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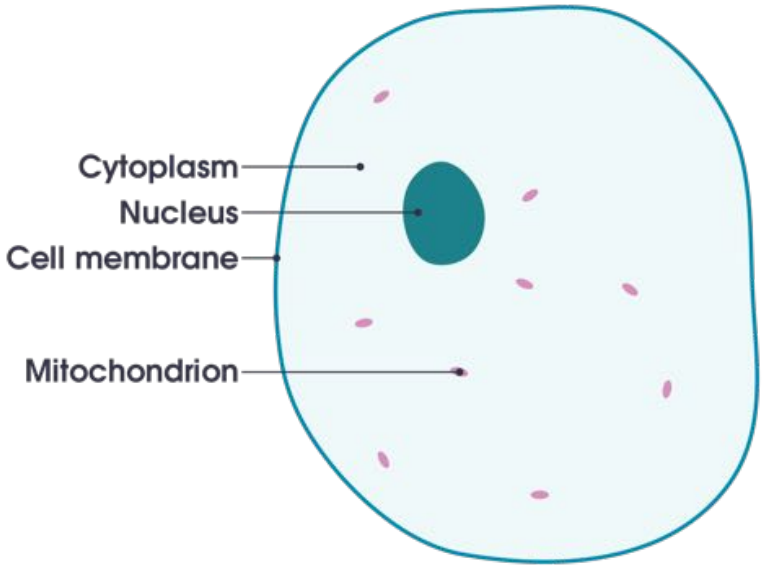

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Editorial Team

Contact us

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BIOMEDPRESS (BMP)
Laboratory of Stem Cell Research and Application
University of Science, Vietnam National University, Ho Chi Minh city
227 Nguyen Van Cu
District 5, Ho Chi Minh city
Vietnam
Email: contact@bmrat.org

PRINCIPAL CONTACT
Lili Hami
BIOMEDPRESS (BMP)
Laboratory of Stem Cell Research and Application
University of Science, Vietnam National University, Ho Chi Minh city
227 Nguyen Van Cu
District 5, Ho Chi Minh city
Vietnam
Email: managingeditor@bmrat.org

SUPPORT CONTACT
Support Team
Email: support@bmrat.org

EDITOR-IN-CHIEF
Phuc Van Pham
Email: pvphuc@bmrat.org

Table of Contents

Vol 4 No 5 (2017): 1281 - 1340

Research articles

Corrections

Biomedical
Research & Therapy

ISSN: 2198-4093
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Original Research

Geographic distribution of breast cancer incidence in Iran

Fatemeh Khosravi Shadmani[1], Kamyar Mansori[2,3], Salman Khazaei[4], Shiva Mansouri Hanis[5], Somayeh Khazaei[6], Mohadeseh Sani[7,*], Erfan Ayubi[8,9]

[1]Modeling in Health Research Center, Institute for Futures Studies in Health, Kerman University of Medical Sciences, Kerman, Iran

[2]Social Development & Health Promotion Research Center, Gonabad University of Medical Sciences, Gonabad, Iran

[3]Department of Epidemiology, School of Public Health, Iran University of Medical Sciences, Tehran, Iran

[4]Department of Epidemiology, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran

[5]School of Public Health, Dezful University of Medical Sciences, Dezful, Iran

[6]School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran

[7]School of Medicine, Zabol University of Medical Sciences, Zabol, Iran

[8]Department of Epidemiology, School of Public Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[9]Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

***For correspondence:**

aubi65@gmail.com

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Abstract

Background: A geographic disparity for breast cancer (BC) incidence by provinces is introduced in Iran. Present study aimed to clarify the geographic disparity of BC incidence after considering the age and gender. **Methods:** In this ecological study data about BC incidence extracted from reports of national registry of cancer (NCR), and Disease Control and Prevention in 2008. BC incidence mapping was conducted in geographic information system (GIS). **Results:** The results were consistent with previous reports but extend the previous knowledge with regarding the age and gender. Highest age specific rates (ASRs) of BC occurred in the provinces located in Central and Northern of Iran. Tehran and Sistan & Balochestan had highest and lowest ASR for male BC and female BC respectively. **Conclusion:** given that BC occurs more in Central and Northern provinces that are mainly with high socioeconomic status (SES) levels, so it is suggested that disparity in BC incidence can be reduced through planning special programs such as education, screening, and preventive policy in provinces with high priorities.

