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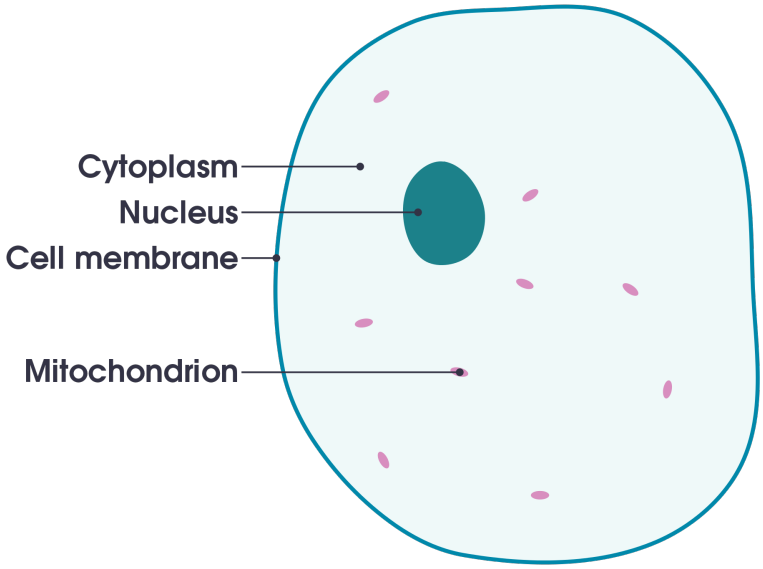

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Review

Hearing impairments, presbycusis and the possible therapeutic interventions

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Abstract

The hearing is an important sensation of all living organism to maintain his or her life mainly in human. Any distortion to it leads to hearing impairment. Hearing loss (HL) can be congenital or acquired. It can be syndromic; HL associated with other abnormalities or non-syndromic, HL is not associated with any other anomalies. Hearing impairment have mostly autosomal recessive loci, but can be autosomal dominant, X-linked, and mitochondrial. Acquired HL can be genetic or environmental factors based. Age-related HL is acquired hearing loss occur in aged population. Its prevalence rate increases with age. Genetic of presbycusis is not well-known, but NAT2*6A polymorphism, SNPs in KCNQ4, grainy head-like 2 gene, Glutamate receptor-7 gene, 4977-bp mt-DNA deletion in human and 4834-bp mtDNA deletion in rodents were identified. Different strains of mice are developed like C57BL/6J, CBA/CaJ, DBA/2J, BALB/cJ and Fisher 344 albino rats mainly used as models to study HL and presbycusis. Like other disorders have complete treatment, but HL cannot be completely treated. However some attempts can be made for its betterment by using hearing aid devices, surgical and pharmaceutical treatments. In future stem cells and gene therapy will be the affective methods to treat congenital hearing impairment and presbycusis.

Keywords

Hearing Loss, Prevalence, Factors, Presbycusis Genetics, Animal models, and Treatments

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Introduction

Hearing is the process through which all living organisms understand different sound waves and respond according to it. It has significant contribution in human life, as it maintain the safety and wellness of human beings. Like if someone has good sense of hearing, crossing road he/she will be safe otherwise become a victim of road accident. Ear is an important organ of hearing. It has three parts, the outer, middle and the inner ear. Any defects in these parts lead to hearing loss. Hearing loss is the partial or total inability of either single or both ears to hear. Hearing impairment deteriorates the quality of human life. Affected persons become socially isolated from their society (Martin, 2003).

Hearing loss has different classification based on different criteria like based on severity it can be mild, moderate, severe and profound. It can be conductive, sensorineural and mixed based on defects in different portions of the ear. Hearing impairment can be congenital (prelingual) or acquired (post-lingual). It can be syndromic (30%) or nonsyndromic (70%) (Schrijver, 2004). The nonsyndromic HL can be autosomal dominant (DFNA), autosomal recessive (DFNB), X-linked (DFN) and mitochondrial (Smith et al., 2005).

Presbycusis is acquired hearing loss which progresses with the age, also called age-related hearing loss (AHL). In elderly group the incidence of various disabilities related to age is known to be high. The aged people rely on hearing to face these disabilities. In both elders' and youngers' hearing is an important sensation (Zhang et al., 2013). If hearing sense of aged people is good their quality of life will be good. In contrast, if the elderly group becomes a victim of hearing loss, then their life quality would be substantially reduced and contributes to depression, social isolation and possibly dementia in the elderly (Woodcock & Pole, 2008). Age-related hearing loss (Presbycusis) is a progressive bilateral symmetrical sensorineural hearing loss result from aging (Zhang et al., 2013). Presbycusis is describe as; patient in noisy environment cannot understand speech, cannot locate sound sources, his/her acoustic information central process become slowed and hence his/her hearing deteriorated (Gates and Mills, 2005; Zhang et al., 2013). It can be sensory, metabolic or neural depends on parts of the ear to be damaged (Mills et al., 2006; Schuknecht and Gacek, 1993).

Different factors are involved to cause presbycusis, can be intrinsic (genetic) or extrinsic (environmental) (Zhang et al., 2013). It occurs in elder people having age 60s or over 70s (Woodcock and Pole, 2008). Approximately 30% of elder people suffer from presbyscusis (Corna et al., 2009). The Center for Disease Control (U.S), reported in 2003 that in aged population next to arthritis, age-related hearing loss was the second most common disorder (Schuknecht and Gacek, 1993; Zhang et al., 2013). Presbycusis along with the cognitive, emotional and physical activities also affects the social functioning of the patients. Hence due to various symptoms such as lowered self-esteem,

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depression and social isolation the life of patients deteriorated (Wallhagen et al., 1997).

Approximately ten mutant genes, gene modifiers and mtDNA mutations have been discovered (Van Eyken et al., 2007). Different strains of mice have been developed for the study of congenital and acquired hearing loss. Although the treatment of hearing loss is not completely understood, but can be managed by cochlear implant, use of hearing aid device (Huang and Tang, 2010), pharmaceuticals (Salami et al., 2010). Stem cells and gene therapy (Tadros et al., 2008) are the future treatment that can recover acquired and congenital hearing loss (Ito et al., 2001). To study hearing process and its disorder, causing factors are too much necessary, as it leads towards its therapeutic intervention. I studied multiple research and review articles but included those, have pathophysiology, prevalence, causing factors, genetics, and treatment of mainly age-related hearing loss.

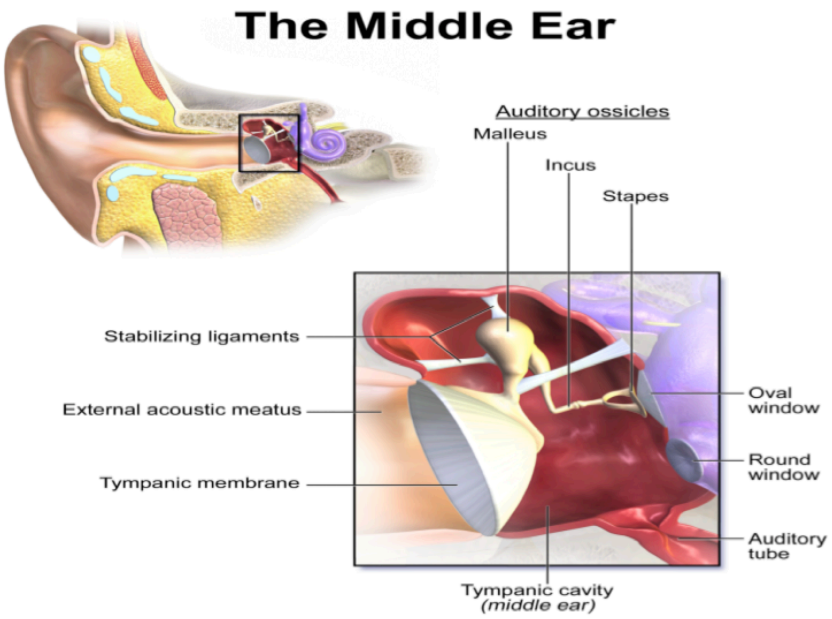

Figure 1. Anatomy of Middle Ear. It starts from tympanic membranes that transfer sound energy into vibrational energy, then ossicles (malleus, incus, and stapes) bones that convert vibrational energy into mechanical energy (Hudspeth, 1989).

Anatomy and Physiology of the Ear

The most important sensation to the people of the World, which maintains their safety and wellness and promotes their quality of life, is hearing. One of the

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important organs of the human body is ear. It plays a very important role in their life by hearing different sound waves. The human ear has three parts, the outer, the middle and the inner ear. The outer ear consists of the auricle or pinna and the ear canal (external auditory meatus). The middle ear includes eardrum and three small bones malleus, incus and stapes, which are, assembled together, the ossicles as shown in **Fig. 1** (Martin, 2003).

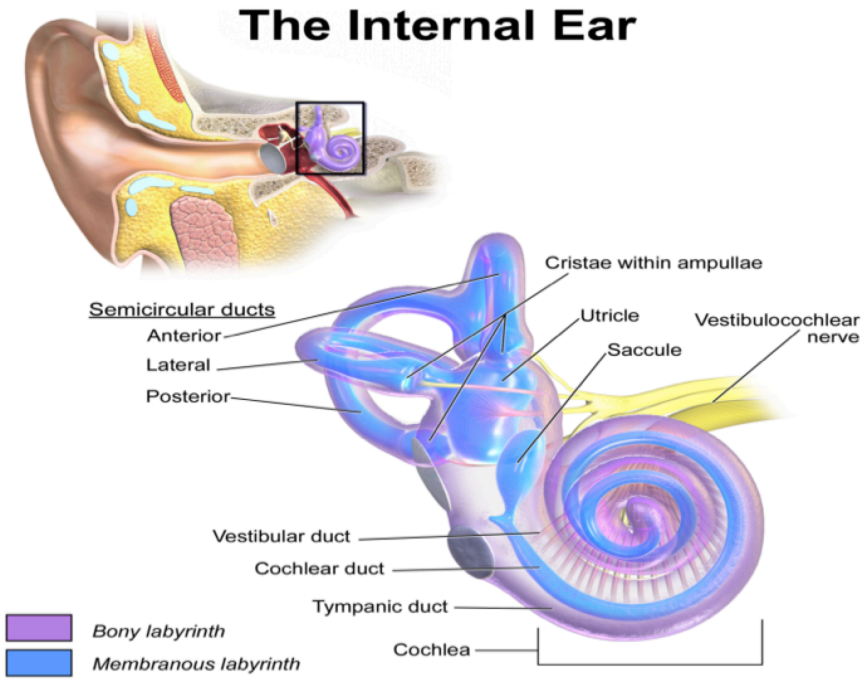

Figure 2. **Anatomy of Inner Ear**. It consists of six mechanoreceptor structures: three semicircular ducts, utricle, saccule, and the cochlea. The bony labyrinth has continuous stone like cavities along with the temporal bone while membranous labyrinth is present within the bony labyrinth that is a series of communicating ducts and sacs. These portions have role in sensation and balance (Hudspeth, 1989).

These bones connect the outer ear with the inner ear. The inner ear comprises of the semi-circular canal (vestibule), the cochlea and the cochlear nerves as shown in **Fig. 2**. Hair cells reside in inner ear, which is the sensory portion of the ear. From the environment, the outer ear pinna collects different sound waves, which passes through the ear canal and reaches the tympanic membrane (eardrum), it starts vibrations. The middle ear bones move like a pistol due to these vibrations, which will lead to the movement of the sensory portion of the ear that is fluids inside the inner ear (Martin, 2003). The human inner is a highly complicated organ composed of a cochlear duct responsible for hearing and

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vestibular portion for balance. When sound energy and balance information reaches the cochlea, the hair cells convert it into electrical signals, which are then transmitted to the brain through their associated neurons hence result to understand the sound waves and responded to balance information's. The inner ear hair cells development is a result of a series of differentiation and cell fate decision process which are tightly regulated by a combination of extrinsic and intrinsic factors (Kelley, 2006). Various causes can be easily damaged these hair cells including genetic mutations, aging, drugs and noise. In most cases the damage is irreversible, lead to permanent hearing loss (Dror and Avraham, 2009). In this regards efforts using gene therapy or stem cells have met with only limited success to regenerate or preserve these hair cells (Izumikawa et al., 2005; Li et al., 2003a; Oshima et al., 2010). Normal hair cells development better understanding may help to overcome such limitations.

