.

A.2 PRIMERS FOR IFN-INDUCED GENES

These primers include the followings:

A.2.1 Primers for other types of IFNs

1. Primer for IFN-α1 gene
    - Forward r: 5’-CTGATGGTCCTGGCGGTGCT-3’
    - Reverse: 5’-CCTTCTCCTGCGGGAATCCAA-3’.
2. Primer for IFN-β gene
    - Forward: 5′-GACCTGTCAGTTGATGCC-3’
    - Reverse: 5′- AGTAGATTCACTACCAGTCCC-3’
3. Primer for IFN-γ genes
    - Forward: 5′-AGTCTCTTCTTGGATATCTGGAGG-3′
    - Reverse: 5′-GTGTGATTCAATGACGCTTATGTT-3′

A.2.2 Primers for inflammatory genes

4. Primer for ICAM-1 gene
    - Forward 5′-CTGTGGCACCGTGCAGTCGTC-3′
    - Reverse 5′-TGGCGGCTCAGTATCTC-3′.
5. Primer for TNF- α gene
    - Forward 5′- GTCAACCTCCTCTCTGCCGTCAAG -3′
    - Reverse 5′- TCCAAAGTAGACCTGCCCGGACTC -3′.

A.2.3 Primers for immune response gene

6. Primer for MHC class-I gene
    - Forward: 5′- CAGAGAGACTCAGGGCCTACC -3′
    - Reverse: 5′- GCGTCGCGTTCCCGTTCTT -3′

A.2.4 Primers for apoptotic genes

1. Primer for Fas gene
    a. Forward: 5′- CCCAGAATACCAAGTGCAAG -3′
    b. Reverse: 5′- AGGGTTCCATGTTCACACGAG -3′
2. Primer for Caspase-3gene
    a. Forward: 5′- TCTGACTGGAAAGCCGAAACT -3′
    b. Reverse: 5′- GGATGAACCACGACCCGTCCT -3′

3. Primer for Bax gene

    - Forward: 5′-TTGCCCTCTTCTACTTTGCTA-3′
    - Reverse: 5′-CCTCAGCCCATCTTCTTCCAG-3′

A.2.5 Primers for 18S rRNA gene

- Forward: 5′-AAGCCATGCATGTCTAAGTACGCA -3′
- Reverse: 5′-CAAGTAGGAGAGGAGCGAGCGACC -3′

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