Classification of Hearing Loss

In developing countries, HL is a major public health concern which rate is two thirds of the worldwide HL patients (Tucci et al., 2010). HL can be classified into different criteria involve based on severity, HL can be classified as; mild: 20 to 39 dB, moderate: 40 to 69dB, severe: 70 to 89 dB, or profound: ≥90 dB, based on age of onset as; congenital (pre-lingual) or acquired (post-lingual), based on affected portion of the ear as; conductive, sensorineural or mixed and based on associated or not with abnormalities in other parts of the body as; syndromic or nonsyndromic (Schrijver, 2004). The genetic basis of pre-lingual HL is estimated to be at least 50% and for nonsyndromic hearing loss (NSHL) it is 70%, whereas the remaining 30% are syndromic. Over 400 syndromic forms have been described till to now; the most common examples are; Pendred syndrome and Usher syndrome (Hilgert et al., 2009). The nonsyndromic HL have different mode of inheritance; can be autosomal dominant (DFNA) comprises ~20%, can be autosomal recessive (DFNB) encompasses ~ 80% of cases, can be X-linked (DFN) transmit ~1% cases or can be mitochondrial comprises <1% cases. The autosomal recessive nonsyndromic hearing loss (ARNSHL) prevalence frequency is even greater in countries have high rate of consanguineous marriage (Smith et al., 2005). It is estimated that up to 1% of human genome is to be involved in auditory process. For NSHL over 130 loci have been identified and is clinically and genetically heterogeneous disorder (Hilgert et al., 2009), of which more than 70 DFNB loci have been identified for ARNSHL (Smith et al., 2014).

Presbycusis

In Greek, Presbycusis means old and hearing (Zwaardemaker, 1891). In aged population it is very common. In various studies it is investigated that hearing impairment (HI) is associated with multiple factors, among older adults age is the

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main factor with the highest epidemiology rate (Wallhagen et al., 1997; Yueh et al., 2003).

Based on etiology, Presbycusis have been divided into three subtypes.

I. Sensory Presbycusis; its causing factor is noise which permanently distort and degenerate the hair cells of the cochlea.

II. Strial or metabolic presbycusis; is due to the decline in the cochlea metabolic function.

III. Neural Presbycusis; in this the degeneration of the auditory nerve occurs (Mills et al., 2006; Schuknecht and Gacek, 1993).

However, in the prevalence of Presbycusis, gene-associated hereditary susceptibility has been observed to play an important role (Rodriguez-Paris et al., 2008; Ünal et al., 2005).

Factors of Presbycusis

There are mainly two factor involved in age-related hearing loss:

Intrinsic factors

It included genetic disorders such as *Ahl,* mutation in mitochondrial DNA, systemic diseases such as sickle cell anemia, metabolic disease and diabetes.

Extrinsic factors

It involved ototoxic medication, diet and noise. Both factors cannot separated from each other (Schuknecht and Gacek, 1993; Zhang et al., 2013).

The aging process contributed to different changes in the body, which may be physiological, molecular, and biochemical. In physiological changes, decreased elasticity of cellular membranes occurs, the molecular changes reduce mitochondrial function, increases in DNA damage and the biochemical changes involved decreases in ionic changes and cellular water concentrations (Prazma et al., 1990; Seidman et al., 1996). In humans the aging process occurs in every organs of the body. The aging process can also occur in the external, middle ear and inner ear. In inner ear, cochlea is the hearing portion consisting of hair cells (Schuknecht and Gacek, 1993). In cochlea aging process occur that leads to loss of hair cells (Nelson and Hinojosa, 2006). The hair cells are very vulnerable as they change sound waves into nerve impulses that are critical to normal hearing function (Zhang and Surles, 2008). The hair cells once degenerated; the degeneration is permanent because in human inner ear cochlea the hair cells live only once. As the eye has eye-lid and eye does not work during sleeping,

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but due to absence of ear lid, it works 24 hours without any rest. It does not depend on that a person is either healthy or not, can develop presbycusis sooner or later due to progressive loss of hair cells (Zhang et al., 2013).

Prevalence

The epidemiology of presbycusis can be expressed in percentage and the majority of aged population have hearing impairment (Rothman, 2012). Hearing impairment is one of the most common sensory disorders observed in the population. In the US it is estimated that 1 in 1000 people is born deaf or acquired in early childhood (Morton, 1991). But in Pakistan its prevalence is 1.6 in 1000 people (Elahi et al., 1998).

As one of the most important factors of hearing impairment is the aging process (Ryan, 2000). Therefore 10% of the elderly have age 65 or above, are suffer from hearing loss with the highest prevalence rate in North America (Wallhagen et al., 1997). Hearing impairment occurs in later life either have good or bad socio-demographic factors like education, income and marital status (Corna et al., 2009). The results of age-related hearing loss prevalence may vary among various literature reports. The American Speech Language Hearing Association statistically reported in aged Americans, hearing loss is the 3rd have high prevalence rate. In older people hearing impairment is the most common communicative disorder, for example hearing loss occurs of about 25% to 40% of the population have age 65 or above (Frisina and Walton, 2006; Yueh et al., 2003).

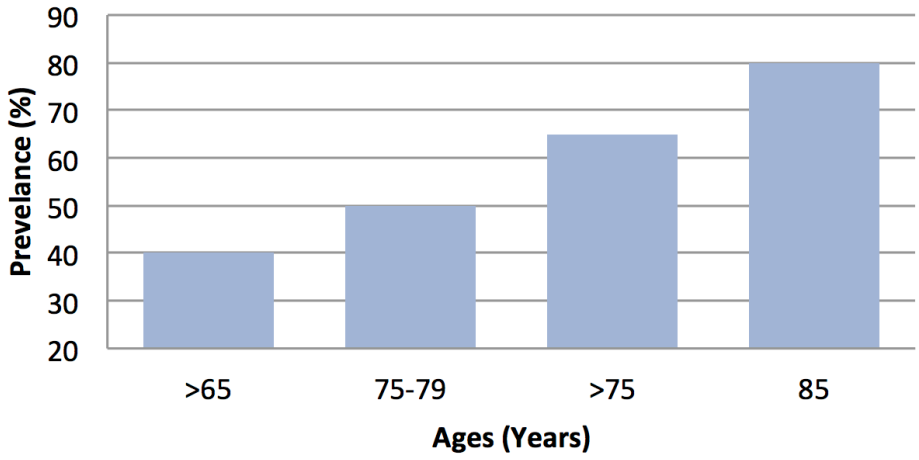

Figure 3. Prevalence of presbycusis as a function of age, it increases with age. In each group, the prevalence of hearing loss shows as percentage of the population (Zhang et al., 2013).

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Like the USA, the Canadian Association of Speech Language Pathologists and Audiologists also confirms the prevalence of hearing loss. It reported 20% of population are affected have >65 years, while 40% are affected have >75 years of age (Zhang et al., 2013). With the age the prevalence rate of hearing impairment will be high. The people have age 70-74 years, its presbycusis prevalence range can be between 25%, 50% at 85 years old, and it can be >80% at age of 85 or above (Bogardus Jr et al., 2003; Desai et al., 2001; Yueh et al., 2003) as shown in **Fig. 3**. Due to this high prevalence rate, presbycusis is one the critical issue in our society.

From different multiple studies, the presbycusis prevalence data obtained are not contradictory, but can be overlapped to each other in terms of progressive rate of presbycusis. As it can be shown in **Fig. 3** based literature survey (Corna et al., 2009; Woodcock and Pole, 2008).

The world wide studies of World Health Organization showed that, in 1999, >120 million, in 2005, 278 million, and in 2011, 642 million people suffered from hearing loss. The estimation that how many people will be affected by 2017, 2025 and even by 2031 will be surprising. It is estimated that the percentage of presbycusis prevalence is increase to 21.8% up to 2031 as shown in **Fig. 4** (Brencla, 1997; Carson and Pichora-Fuller, 1997; Secretariat, 1993). Here is a proposed projection which estimated that 1248 million people in 2017, 2568 million people in 2025, and 5136 million people in 2031 will suffered from hearing loss.

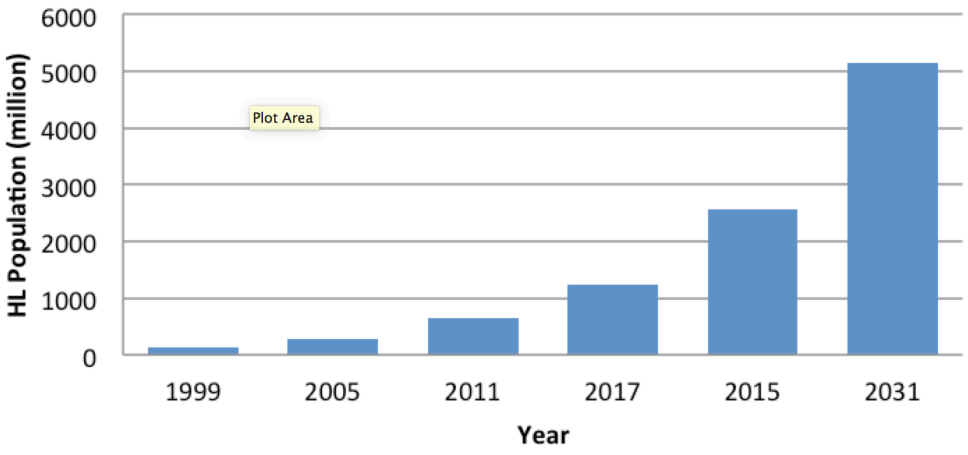

Figure 4. Showed the prevalence of hearing impairment as a function of year, the total number of hearing loss patients worldwide and HL increase each year (Zhang et al., 2013).

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Genetics of Presbycusis

Genetic is one of the most critical factors of presbycusis. Different genes contribute to hearing impairments, which lead to Presbycusis. The genes related to presbycusis have been discovered, including gene modifiers, ten presbycusis genes and mitochondrial DNA mutation. Through genome wide studies association age-related hearing loss genes include NAT2*6A polymorphism, SNPs in KCNQ4, common 4977-bp mitochondrial DNA deletion, grainy head-like 2 gene and Glutamate receptor-7 gene were identified (Van Eyken et al., 2007).

In mitochondrial respiratory chain the ATP is generated by oxidative phosphorylation, but due to mitochondrial dysfunction which affect oxygen free radical, apoptosis and calcium imbalance, leads to hearing loss. The mtDNA mutation in mice showed early presbycusis (Niu et al., 2007). In human mtDNA 4977 mutation related to presbycusis and mtDNA 4834 in rodents (Lee, 2013).

Animal Models Used to Study Presbycusis

It is explored that 19 inbred strains of mice model have presbycusis (Johnson et al., 2003; Johnson et al., 2008; Johnson et al., 2006). Out of these, 10 strains had age-related hearing loss (Ahl) locus on chromosome 10 (Johnson et al., 2000). Ahl is a core gene in C57BL/6J mice as it encodes cadherin-23 and for presbycusis. C57BL/6J mice were used as a key model because they exhibited at age of 6 months high frequency hearing loss. At age of 12 months, low-frequency hearing loss and at age of 15 months, whole frequency hearing loss was observed (Fetoni et al., 2011). In the cochlea histological changes occur due to the progression of hearing impairment. In C57BL/6J, the degeneration of the abaxial cochlea neurons have association with the degeneration of peripheral hair cells as both leads to presbycusis (Idrizbegovic et al., 2001). In C57 mice in addition to early presbycusis, in the nucleus of ventral cochlea changes in sensitivity were also observed, as for the analysis of sound features like temporal cue, frequency and sound intensity in the central hearing system, the cochlear nucleus is responsible (Caspary et al., 2008). Another animal model CBA/J mouse was developed, in this model, Ahl resistance alleles such as Ahl 2, Ahl 4 and Ahl 8 were also explored in addition to Ahl, Ahl 3. Ahl resistant allele was discovered in CBA/J mice model and has observed at age of 12 months, developed high-tone hearing impairment. It was investigated at age of 18 months that the mice model has to begin the outer hair cells loss, and also observed until the age of 25 months that the model did not develop any abnormality in stria vascularis. For neural and sensory presbycusis therefore these are used as models (Sha et al., 2008). Another model CBA/CaJ mice was developed and widely used as a model for strial and metabolic presbycusis. As it was observed that this model has hearing impairment early with declined endocochlear potential. DBA/2J mice used as a model and shown loss of

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cochlear ganglion and outer hair cells at age of two months led to hearing loss. Ahl genes were also present in this model (Fetoni et al., 2011). A model BALB/cJ mice for metabolic presbycusis was observed at age of 19 months to have a decrease in endocochlear potential and presbycusis (Popelar et al., 2006). At age of 12 months, Fisher 344 albino rats developed a rapid presbycusis due to degeneration of stria vascularis and outer hair cells (Fetoni et al., 2011; Popelar et al., 2006). It has been studied in Fischer 344 rats, the aging process lost the glycinergic inhibition in the nucleus of ventral cochlea. It also changed the dorsal cochlear nucleus coding intensity (Caspary et al., 2008). Mongolian gerbils developed models for metabolic age-related hearing impairment and shared similar pathological characteristics as humans. In these models, inside the gerbils, a decline of endocochlear potential, the atrophy of spinal ligaments and stria vascularis were investigated (Gratton et al., 1997; Mills et al., 1990). Similar to humans, Chinchillas have aged-related hearing loss, but poses a limitation as it has long life span of 20 years, therefore does not use for the studies of presbycusis (Lee, 2013).

Treatment

As presbycusis is a sensorineural hearing loss, so cannot be cured or prevented and there are no recommended or approved pharmaceutical treatments. However some attempts can be used for its betterment. The attempt options of treatment for presbycusis fall into three categories: surgical, management and pharmacological.

Surgical Treatment

It involves surgical cochlear implant and is possible in cases of profound or severe hearing loss. A cochlear implant is a complex electronic device that replaces the inner ear cochlea. This device may enable the presbycusis patient to understand the environmental sound and speech waves. But there is a difference between natural hearing sound spectrum and the sound spectrum produces by the cochlear implant (Huang and Tang, 2010).

Management

It involves hearing aids devices and aural rehabilitation. Hearing aids now help improve the hearing of many aged people and can be tuned to specific frequency ranges of hearing loss. Aural rehabilitation may reduce the impact on communication between the affected person and their communication partners. It includes different techniques like reduce environmental noise, communicates directly, pronounced clearly with the affected person and used of contextual signs to improve comprehension (Huang and Tang, 2010).

Pharmaceuticals

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Until now the pharmaceutical treatment for presbycusis remain limited and clinically unproven. The limited treatments for age-related hearing loss are: water-soluble coenzyme Q10 (CoQ10) formulation, the prescription drug Tanakan, and combination antioxidant therapy. In 2010 it was explored that coenzyme Q10 a water-soluble formulation improved the air laminar tonal audiometry and bone thresholds at 1000 Hz, 2000 Hz, 4000 Hz, and 8000 Hz (Salami et al., 2010). Coenzyme Q10 has antioxidant properties similar to a vitamin. It produces in the body, but with the age its levels fall (Yamasoba et al., 2013).

Tanakan is a vasodilator and is an international brand name of prescribed drug extract of Ginkgo biloba. Its pharmaceutical effects were observed when treating aged women with tympanophonia. It was found that Tanakan improved hearing and speech in adult patients, decreases tympanitis intensity, and gives idea to recommend the presbycusis treatment (Boboshko et al., 2010). Antioxidant therapy is a combination of six antioxidant agents, i.e. ribose-cysteine, L-cysteine-glutathione mixed disulfide, NW-nitro-L-arginine methyl ester, folate, vitamin B12, and vitamin C, within the oxidative pathway it target four sites (Boboshko et al., 2010).

Ebselen is a synthetic drug molecule composed of organic selenium with antioxidant, anti-inflammatory and cytoprotective activity (Schewe, 1995). It mimics glutathione peroxidase (GPx), a crucial enzyme that protects the inner ear from damage caused by noise or loud sounds (Dudbridge, 2008). It is found that ebselen can be used as a possible treatment for hearing loss and tinnitus (Kil et al., 2007).

Treatment in Future

Transplant therapy of stem cells

It has been investigated in vitro that the mature central nervous system does have the potential to regenerate nerve. In this observation scientists successively in vitro departed neural stem cells from various sites of brains of human and mammals. It was demonstrated in a research carried out by Ito (Ito et al., 2001) that cochlea environment is compatible to the stem cells of neurons. Kojima explored that immature progenitors neurons have the potential to differentiate into the phenotypes of hair cells (Kojima et al., 2004). This research gives hope to treat the damaged cochlea and presbycusis. In animal models such as guinea pig or murine, it has been reported that embryonic stem cells (Li et al., 2003b) or progenitor cells (Rivolta et al., 2006), the adult utricular sensory epithelium stem cells (Li et al., 2003a), and even stem cells of marrow (Ge et al., 2005) also have some potential to differentiate into audiometric cells. These researches help us to remedy age-related hearing impairment patients at the cell replaced therapy level with neural stem cells, xenogeneic or autologous embryonic stem cells, and

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even the stem cells of marrow. This treatment is still limited to animal models and theory. For human application this technology remains years or even decade's away.

Gene therapy

For gene therapy various number of administration routes have been suggested. It has been investigated that the apoptosis of related cells due to gene mutation, aging and ototoxic medications is the final way to cause deafness. In aged gerbil cochlea that the bcl-2 protein expression is suppressed due to activation of caspase-3, leads to apoptosis induced presbycusis (Alam et al., 2001). Matsui found that after the treatment of amino glycoside to inhibit the caspases activation promotes the hair cells survival (Matsui et al., 2003). These results showed that to activate the apoptosis inhibitors like caspase-3 may provide protection to presbycusis related cells. Tandros had observed that in inner ear aging 31 genes play important roles. The genes include are: B cell leukemia/lymphoma 2, activating transcription factor 3, Calpain2, Bcl2-like1, tumor necrosis factor receptor superfamily member 12a, tumor necrosis factor superfamily member 13b, caspase 4 apoptosis-related cysteine protease 4, dual specificity phosphatase 9 (Tadros et al., 2008). Thus, the apoptosis related genes should be the candidate gene for gene therapy. Beside the apoptosis related genes, in the past few years, the cell cycle regulatory genes (Chen et al., 2003a; Löwenheim et al., 1999; Mantela et al., 2005), neurotrophin-based gene (Chen et al., 2003b) and cellular and functional restoration gene (Izumikawa et al., 2005) were so much promising and hot fields of gene therapy.

Suggestions

- Different research should be conducted to determine the actual cause and frequency prevalence of hearing loss in the country.
- The information's resulted from these research should develop different strategies to help people with hearing impairment.
- The society should be well aware about the etiology of hearing loss.
- If the disorder is due to genetics then the families should be well aware to avoid consanguineous marriages.
- To remove the psychological problems there should be no social discrimination among people with hearing disorder.
- The health care providers at hospitals and audiology clinics should be well trained about hearing loss.

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- The people with hearing loss should wear hearing aid device to protect himself from road accident.

- To aid the people with hearing loss the government should develop different programs, which can encourage the community to be self-supported

Conclusion

Hearing impairment is the most common neurosensory defect in human. In developing countries it is two-third of worldwide hearing loss. Genetic basis of congenital hearing loss is 50%, nonsyndromic hearing loss is 70%, and remaining 30% is syndromic. Pendred syndrome and Usher syndrome are common examples of syndromic hearing loss. The non-syndromic HL have different mode of inheritance; can be autosomal dominant (DFNA) comprises ~20%, can be autosomal recessive (DFNB) encompasses ~ 80% of cases, can be X-linked (DFN) transmit ~1% cases or can be mitochondrial comprises <1% cases. The autosomal recessive non-syndromic hearing loss (ARNSHL) prevalence frequency is even greater in countries have high rate of consanguineous marriage. It is estimated that up to 1% of human genome is to be involved in auditory process. For NSHL over 130 loci have been identified and is clinically and genetically heterogeneous disorder of which more than 70 DFNB loci have been identified for ARNSHL. The population above 40 years of age in those where the average is 50 years and have hearing loss called presbycusis. As the people become aged with of AHL in the aged people become higher and higher. Presbycusis can be due to environmental or genetic factor. Some genetic mutations related to presbycusis have been identified. It is important to give awareness to the affected family to avoid consanguineous marriages, to prevent their future family members from hearing impairments. It is also very important and clinically significant to develop and identify therapeutics treatments, potential protective measures to avoid presbycusis and HL. The above all reported treatment is still limited to animal models and the scientists continue their efforts to apply gene therapy to human beings. In future, we assure that due to the development in technology, scientists' efforts and interest that the stem cell and gene therapy will be proved helpful and fruitful to be effectively applied in clinic to cure presbycusis patients. Here the variety of techniques are reviewed by using animal models is the future of therapeutics treatment development. But for human clinical applications best results, these techniques are going to refine and evaluate. In near future the presbycusis might not be completely cured, but can be minimized and prevented.

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Abbreviations

AHL: Age-related Hearing Loss; ARNSNL: Autosomal recessive non-syndromic hearing loss; ATP: Adenosine Triphosphate; DFN: Non-syndromic, X-linked deafness; DFNA: Non-syndromic, autosomal dominant deafness; DFNB: Non-syndromic, autosomal recessive deafness; HI: Hearing Impairment; HL: Hearing Loss; KCNQ4: Potassium Voltage-Gated Channel subfamily Q Member 4; mt-DNA: mitochondrial DNA; NAT2*6A: N-acetyltransferase 2 Polymorphism; NSHL: Non-syndromic Hearing loss; SNPs Single Nucleotide polymorphisms

Author Contribution

All authors contributed to the Manuscript. BH collected data and wrote the manuscript. MA, MQ, & MSM edited the first draft. LK contribute to the design and revising the manuscript. All authors reviewed and commented on final draft.

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Original Research

Artemisinin-induced delayed hemolysis after administration of artesunate and artesunate-amodiaquine in malaria-free Wistar rats

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Abstract

Background: Hemolysis is common in malaria infection and during the course of treatment. Previous studies have reported delayed post-artemisinin hemolysis occurring in naïve and immune individuals treated with parenteral or oral artemisinin-derivatives. This study aims to understand if delayed hemolysis occurs in the absence of malaria parasites and the underlying mechanisms for the hemolytic effects after administration of two antimalarial drugs to malaria-free Wistar rats. **Methods:** Forty animals were randomized into 5 groups of eight animals each; they received 4 mg/kg artesunate for 7 days (AS), 4 mg/kg artesunate plus 10mg/kg amodiaquine for 3 days (ASQ), 10mg/kg amodiaquine for 3 days (AQ), distilled water [normal control], (Control) and 1mg/kg phenylhydrazine for 1 day to induce hemolysis (PHZ) groups respectively. Packed cell volume (PCV), white blood cell differential count and serum haptoglobin (Hpt) levels were determined in all groups on day 4 and 18 to detect hemolysis. Mean values were compared using t-test and ANOVA with p values <0.05 taken to be significantly different. **Results:** Post-treatment mean PCV on day 4 was significantly lower than day 18 in all groups except AS group which had similar PCV all through evaluation. Although AQ had lowest mean PCV on day 4, by day 18, mean PCV returned to normal and hpt levels was significantly higher than AS and ASQ groups ($p<0.001$). Hpt level (mean ± sem) for AS, ASQ, AQ, and control on day 18 were: 18.67 ± 0.004, 50.66 ± 0.014, 73.06 ± 0.003, and 74.13 ± 0.032 mg/dl respectively ($p < 0.0001$). On day 18, AS and ASQ had significantly lower Hpt level compared to day 4 ($p <0.001$). No neutropenia was observed during the study. **Conclusion:** Artesunate induces delayed hemolysis in malaria-free animals possibly through an oxidative toxic effect on the red blood cell membrane. Delayed post-treatment hemolysis was not observed with artesunate-amodiaquine or amodiaquine alone.

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Keywords

Antimalarial, Artemisinin, Delayed hemolysis, Drug-induced hemolysis

Introduction

Hemolysis, the immature destruction of red blood cells (RBC) at a rate that exceeds production of the RBC, commonly occurs in malaria infection. The causes of hemolysis is multifactorial, it may be a consequence of merozoite release from red blood cells, suppression of erythropoiesis by cytokines which are released along with, nutritional deficiencies, immunity, hemoglobinopathies or oxidative effects of drugs. In the food vacuole of malaria parasite, heme increases the activation of the endoperoxide moiety of the drug (Meshnick et al., 1993) to generate reactive oxygen species, glutathione peroxidase and malondialdehyde which causes damage to the parasite membranes and proteins (Meshnick et al., 1989; Scott et al., 1989). The resulting degree of hemolysis depends on the duration and course of malaria infection (Price et al., 2001; Yeo et al., 2009).

Artemisinin based combination treatments are the most rapidly acting and effective antimalarials for treatment of falciparum malaria (WHO, 2013). Artemisinin drugs are known to act by inducing oxidative stress after activation of the peroxide bridge which generates reactive metabolites (Meshnick et al., 1993); this is their major mechanism of killing malaria parasite. Despite generating oxidative radicals, artemsinins are known to conserve the red blood cells, this is demonstrable by moderate falls in hematocrit (Gbotosho et al., 2014; Price et al., 2001) and presence of once infected red blood cells in circulation after parasite clearance (Angus et al., 1997; Chotivanich et al., 2000; Newton et al., 2001). The red cell conservation by artemisinin drugs is however transient because the erythrocytes are eventually destroyed by the spleen leading to the "delayed post-artemisinin hemolytic" syndrome (DPAH).

Delayed hemolysis was first recognized after parenteral artesunate in naïve patients, studies have also reported delayed hemolytic anemia after administration of oral artemisinin derivatives in immune and non-immune subjects with severe malaria or hyperparasitemia (Control and Prevention, 2013; Jauréguiberry et al., 2014; Molta et al., 2003; Rehman et al., 2014; WHO, 2013). Different hypothesis have been proposed to explain the underlying cause of hemolysis after artemisinin treatment, however the hypothesis that pitted erythrocytes are destroyed during recovery best explains post-artesunate delayed hemolysis after treatment of malaria infection (Jauréguiberry et al., 2014). Hemolysis may occur after treatment with different classes of antimalarials

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(Ngole et al., 2010; Price et al., 2001; Sowunmi et al., 2009), however studies have not reported delayed post-treatment hemolysis occurring with antimalarials other than artemisinin derivatives.

This study aims to better understand the mechanism of post-delayed hemolytic effects of artemisinin and a commonly used partner drug – amodiaquine. This study evaluates post-artesunate or ACTs delayed hemolytic effects in uninfected animal model, providing a clear understanding of the mechanism of delayed hemolysis after antimalarial treatment.

Materials - Methods

Study design

Forty female albino rats were weighed and divided randomly into five groups of eight animals each. There were 4 treatment groups (3 groups received antimalarials, 1 group received a known hemolytic drug) and one untreated group (control). Treatment outcomes were compared within treatment groups or with positive or normal control group.

Drug treatment

Two commercially available antimalarials were purchased and used for the study; 200mg base amodiaquine (Camoquin®, Pfizer, USA) and 50mg artesunate (Artesunat®, Mekophar, Vietnam) and were crushed and dissolved in distilled water. Standard doses of the drugs were administered based on weight of the animals and given orally using cannula. Animals in group 1 received 4mg/kg body weight artesunate for 7 days (AS), group 2 received 10mg/kg amodiaquine (AQ) for 3 days, group 3 received 4mg/kg artesunate plus 10mg/kg amodiaquine (ASQ) for 3 days, group 4 received distilled water and served as normal control (Control) and group 5 received 1ml/kg phenylhydrazine for 1 day and served as positive control for hemolysis (PHZ).

Animals

The animals were purchased and housed separately per group; they were kept at room temperature in the animal house to acclimatize for one week. They were fed animal feeds *ad-libitum* and allowed free movement. All the animal experiments and handling were carried out following the standard ethics for Animal studies.

Laboratory tests

Blood samples for hematological tests were collected by pricking the tail of the rat on day 0, 4 or 8 and 18 after treatment. The blood was collected in micro-hematocrit tubes and centrifuged at 12,000 x g for 5 minutes to determine

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packed cell volume (PCV). Post-treatment, half of the animals were sacrificed on the day of test; 3 mL blood was collected through cardiac puncture under mild anesthesia using diethyl ether. The blood samples were collected into heparinized tubes or plain bottles and centrifuged at 12,000 x g for 15 mins to obtain the serum and plasma. Blood for reticulocyte and white blood cell count was diluted with normal saline and fixed with methanol before counting.

They were stained with Leishman, and counted against 1000 white blood cells using Naubauer hemocytometer under the microscope at magnification of x 100. Differential count of the white blood cells was determined by absolute neutrophil, basophil, eosinophil, monocyte and reticulocytes count, thereafter the percentage of each was calculated from a total of 100 cells counted. Haptoglobin test was done on day 4 in half of the animals and on day 18 in the other half. Hemolysis was induced in group 5 by oral administration of 1ml/kg body weight of phenylhydrazine intravenously on day 4. This served as positive control for hemolysis.

Haptoglobin assay

Rat haptoglobin (Hpt) level was assayed using the biotin labelled ELISA kit (EASTBIOPHARM®) and was carried out according to manufacturer's instructions as follows. Serum (40 µl) sample was added to each Hpt pre-coated well, incubated with anti-Hpt antibody labeled with biotin or streptavidin-HRP at 37°C for 60 minutes. The reaction was terminated using chromogen reagent and absorbance was read on a microplate reader at 450 nm wavelength and compared with standard.

Hematological evaluation

Hemolytic effects of drug treated groups were compared with normal control and positive control groups. Anemia was taken to be present if PCV value was <30% and hemolysis was present if Hpt level is very low or similar to that of positive control. Overall neutrophil counts lower than 100 (against 1000 WBC) was taken to indicate neutropenia. At recovery phase, hematological values in drug treated groups were compared with control groups to determine extent of hemolysis and recovery.

Statistical Analysis

The data was analyzed using the SPSS software version 16 (SPSS Inc., Chicago, USA). Graphs were done using excel or Graph pad prism software (Graphpad version 4.0, 1999). Values are reported as mean values ± standard error of mean or proportions. Mean values were compared using t-test or ANOVA, followed by Duncan's multiple range test, proportions were compared using chi-square test or fisher exact test, the level of significance was set at $p<0.05$.

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Results

Before treatment, the weight range of the animals was between 170-250 g. The average weight of all animals on day 0 was 201.25 g and mean packed cell volume on day 0 was 48.1%. Severe hemolytic effects were not recorded in any animal.

Packed cell volume

Packed cell volume in all but 1 animal was >30% prior to treatment, this one animal was excluded from evaluation. Following treatment, anemia was not recorded in any animal. Table 1 shows the hematological outcomes in all groups. Post-treatment and recovery PCV values were similar in AS group ($p = 0.10$). In ASQ and AQ groups, there was 28-38% fall in PCV by day 4 after treatment, this was significantly lower than PHZ group, however mean PCV on day 18 was significantly higher than day 4 ($p < 0.03$). In PHZ group, PCV continued to decline until day 18 (Fig. 1). In ASQ and AQ, mean PCV on day 4 was significantly lower than other treatment groups ($p < 0.0001$) (Fig. 1). After completion of treatment, mean PCV was significantly higher in AS than ASQ or AQ ($p < 0.0001$). PCV on day 18 in all groups was similar, but significantly higher than PHZ ($p < 0.001$) indicating full recovery from the moderate and non-severe hemolytic effects of the antimalarials.

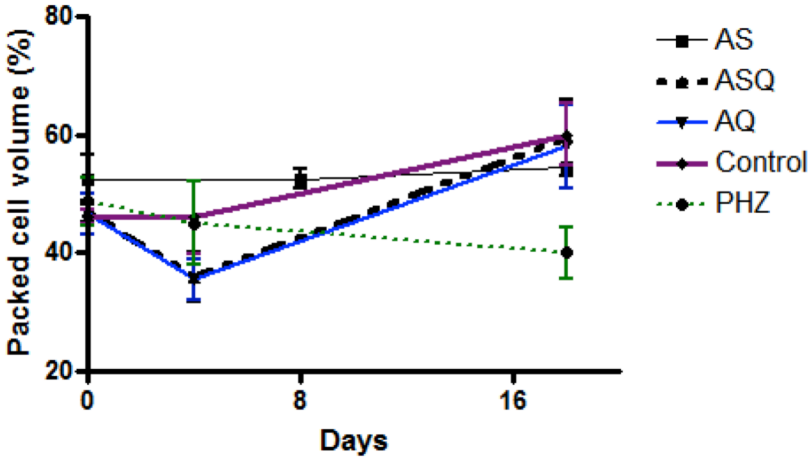

Figure 1. Mean packed cell volume in artesunate (AS), artesunate-amodiaquine (ASQ), amodiaquine (AQ), and control (CTRL) groups of malaria-free rats.

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Table 1. Hematological profiles of treated animals during the study

PCV (%)	AS	ASQ	AQ	PHZ	Control	P value
D0 Mean ± sd (%)	52.4 ± 4.15	46.75 ± 1.70	47.25 ± 3.40	48.6 ± 4.08	46.2 ± 1.25	0.13
After treatment Mean ± sd (%)	52.5 ± 1.68 §	36. 0 ± 4.24*	35.5 ± 3.50*	45.0 ± 7.07	46.0 ± 6.25	<0.0001
At recovery Mean ± sd (%)	54.5 ± 0.71	59.5 ± 6.36	58.0 ± 7.01	40.0 ± 4.24**	60.0 ± 5.25	<0.0001
Neutrophil count D4 (%)	17	16	18	ND	14	-
Neutrophil count D18 (%)	17	19	15	ND	13	-
P value (within group)	0.10	0.0001	0.001	0.56	0.003	-

** Significantly lower than other treatment groups after treatment, ** significantly lower than other treatment groups at recovery, §evaluation done on D8, ND – not done*

Haptoglobin level

In AS group, mean Hpt level on day 8 was 67.73 mg/dl, this was significantly higher than Hpt level on day 18 (18.67 mg/dl), $p < 0.0001$. In ASQ, mean Hpt level on day 4 (73.06 mg/dl) was significantly higher than day 18 (50.66 ± 0.013 mg/d), $p = 0.001$ while in AQ group, mean haptoglobin level was significantly lower on day 4 (58.13mg/dl) than day 18 (73.46 mg/dl), $p = 0.01$. All treatment groups were compared with control at the recovery phase to determine delayed hemolysis. Hpt level (mean ± sem) for AS, ASQ, AQ, and control on day 18 were: 18.67 ± 0.004 (n = 2), 50.66 ± 0.014 (n = 4), 73.06 ± 0.003 (n = 4), and 74.13 ± 0.032 mg/dl (n = 3), respectively. There was a significantly lower Hpt level in AS and ASQ groups compared to AQ or control groups on day 18 ($p < 0.05$) (Fig. 2).

White Blood Cell Differential Count

Total white blood cell (WBC) count on day 0 was within normal range (5 x 10^9/mm^3), this was similar in all groups at baseline, after treatment (day 4) and during recovery (day 18). Neutrophils were compared within and between groups to determine if neutropenia occurred following treatment and during recovery. Neutropenia and agranulocytosis was not observed. In AS and AQ groups, no significant difference was observed between day 4 and day 18 ($p = 0.703$). Overall, there was no significant difference in differential count observed in all groups compared to PHZ group.

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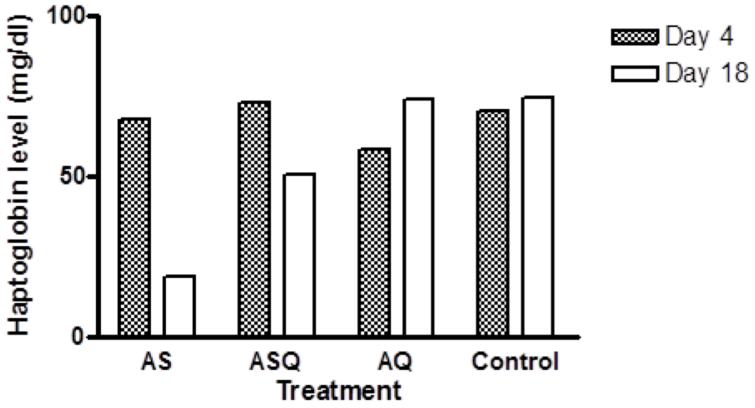

Figure 2. Post-treatment haptoglobin levels in artesunate (AS), artesunate-amodiaquine (ASQ), amodiaquine (AQ), and control on day 4 and 18 in malaria-free treated animals.

Discussion

The recent reports of delayed post-artemisinin hemolysis have brought to light the unrecognized burden of drug-induced hemolysis occurring especially after treatment of infections. Studies have reported immunosuppressive or inhibition of erythropoiesis (Finaurini et al., 2010; Wang et al., 2007; Yang et al., 2005), and other adverse effects of artemisinin derivatives both in the presence or absence of malaria parasites including hemolytic effects (Alzoubi et al., 2014; Anaba et al., 2012; Garba and Ubom, 2005; Gu et al., 1986; Kurth et al., 2016; Lee et al., 2015; Orjih, 1996; Zhou et al., 2005), many of which appear to be non-severe. The mechanism for inducing delayed post-artemisinin hemolysis when administered as monotherapy or co-administered with a partner drug such as amodiaquine remains to be understood. Artemisinin-based combination treatments (ACTs) remains the preferred first-line and safe treatment of falciparum malaria because of its rapid clearance of parasitemia, and reduction of chances of deaths in cases of severe malaria (Sinclair et al., 2012).

In the present study, animals in AQ and ASQ groups recovered from the hemolytic effects of all drug treatment. Packed cell volume and differential count by day 18 were similar compared to untreated animals. The lowest PCV values recorded on day 4 (after treatment) is a well explained phenomenon of drug induced hemolysis (Price et al., 2001; Sowunmi et al., 2009). This lends support to findings from other studies reporting decrease in hematocrit level after treatment with ACTs (Gbotosho et al., 2014; Omotuyi, 2008; Price et al., 2001; Rolling et al., 2015).

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After completion of treatment, drug-attributable fall in hematocrit is common, this decline in hematocrit is attributable to the hemolytic effects of longer acting antimalarials (Price et al., 2001; Sowunmi et al., 2009). Haptoglobin is known to facilitate the removal of hemoglobin from the extravascular compartment via the CD163 macrophage scavenger receptor and is usually present in the presence of inflammation, or inflammatory diseases, or hemolysis to block hemoglobin-induced oxidative damage by forming haptoglobin-hemoglobin complex with decreased level reported in patients with acute inflammatory disease or hemolysis (Rolling et al., 2015). Reduced serum haptoglobin observed in AS group on day 18 shows hemolysis occurred in the absence of malaria infection, although PCV level appeared normal.

Surprisingly, delayed hemolysis did not occur in ASQ group, this could have been masked by the effects of amodiaquine in the combination. In the absence of malaria parasites, free iron bioactivation of the endoperoxide bridge of artemisinin drugs can be used to modulate its effects (Conran, 2014; Krishna et al., 2004; Meshnick et al., 1989; Meshnick et al., 1993; Muhia et al., 1994; Tangnitipong et al., 2012). Delayed hemolysis in AS group follows a pattern similar to that reported in malaria infected persons (Arguin, 2014; Boillat et al., 2015; Chavada et al., 2015; Conran, 2014; Jauréguiberry et al., 2014; Kurth et al., 2016; Lee et al., 2015; Rehman et al., 2014; Rolling et al., 2015; WHO, 2013), although no anemia was recorded throughout this study.

The mechanism of delayed hemolysis occurring in the absence of malaria parasite is likely due to toxic effects of artemisinin owing to generation of oxidative radicals. This can result in suppression of erythropoiesis (Finaurini et al., 2010; Wang et al., 2007; Yang et al., 2005) and toxicity of the red cell membrane through generation of reactive species which induce oxidative stress causing lipid peroxidation, gluthathione oxidation and oxidation of membrane protein thiols (Meshnick et al., 1993; Scott et al., 1989). Drug-induced oxidative stress worsens hemolysis in the presence of hemoglobinopathies or inadequate protection by the antioxidant defense system of the red blood cells which is often overwhelmed in disease states such as malaria. In the absence of the protective enzyme glucose-6-phosphate dehydrogenase; which maintains the supply of reduced glutathione used to mop up free radicals, oxidative damage of the vascular epithelium occurs (Beutler et al., 2007; Chikezie, 2014). In this study, G6PD deficiency or other hemoglobinopathies cannot be implicated.

Indeed studies have shown that oxidative stress precedes hemolysis of the red blood cells (Jeney et al., 2002; Jollow and McMillan, 2001) following a similar mechanism in both human and rats when exposed to highly oxidative compounds (Vickers et al., 2010). Shortened survival and removal of pitted erythrocytes explains only part of hemolysis in presence of malaria infection (Arguin, 2014; Jauréguiberry et al., 2014; Kurth et al., 2016; Rolling et al., 2015), unparasitized red blood cells are also cleared (Newton et al., 2001). Increased splenic clearance and lysis of the erythrocytes is also attributed to altered symmetry of phospholipid layer and reduced red cell deformability due to

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membrane rigidity caused by merozite invasion (Cooke et al., 2004; Nuchsongsin et al., 2007; Park et al., 2008; Pasvol et al., 1992).

In this study, the haptoglobin levels in all groups were moderately low by day 4, indicating potent hemolytic effect of both artesunate and amodiaquie. Clark et al. (1991) indicated that nitric oxide production increases in a state of infection such as malaria leading to poor deformability of red blood cells membrane (Clark et al., 1991). Free hemoglobin also depletes nitric oxide and increases oxidative inflammatory process. The nitric oxide is thought to inhibit Na+/K+ ATPase and causing oxidization of lipids in the red blood cell membrane through generation of peroxynitrate – a highly potent oxidant (Yeo et al., 2009). Most antimalarials reduce the deformability of the red blood cell membrane which makes the red cell rigid and unable to pass through the microcirculatory bed of the spleen, thus they are marked for splenic clearance (Dondorp et al., 2004).

Drug induced hemolysis can be easily implicated in a case of multiple drug intake, because hemolysis could be triggered via immune complex formation with the drugs on RBC surface or auto-antibody production. In humans, other possible causes of hemolysis include genetic susceptibility such as polymorphisms in drug metabolizing enzymes – CYP2A6 (Roederer et al., 2011), hemoglobinopathies such G-6-P-D deficiency which is common in malaria endemic areas (Beutler et al., 2007; Howes et al., 2012; Price et al., 2001; Van Malderen et al., 2012) and suppressed erythropoiesis after administration of artemisinin, in the presence of other hemolytic factors such as malaria parasite, it would result in anemia as seen in hyperparasitemic individuals.

The hypothesis that explains that post-artemisinin delayed hemolysis after treatment of malaria infection is attributable to eventual destruction of pitted erythrocytes which have a shortened lifespan (Arguin, 2014; Clark, 2014; WHO, 2013) could also explain the case with malaria-free animals in which case exposure to artemisinin drugs cause oxidative stress on the red cell membrane, thus a reduced lifespan or clearance by the spleen. Haptoglobin levels in the experimental animals indicate that hemolysis occurred during treatment in all groups but was more pronounced on day 18 in AS group. Although Hpt level observed in ASQ group was low, it was not as low as in artesunate-treated animals on day 18. Only 2 animals were evaluable in AS group on day 18, this may have accounted for a skewed value recorded for the group.

Amodiaquine is implicated as a cause of neutropenia, which is characterized by a decrease in granular white blood cells after prolonged use for prophylaxis (Olliaro and Mussano, 2003). Our findings are in consonance with other reported animals studies (Omotuyi, 2008) and that observed in human treated with amodiaquine, they showed normal limits of hematological profile with no agranulocytosis (Molta et al., 2003). Artesunate have been previously reported to be associated with neutropenia at doses higher than doses administered in our study (Bethell et al., 2010), however neutropenia was not observed with AS, ASQ or AQ during the study. This study lends support to a case report of

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artesunate induced auto-immune post-treatment delayed hemolysis, although this occurred in a malaria patient (Raffray et al., 2014).

Limitations of the study

Artesunate treatment was given for 7 days following recommendation for artesunate monotherapy while other treatments were administered for 3 days. This study did not evaluate delayed hemolysis after 3 days administration of artesunate to see if the outcome will differ from the reported result. Other antimalarial drug combinations were not evaluated for this study. Comparison was done in half of the animals in each group sacrificed after treatment and the remaining half at the end of the study. Thus these data were taken to be representative of the hemolytic effects occurring in individual animals. It is not known if this is the case. Lactate dehydrogenase assay was not done and no malaria infected group was evaluated along with the study groups, this group would have shown hemolysis during untreated malaria infection in mice.

Conclusion

Hemolysis occurred with all treatment in the absence of malaria parasites but rather occurred late; during recovery in AS and ASQ groups, PCV levels did not indicate hemolysis, > 30% anemia cut off but hpt levels were lower. This study reports that delayed post-artemisinin hemolysis can also occur in the absence of malaria parasites possibly through an oxidative toxic effect on the red blood cell membrane. There was no delayed hemolysis occurring after amodiaquine administration, rather hemolysis occurred immediately after completion of treatment.

Abbreviations

ACTs: artemisinin-based combination treatment
AQ: amodiaquine
AS: artesunate
ASQ: amodiaquine-artesunate
ELISA: enzyme-linked immunosorbent assay
G6PD: glucose-6-phosphate dehydrogenase
Hpt: haptoglobin
PCV: packed cell volume
RBC: red blood cells

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Author contribution

TMD designed the study and wrote the manuscript, AA carried out the laboratory evaluation, all authors participated in the conduct, analysis and contributed to the success of the study.

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Original Research

Hepatoprotective effect of a polyherbal formulation and ascorbic acid in paracetamol induced hepatic damage in rabbits

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Abstract

Background: The hepatoprotective effect of a polyherbal formulation was evaluated in female rabbits. The herbal formulation was used alone and in combination with ascorbic acid (AsAc) in animals with paracetamol (ParCM)-induced hepatic damage. **Methods**: The study design included five groups, each comprised of five animals. Group A was the control group (untreated; given only routine diet) while group B was given a single oral dose of ParCM (2 g/kg) on day 9. Groups C, D and E were pretreated with polyherbal formulation (PoHF; 500 mg/kg), ascorbic acid (AsAc; 200 mg/kg) and PoHF (500 mg/kg) combined with ascorbic acid (AsAc; 200 mg/kg), respectively for 9 consecutive days. On the last day (day 9), after 30 minutes of routine treatments, a single dose of ParCM (2 g/kg) was administered in groups C, D and E. Animals were sacrificed 24 hours after the last treatment. Blood and liver samples were collected from all animals. Serum was separated from the blood samples and subjected to biochemical tests for liver biomarker analysis. The biomarkers included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin. Elevation of enzyme markers was considered an indicator of hepatocellular injury. **Results**: Serum levels of liver enzymes and total bilirubin were elevated significantly in group B when compared to group A. The level of serobiochemicals significantly dropped in group C but increased significantly in group D, as compared to group B.

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No statistically significant effect on liver enzymes was observed in group E when compared to group B. Groups D and E showed a significantly higher level of serological parameters as compared to group C. The biochemical findings were further corroborated with histopathological analyses of the liver tissue samples. Histopathological examination of the livers of rabbits in group A showed normal hepatic cell architecture. However, groups B and D revealed severe congestion of the central vein and sinusoids, periportal fibrosis and infiltration of inflammatory cells; these parameters were of mild and mostly moderate severity in groups C and E, respectively. The histopathological findings strongly supported the results of the biochemical analyses. **Conclusion**: Thus, our study herein demonstrates that herbal formulation remains an effective means to ameliorate ParCM-induced elevation of serum biochemical parameters and changes to the liver histology. Ascorbic acid induced deteriorating effects in a ParCM-intoxicated rabbit animal mode. Moreover, combination of herbal product and ascorbic acid failed to yield liver protecting effects in ParCM-poisoned animals. The damaging effects of ascorbic acid might be attributed to its pro-oxidant attitude, as reported by many research studies. To expand on these findings further studies are warranted, including evaluating other hepatotoxicity inducers (besides ParCM), testing different doses of ascorbic acid and ParCM, and testing a greater number of animals along with other animal models.

Keywords

Ascorbic acid, Hepatoprotective, Paracetamol, Polyherbal formulation

Introduction

The liver is a dynamic organ important for a multitude of functions, namely the assimilation and conversion of nutrient materials absorbed from the gastrointestinal tract to a more useful form of energy, and the neutralization and removal of a number of drugs, xenobiotics and various other substances from the body (Nadeem et al., 1997). The liver is a vibrant organ in the human body, playing an indispensable job of detoxification of countless endogenous and exogenous harmful substances (Yang et al., 2010). As the liver is consistently exposed to environmental poisonous elements (such as drugs), various hepatic disorders can result; liver damage can lead to hepatic failure and eventual death (Mukherjee et al., 2006).

Modern drugs are not suitable for liver diseases due to the high rate of undesirable effects. It is, therefore, necessary to search for alternative remedies (Kang, 2002). There are some natural plants which show excellence at preventing, treating and curing liver diseases, while imposing only a few side

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effects. The hepatoprotective class of therapeutic regimens possesses the competence to protect liver damage and revitalize hepatocytes. International agencies, such as the World Health Organization, have insisted on the evaluation of herbs and herbal products by advanced scientific standards (Neraliya and Gaur, 2004). About 600 herbal drugs are being marketed all over the world for hepatoprotective effects (Sharma et al., 1991). However, only a limited number of formulations have been scientifically qualified for their prescribed use. Examples of the compound formulations which have been scientifically validated for their intended use in liver diseases are: Amalkadi Ghrit, an herbal formulation (Achliya et al., 2004), Liv-52 Syrup & Livomyn Suspension (Sapakal et al., 2008), Liv-52, Livergen, Livokin, Octogen, Stimuliv and Tefroliv liquid formulations (Girish et al., 2009), Livergen (a polyherbal liquid formulation) (Arsul et al., 2011), Khamira Gaozaban Ambri Jadwar Ood Saleeb Wala (Akhtar et al., 2013), Herbal drug (Feroz and Khan, 2013), Herbal preparations F-I, F-II & F-III (Sivakumar et al., 2014), and Virgoliv Syrup (Ingawale et al., 2015).

Paracetamol (ParCM) is an excellent analgesic and antipyretic. The toxicity of ParCM is related to its biotransformation which mostly follows three steps: sulfation, glucuronidation and oxidation (Mitchell et al., 1974). Oxidation is the most crucial step as far as hepatic toxicity is concerned (Jollow et al., 1973). A small quantity of ParCM undergoes oxidation by cytochrome P450 2E1 to produce an extremely reactive component, N-acetyl-P benzoquinoneimine (NAPQI) (Vermeulen et al., 1992). When ParCM is used in therapeutic doses, NAPQI is conjugated by hepatic glutathione (GSH) to produce a water-soluble product called mercapturic acid (Lee et al., 1996). The rate of production of GSH counterbalances the toxicity of NAPQI synthesized as a consequence of ParCM oxidation so there is no damage to the hepatocytes so long as this balance is maintained (Sabina et al., 2009). As higher doses of ParCM are administered, generation of GSH in the liver cells is diminished after a few hours and hepatic intoxication is evident when the level of GSH in liver is less than 30% of the normal value (Makin and Williams, 1997). Uninhibited NAPQI becomes noxious by binding to macromolecules, including cellular proteins (Vermeulen et al., 1992). Ascorbic acid (AsAc) is a popular antioxidant that can act as pro-oxidant under certain conditions (Duarte and Lunec, 2005).

Polyherbal formulation (PoHF) has been claimed to be effective in liver inflammation and other liver aliments. The composition of PoHF consists of: *Zingiber officinale* (Ginger), *Peganum harmala* (Wild Rue), *Cassia angustifolia* (Senna), and *Operculina turpethum* (Turpeth). PoHF contains flavonoids & phenolic compounds (present in *Zingiber officinale*) (Ghasemzadeh et al., 2010), alkaloids (present in *Peganum harmala*) (Monsef et al., 2004), and glycosidic resins and beta-sitosterol (present in *Operculina turpethum*) (Iweala et al., 2011). The aim of this study was to validate the hepatoprotective effects of PoHF, which is currently being used without any scientific authentication. In our study, PoHF was used alone and in combination with ascorbic acid (AsAc) in ParCM-induced hepatic damage in rabbits. PoHF was evaluated on the basis of various biochemical and histopathological parameters.

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Materials - Methods

Polyherbal formulation (PoHF)

The PoHF evaluated in our study is comprised of parts from four medicinal plants, namely *Zingiber officinale* (Ginger), *Peganum harmala* (Wild Rue), *Cassia angustifolia* (Senna) and *Operculina turpethum* (Turpeth). The product is in semisolid dosage form and each 10 grams of the herbal formulation contains *Zingiber officinale* (2 g), *Peganum harmala* (2 g), *Cassia angustifolia* (2 g) and *Operculina turpethum* (2 g). PoHF was obtained from the local manufacturer of the herbal product.

Animals

Twenty-five female rabbits of local breed (*Oryctolagus cuniculus*) of particular age (2.5-3.5 months) and weight (550-750 g) were used in the study. They were fed a balanced diet. Animals were kept at light and dark cycle (12/12 h) at room temperature (25±2°C) and relative humidity (60-65%). Animals were acclimatized to the atmosphere of their environment for one week prior to the start of treatment. Protocols for animal use were approved by the Committee on Animal Ethics, Hajvery University, Lahore, Pakistan.

Experimental Design

Animals were randomly divided into 5 groups; each group was comprised of 5 animals. The duration of the study was 9 days and the administration of drugs was through oral route.

Group A: This group served as negative control (was given only routine diet).

Group B: Animals in this group were administered with a single dose of ParCM (2 g/kg) on the 9th day of treatment using 1% CMC as a vehicle.

Group C: Animals in this group were administered with a single dose of PoHF (500 mg/kg; dispersed in normal saline) daily for 9 days, followed by a single dose of ParCM (2 g/kg) on the 9th day, after 30 minutes of routine treatment.

Group D: Animals in this group were administered with a single dose of AsAc (200 mg/kg; dissolved in distilled water) for 9 successive days followed by a single dose of ParCM (2 g/kg) on the 9th day, after 30 minutes of routine treatment.

Group E: Animals in this group were administered with PoHF (500 mg/kg; dispersed in normal saline) & AsAc (200 mg/kg; dissolved in distilled water) for 9 days, and then a single dose of ParCM (2 g/kg) on the last day, after 30 minutes of routine treatment.

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Determination of Liver Enzymes (ALT, AST and ALP) & Total Bilirubin

Animals were sacrificed at 24 hours after the last treatment. Samples of blood were collected in blood collection glass tubes from the control and treated rabbits. Blood samples were stored at room temperature for 30 minutes for coagulation and then centrifuged at 4000 rpm for 8 minutes to separate the serum, which was collected in aliquots for biochemical testing. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were analyzed by Micro Lab 300 (Semi-automated clinical chemistry analyzer) using standard kits (Merck, France). Total Bilirubin was measured using standard kits (DiaSys Diagnostic Systems, Germany).

Tissue Processing of Liver Samples for Histopathological Evaluations

The liver of each rabbit was excised and extra connective tissues adhered to the organ were removed. Organs were washed with distilled water and small pieces of liver were cut and immersed immediately in 10% Neutral Buffered Formalin (NBF).

Standard techniques for Fixation, Washing, Dehydration, Clearing, Embedding, Sectioning and Staining (Carleton et al., 1967) of liver tissue samples were adopted. The Hematoxylin & Eosin stained slides were then examined under different resolutions of microscope in a photographic facility and photomicrographs were taken.

Statistical Analysis

One-way ANOVA followed by post hoc, Tukey HSD were used to determine the statistical differences between the means of study groups, using the SPSS computer software (version 23). The level of significance was set at $p < 0.05$.

Results

Analysis of Biochemical Parameters

For group B, the administration of a single dose of ParCM increased the serum concentrations of ALT, AST, ALP and total bilirubin to significantly ($P < 0.05$) higher values as compared to group A (the negative control group). Pretreatment with PoHF in group C caused liver enzymes and total bilirubin to drop to significantly ($P < 0.05$) lower levels as compared to group B. Oral administration of AsAc in group D caused an elevation of serobiochemicals to a significantly ($P < 0.05$) higher number in comparison to group B. The level of serobiochemicals in group D was also significantly higher than group C.

PoHF and AsAc were co-administered in group E. Comparison of hepatic enzymes in groups B & E showed a statistically non-significant ($P > 0.05$) relation.

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Serum concentrations of liver enzymes in group E rose to a significantly higher level ($P < 0.05$) when compared to group C (Table 1; Figs. 1, 2, 3, 4).

Table 1. Effect of treatments on serum ALT, AST, ALP and Total bilirubin in Par-CM induced liver damage

Groups	Treatments	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)
A	Negative control	37.20 ±3.19	26.40 ±3.57	71.60 ±2.19	0.42 ±0.04
B	Par-CM (2g/kg)	68.20 ±3.27#	68.20 ±5.54#	159.60 ±3.65#	1.52 ±0.04#
C	Po-HF (500mg/kg + Par-CM (2g/kg)	46.40±2.40*	39.00±1.58*	83.20±3.03*	0.54±0.05*
D	As-Ac (200 mg/kg) + Par-CM (2g/kg)	78.40 ±4.39*,^	82.40 ±3.05*,^	191.00 ±5.78*,^	1.76 ±0.05*,^
E	Po-HF (500mg/kg) + As-Ac (200 mg/kg) + Par-CM (2g/kg)	60.60±5.98^	59.00±8.27^	145.20±16.11^	1.44±0.05^

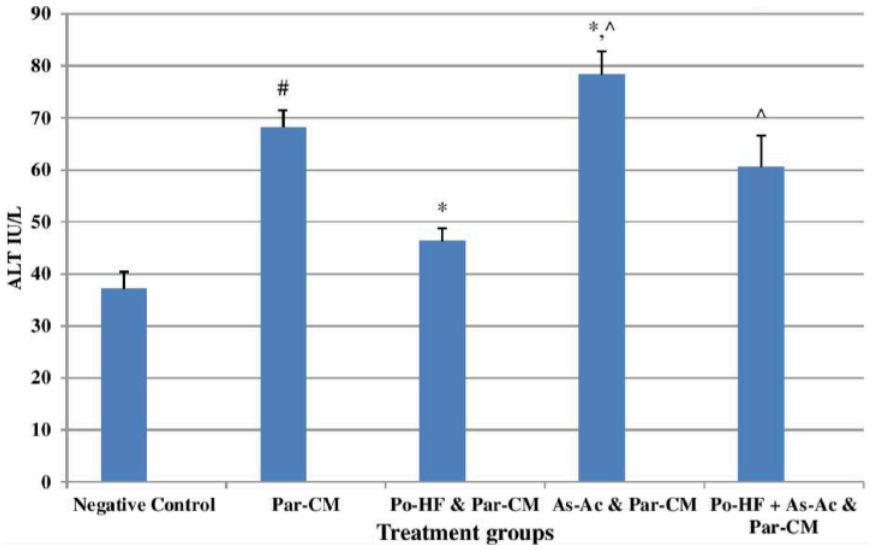

Figure 1. Effect of treatments on serum ALT in Par-CM induced liver damage. Each bar represents the Mean±SD of five rabbits. The sign (#) indicates a significant difference at p<0.05 level from the negative control group. (*) indicates a significant difference at p<0.05 from the Par-CM intoxicated group. (^) indicates a significant difference at p<0.05 from only Polyherbal formulation (Po-HF) treated group. Po-HF & Ascorbic acid (As-Ac) were given for 9 days and Par-CM for the last day only.

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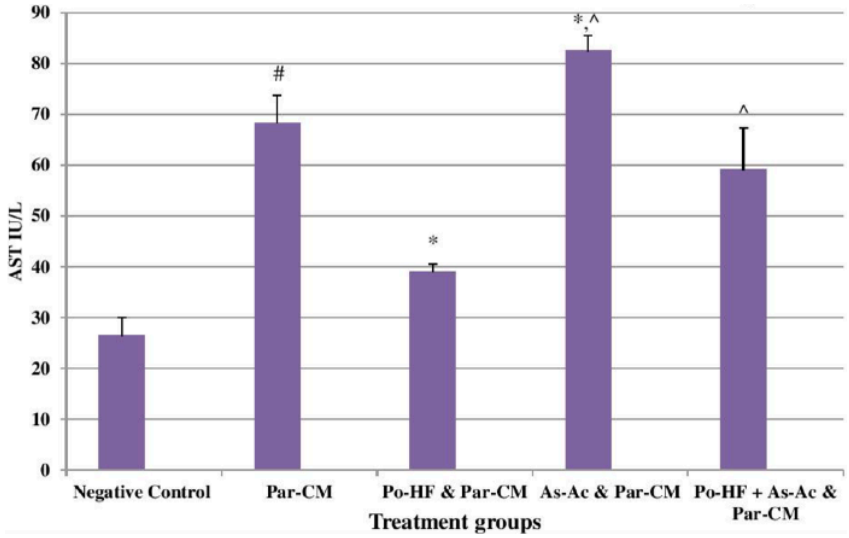

Figure 2. Effects of treatments on serum AST in Par-CM induced liver damage. Each bar represents the Mean±SD of five rabbits. The sign (#) indicates a significant difference at $p<0.05$ level from the negative control group. (*) indicates a significant difference at $p<0.05$ from the Par-CM intoxicated group. (^) indicates a significant difference at $p<0.05$ from only Polyherbal formulation (Po-HF) treated group. Po-HF & Ascorbic acid (As-Ac) were given for 9 days and Par-CM for the last day only.

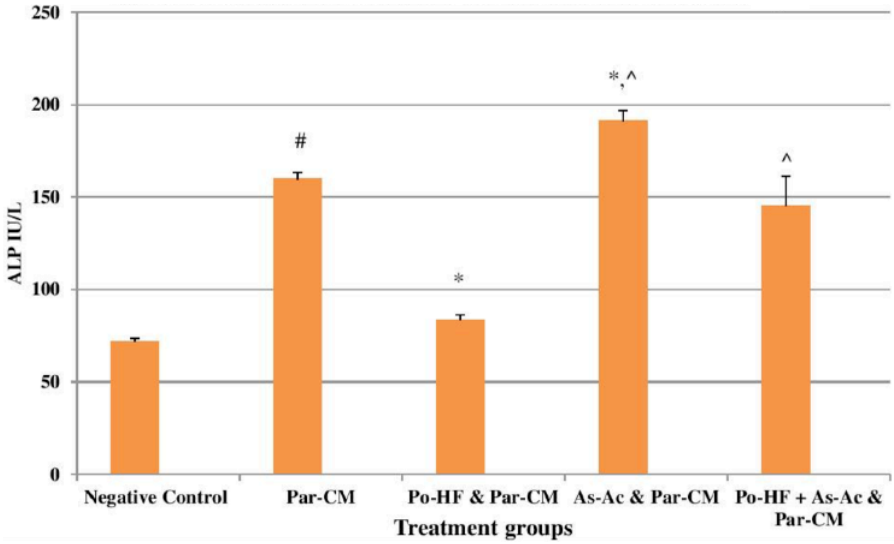

Figure 3. Effects of treatments on serum ALP in Par-CM induced liver damage. Each bar represents the Mean±SD of five rabbits. The sign (#) indicates a significant difference at $p<0.05$ level from the negative control group. (*) indicates a significant difference at $p<0.05$ from the Par-CM intoxicated group. (^) indicates a significant difference at $p<0.05$ from only Polyherbal formulation (Po-HF) treated group. Po-HF & Ascorbic acid (As-Ac) were given for 9 days and Par-CM for the last day only.

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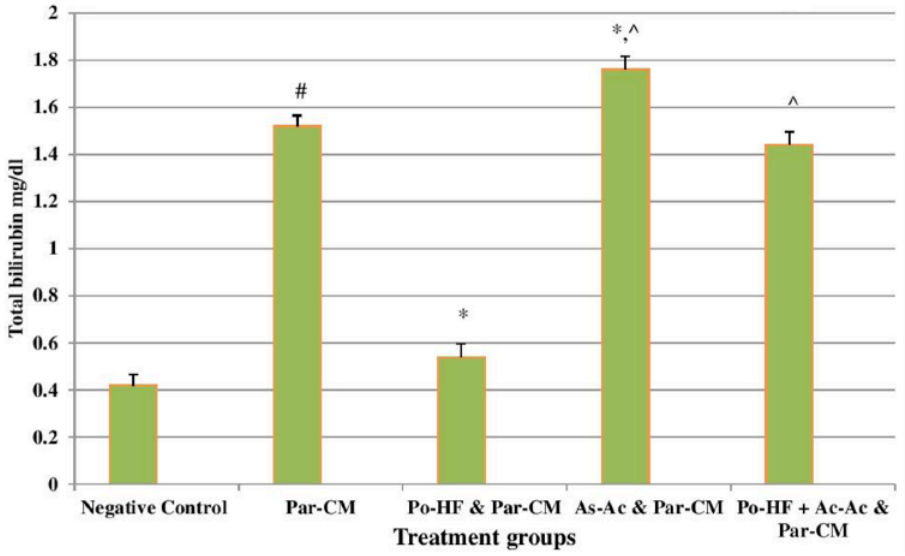

Figure 4. Effects of treatments on T. Bilirubin in Par-CM induced liver damage. Each bar represents the Mean±SD of five rabbits. The sign (#) indicates a significant difference at $p<0.05$ level from the negative control group. (*) indicates a significant difference at $p<0.05$ from the Par-CM intoxicated group. (^) indicates a significant difference at $p<0.05$ from only Polyherbal formulation (Po-HF) treated group. Po-HF & Ascorbic acid (As-Ac) were given for 9 days and Par-CM for the last day only.

Histopathological Findings

Hematoxylin & Eosin stained sections of liver tissues of rabbits from all study groups, except negative control group A, exhibited various histopathological changes as seen by microscopy. Histopathological analysis revealed that group A (negative control) had the most prominent central vein. There was a severe congestion of the central vein in groups B & D, moderate congestion in group E, and mild congestion in group C (Fig. 5). Microscopic examination of paraffin embedded and H&E stained slides of rabbit livers showed that there was no sinusoidal congestion in group A while there was a severe, mild, severe and moderate congestion of sinusoids observed in groups B, C, D and E, respectively (Fig. 6). Microscopic evaluation of processed slides of rabbit liver tissues showed no inflammatory cell infiltration for group A. However, there was a severe degree of infiltration of inflammatory cells for groups B & D, a moderate level of infiltration for group E, and a mild level of infiltration for group C (Fig. 7). Microscopy of liver sections was represented by different levels of periportal fibrosis; Group A had a normal periportal area, groups B, D and E showed a severe collection of connective tissues around the portal area, while group C showed no presence of fibrosis (Fig. 8).

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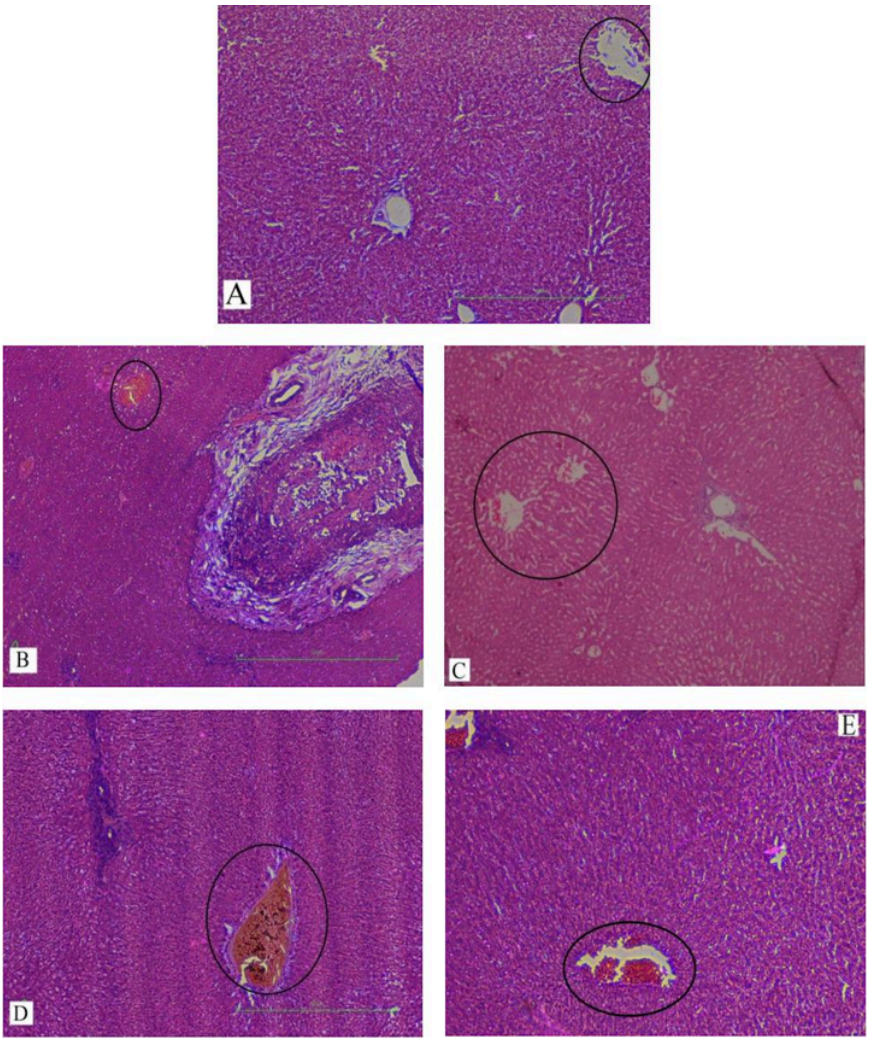

Figure 5. H&E staining of liver sections. H&E stained sections of liver tissue shows severe (group B, Par-CM intoxicated), mild (group C, Po-HF & Par-CM), severe (group D, As-Ac & Par-CM), moderate (group E, Po-HF + As-AC & Par-CM) congestion of central vein. Group (A, negative control) shows normal central vein. The degree of congestion of central vein represents the extent of hepatic damage.

Discussion

ParCM-induced hepatotoxicity is an authentic method for evaluation of hepatoprotective effects of various medicinal plants and herbal formulations. Numerous studies have demonstrated that at high doses, ParCM produces hepatic harm or necrosis (Vermeulen et al., 1992). Bonkovsky et al. (1994) reported that ParCM, in a single overdose or continual low doses, causes

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hepatic damage (Bonkovsky et al., 1994). The extent of liver damage can be accessed from the level of enzymes like ALT, AST and ALP in the serum, as indicators of the physiological status of the liver. Liver enzymes residing in hepatic cells are released in the bloodstream after cellular insult by hepatotoxic dosage of ParCM (Parmar et al., 2010). When hepatocytes are injured, the permeability of the cell membrane and transport mechanisms are altered, resulting in seepage of enzymes from the cells leading to an increase in serum levels of these biomarkers (Jain and Singhai, 2012; Raja et al., 2007).

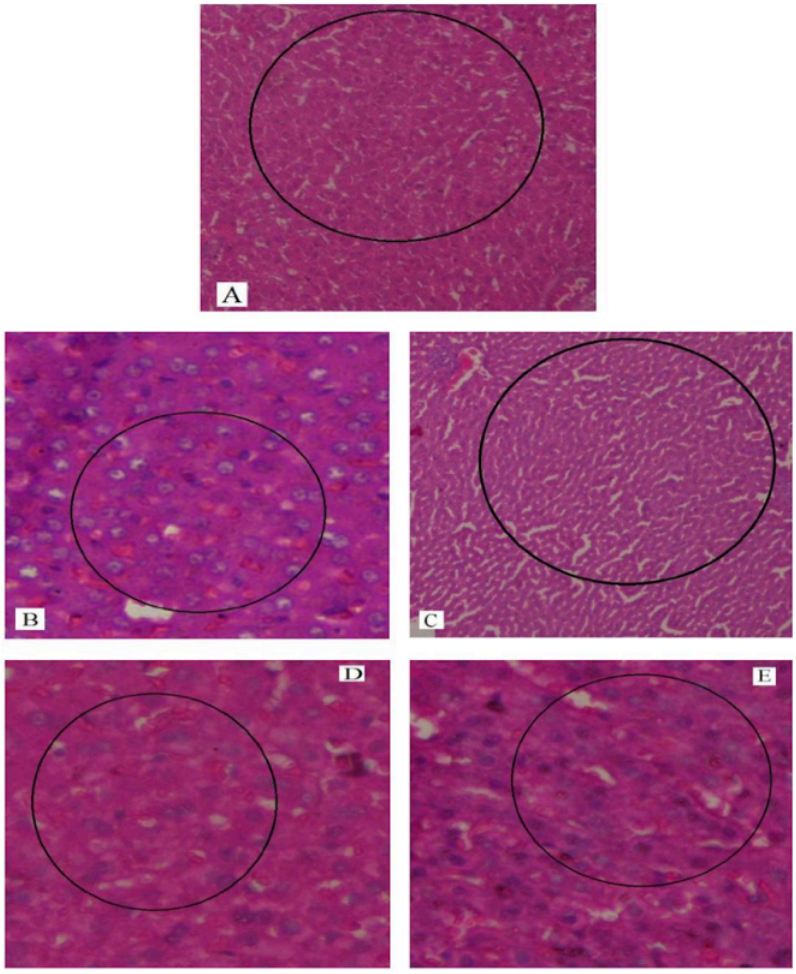

Figure 6. H&E staining of liver sections. H&E stained sections of liver tissue shows severe (group B, Par-CM intoxicated), mild (group C, Po-HF & Par-CM), severe (group D, As-Ac & Par-CM), moderate (group E, Po-HF + As-AC & Par-CM) congestion of sinusoids. Group (A, negative control) shows normal sinusoids. The intensity of sinusoidal congestion illustrates the level of liver damage.

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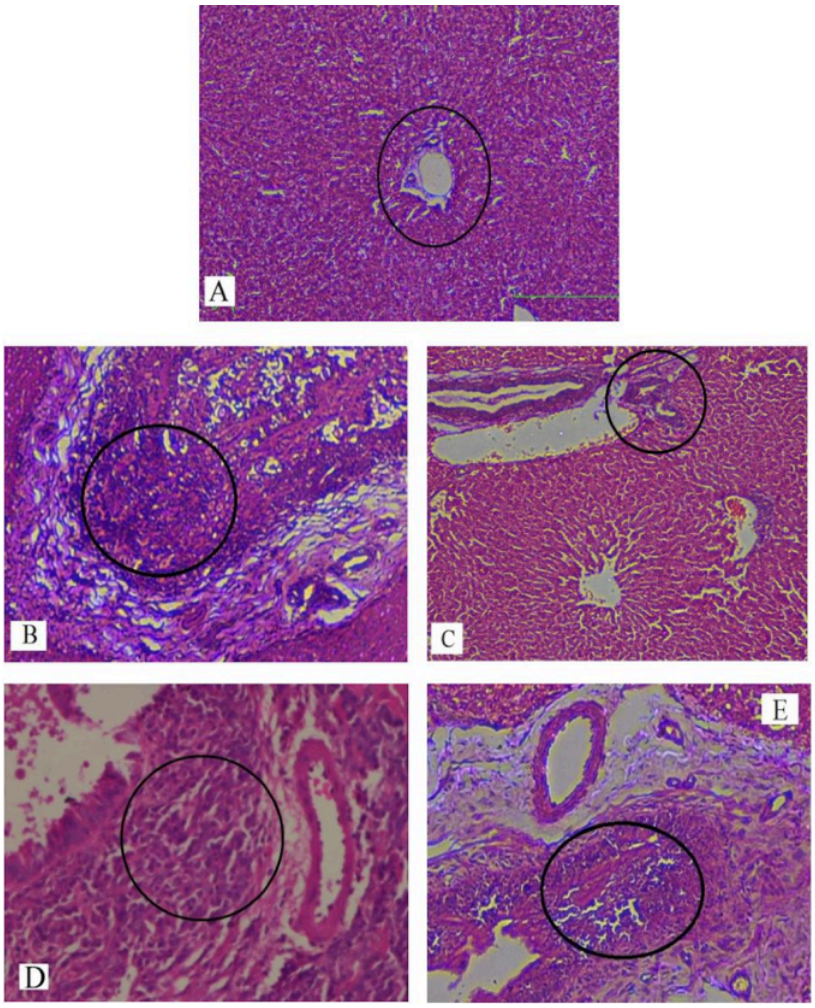

Figure 7. **H&E staining of liver sections**. H&E stained sections of liver tissue shows severe (group B, Par-CM intoxicated), mild (group C, Po-HF & Par-CM), severe (group D, As-Ac & Par-CM), moderate (group E, Po-HF + As-AC & Par-CM) infiltration of inflammatory cells. Group (A, negative control) shows normal portal area. The rate of inflammatory cells infiltration indicates the intensity of hepatic challenge to a foreign intoxicant.

In our study, elevated levels of all the aforementioned bio-indicators were observed in hepatic damage. The measure of ALT, AST, ALP and total bilirubin in ParCM intoxicated group B was significantly ($P<0.05$) higher than in the untreated group A (**Table 1, Figs. 1-4**), demonstrating the damaging effects of ParCM on hepatic cell membranes from enzymes residing within the liver cells and leaking out of cell boundaries in the bloodstream, as reported by Raja *et al*. (2007) (Raja et al., 2007) and Jain & Singha (2012) (Jain and Singhai, 2012). Our results show similar liver damaging effects of ParCM, as which are in accordance with studies by Ahmad et al. (2010), Rehman et al. (2013), Naveed and Ibrar

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(2014), Rehman et al. (2015), and Mumtaz et al. (2015) (Ahmad et al., 2010; Mumtaz et al., 2015; Naveed and Ibrar, 2014; Rehman et al., 2015; Rehman et al., 2013). The hepatotoxic effects of ParCM were further verified by histopathological analyses. Our findings revealed a severe congestion of the central vein and sinusoids, infiltration of inflammatory cells, and periportal fibrosis in liver tissues samples of group B (**Figs. 5-8**). Similar histopathological observations with high dose ParCM have also been reported (Naveed and Ibrar, 2014; Rehman et al., 2015; Rehman et al., 2013).

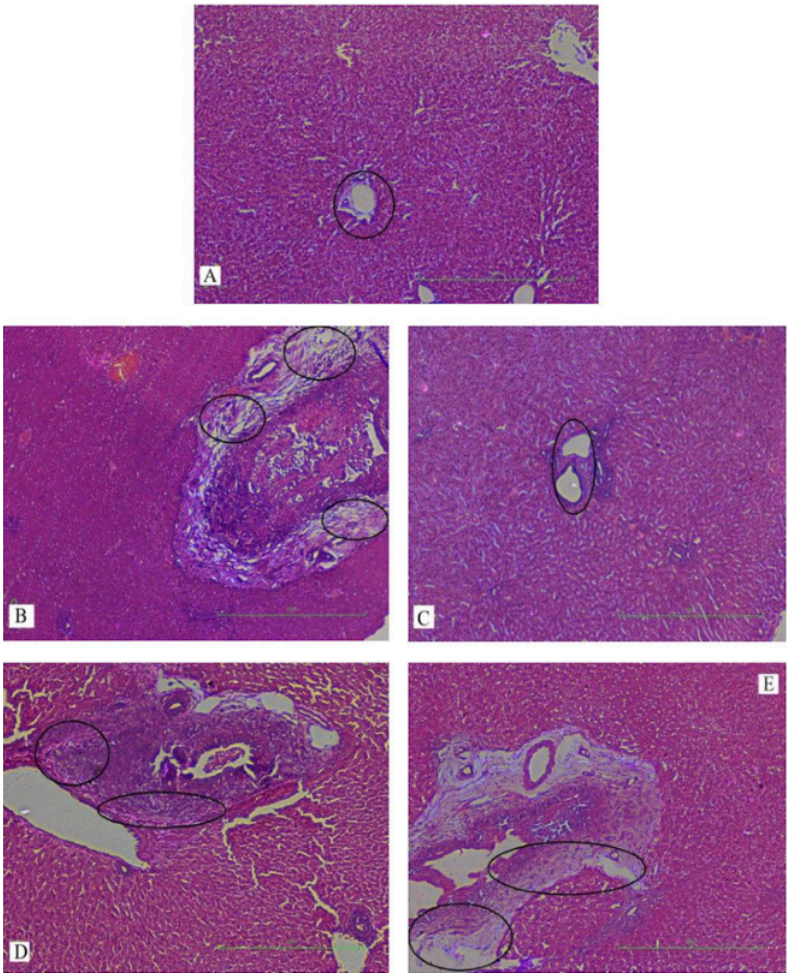

Figure 8. H&E staining of liver sections. H&E stained sections of liver tissue shows severe (group B, Par-CM intoxicated), severe (group D, As-Ac & Par-CM), severe (group E, Po-HF + As-AC & Par-CM) periportal fibrosis. Groups (A, negative control) & (group C, Po-HF & Par-CM) shows no phenomenon of fibrosis. The volume of connective tissue deposition in periportal area explains the severity of hepatic injury.

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The PoHF used in the study for evaluation of hepatoprotective effects caused a significant decline in serum levels of liver enzymes and total bilirubin, compared to levels for group B, indicating that the herbal product positively prevented the deteriorating effects of ParCM on the liver (**Table. 1, Figs. 1-4**). Histopathological outcomes also reinforced the results of the biochemical analyses; central vein & sinusoids were mildly congested, infiltration of inflammatory cells was also of mild nature and no phenomenon of fibrosis was observed in group C (**Figs. 5-8**). The composition of PoHF includes different parts of natural plants and the beneficial effects achieved in our study may be attributed to the various constituents contained in the herbs of herbal formulation.

In group D (AsAc treatment), the level of liver biomarkers was significantly ($P < 0.05$) greater than that for group B and for group C, indicating severe disturbance in the transport mechanisms and permeability of the liver cell membranes (**Table 1, Figs. 1-4**). The biochemical analysis data was fully supported by the histopathological results, as witnessed by the severe congestion of central vein & sinusoids, severe infiltration of inflammatory cells and periportal fibrosis in group D (**Figs. 5-8**). Indeed, Richardson and Ponka (1997) found that in spite of its role as a prototypical antioxidant, AsAc also exhibited pro-oxidant characteristics in different situations (Richardson and Ponka, 1997). Halliwell and Gutteridge (1986) reported that the pro-oxidant action of AsAc depends on the presence of catalytic forms of metals (Halliwell and Gutteridge, 1986). It was explained by Halliwell (1996) (Halliwell, 1996) and Buettner and Jurkiewicz (1996) (Buettner and Jurkiewicz, 1996) that if catalytic metal ions are available in excess as is the case in pathological conditions, ascorbic acid reduces the transition metal ions. This results in excessive production of ferrous ions which by interacting with hydrogen peroxide in Fenton reaction creates more hydroxyl radicals, leading to damaging effects. Bauer et al. (2000) found that in ParCM-poisoned liver, heme oxygenase, an enzyme responsible for breakdown of heme from hemoglobin and ultimately liberation of more free iron, is upregulated (Bauer et al., 2000). In light of this finding, it may be postulated that in our study due to intoxication with high dose of ParCM in group D, the activity of heme oxygenase might have accelerated resulting in frequent availability of free transition iron and presence of AsAc, causing reduction of transition metal ions, boost of the Fenton reaction, and detrimental effects on the liver cells. Wang et al. (2007), Park and Lee (2008), Kamel et al. (2010), and Marim et al. (2015) have all reported the damaging effects of AsAc in their studies (Kamel et al., 2010; Marim et al., 2015; Park and Lee, 2008; Wang et al., 2007).

The values of ALT, AST, ALP and total bilirubin in the blood serum of the animals of group E were significantly greater than group C (**Table 1, Figs. 1-4**). Microscopy of liver sections of group E showed moderate congestion of central vein & sinusoids, infiltration of inflammatory cells and severe fibrosis (**Figs. 5-8**), thereby corroborating the results obtained in biochemical analyses of blood serum. Although PoHF alone showed hepatoprotective effects in group C, here

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in group E (where PoHF & AsAc were co-administered) liver protection was not achieved. AsAc exhibited liver-damaging effects in group D; similar effects seen in group E may be ascribed to AsAc in ParCM poisoned animals.

Overall, administration of PoHF alone exhibited a remarkable hepatic cell protection in ParCM- intoxicated animals. Oral administration of AsAc, in a ParCM poisoned rabbit animal model, surprisingly resulted in more malicious effects. Co-administration of PoHF and AsAc (ideally to achieve synergistic effects to counter ParCM intoxication) was not achieved. This may be due to the possibility that AsAc transitions from an antioxidant to pro-oxidant in ParCM-intoxicated liver, thereby turning the AsAc from a beneficial to a noxious element.

Conclusion

PoHF is a successful hepatoprotective regimen in animals, even those administered with high dose ParCM. In our study, AsAc failed to generate hepatic cell protective effects and concomitant administration of PoHF with AsAc showed no benefit for ParCM-poisoned rabbits. Further studies of other hepatotoxicity inducers (besides ParCM) are warranted. Moreover, dose studies of AsAc and ParCM using a larger sample size and in other animal models are warranted.

Abbreviations

As-Ac: Ascorbic acid; Par-CM: Paracetamol; Po-HF: Polyherbal formulation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; CMC: Carboxy Methyl Cellulose; H & E: Hematoxylin & Eosin

Author contribution

Muhammad Fiaz designed the study, collected the literature, executed the experimental work and wrote the manuscript. Waseem Mehmood biochemically analyzed the blood serum. Ghulam Mustafa and Abdul Rauf performed the processing of the liver tissues, examined the slides of liver samples under microscope and concluded the findings of histopathology. Komal Najam reviewed the final manuscript. Naghma Fiaz statistically analyzed the data. Lubna Shakir and Alamgeer supervised the study.

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Correction

CORRECTION: A mouse model of osteonecrotic femoral head induced by methylprednisolone and lipopolysaccharide

Ha Thi -Ngan Le, Lan Thi Phi, Thuy Thi-Thanh Dao, Ngoc Kim Phan, Phuc Van Pham, Ngoc Bich Vu*

Laboratory of Research and Application Stem Cell, University of Sciences, Vietnam National University, Ho Chi Minh City, Vietnam

The title of the article "**A mouse model of osteonecrotic femoral head induced by methylprednisolone and liposaccharide**" published in 25 March 2016, in 2016 at Vol 3, Issue 3, from pages: 548-556 (DOI: 10.7603/s40730-016-0012-x)

should be read as:

"**A mouse model of osteonecrotic femoral head induced by methylprednisolone and lipopolysaccharide**".

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Correction

CORRECTION: Adipose derived stem cell transplantation is better than bone marrow mesenchymal stem cell transplantation in treating hindlimb ischemia in mice

Ngoc Bich Vu [1], Lan Thi Phi [1], Thuy Thi-Thanh Dao[1], Ha Thi-Ngan Le[1], Van Thanh Ta [2], Phuc Van Pham [1,3,*]

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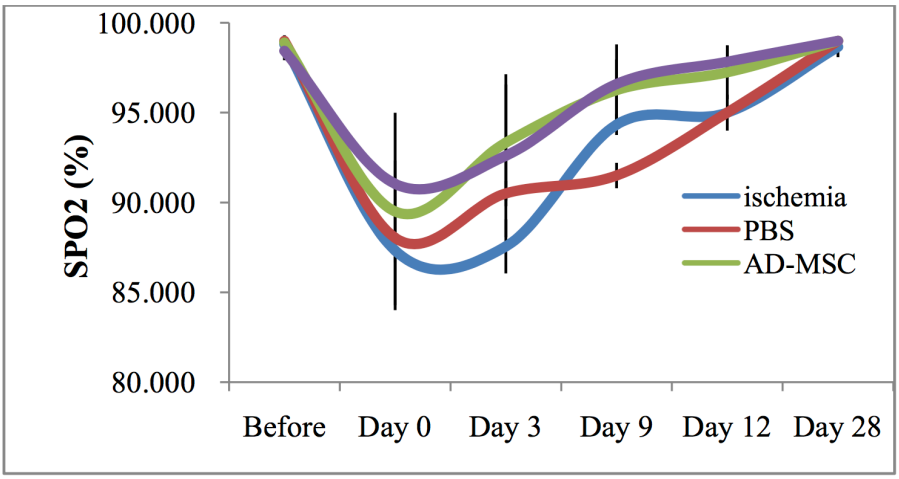
The **Figure 5** of the article "**Adipose derived stem cell transplantation is better than bone marrow mesenchymal stem cell transplantation in treating hindlimb ischemia in mice**" that published in 29 Sept 2016, at Vol 3, Issue 9, from page 844-856 (DOI: 10.7603/s40730-016-0046-0) as below



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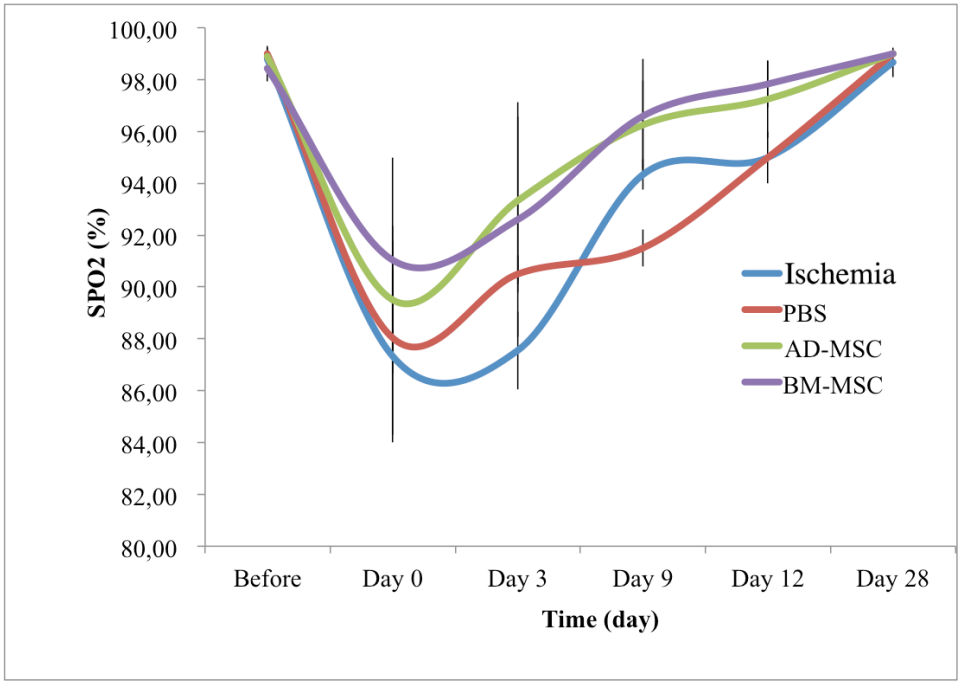
that lacked the legend for the violet line should be corrected as:



The violet line demonstrated "BM-MSC".

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