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Keywords

Geographic distribution, Breast cancer, incidence, Iran

Introduction

Breast cancer (BC) is the most common cancer and leading cause of female mortality in the world (García Martínez et al., 2014), this represents about 25% of all cancers in women (Ferlay et al., 2015). Although BC mortality was decreased approximately 5% during the last decade, it is still a serious concern with 522,000 deaths in 2012 (Ferlay et al., 2015). Statistics showed that BC incidence is increasing in the low and middle-income countries (Tfayli et al., 2010). Iran is a middle-income country located in the Middle East. BC incidence in Iranian women is 24 case per 100,000, which is lower than of high income countries (Mousavi et al., 2007). However, regarding to lifestyle changes, the increase of life expectancy and socio-economic status, prediction models have found that the BC incidence for future decades will be increased (Mousavi et al., 2007).

BC is a multi-factorial disease, epidemiological studies have shown that the genetic, hormonal factors and environmental exposures are associated with incidence of BC (Pakseresht et al., 2009). It found that exposure to the different risk factors can lead to an uneven distribution of BC incidence (Laden et al., 1997). Iran have several geographic, climatic, ethnic, regional, racial, and cultural classifications that cause of exposure to different risk factors. It has been identified that BC incidence can be along with province disparity in Iran (Jafari-Koshki et al., 2014) but it proved that relevant covariates such as age and gender can affect the geography disparity in cancer measures (Henry et al., 2009).

Hence the present study attempts to discover the geographic distribution for age specific incidence rate of BC in both gender in Iran; determine whether geographic variation is in breast cancer incidence could be helpful for the future works; and provide an evidences for policymakers and planners for optimal allocation of resources.

Materials - Methods

This ecological study used re-analysis medical records aggregated to provinces from national registry of cancer (NCR), and Disease Control and Prevention report of ministry of Health and Medical Education for BC in 2008 (Ministry-of-Health-and-Medical-Services, 2008). Data collection by the Iranian Cancer Registry is active and pathology-based and covering the whole country's

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pathology laboratories. Hospital-based and death certificate–based data have not been included. Cleaned data from province after deleting for repeated cases transmitted to Ministry of Health every 3 months.

Registered data classified into three part as follow:

1) patient's identity characteristics including age, gender, race and residence location,

2) patient's clinical history and

3) preclinical findings.

Data on primary location of tumor, date of cancer diagnosis, morphology, histology and diagnosis method is registered.

Statistical analysis

For each province, the average annual age-standardized incidence rate (ASR) per 100 000 person-years was calculated by the direct method using the World Standard Population (Boyle and Parkin, 1991). The data were presented using MS Excel 2010 and GIS ver 10.3.

Results

Figure 1 illustrates the spatial pattern for ASR of BC in Iran. Roughly an apparent geography variation in ASR of BC is occurred in Iran, so that higher ASR is belong to provinces located in central and northern parts of Iran. **Table 1** describes the province specific ASR of BC for both gender. ASR for Semnan, Zanjan and Kohkiloyeh & Boyerahamad was 0 for males. With overlooking three former province, highest and lowest total ASR were for Tehran (ASR=1.1) and Sistan & Balochestan (ASR=0.22) for males respectively. Tehran and Sistan & Balochestan have highest and lowest ASR for male BC and female BC respectively.

According to age distribution of BC in females as shown in **Figure 2**, BC roughly have a binomial distribution and highest incidence rate was for 55 to 59 age group. Basal Cell Carcinoma, NOS of the breast was the most common 'special' morphological subtype of breast cancer in both gender, for female and male were 6452 (76.59) and 136 (70.83) respectively.

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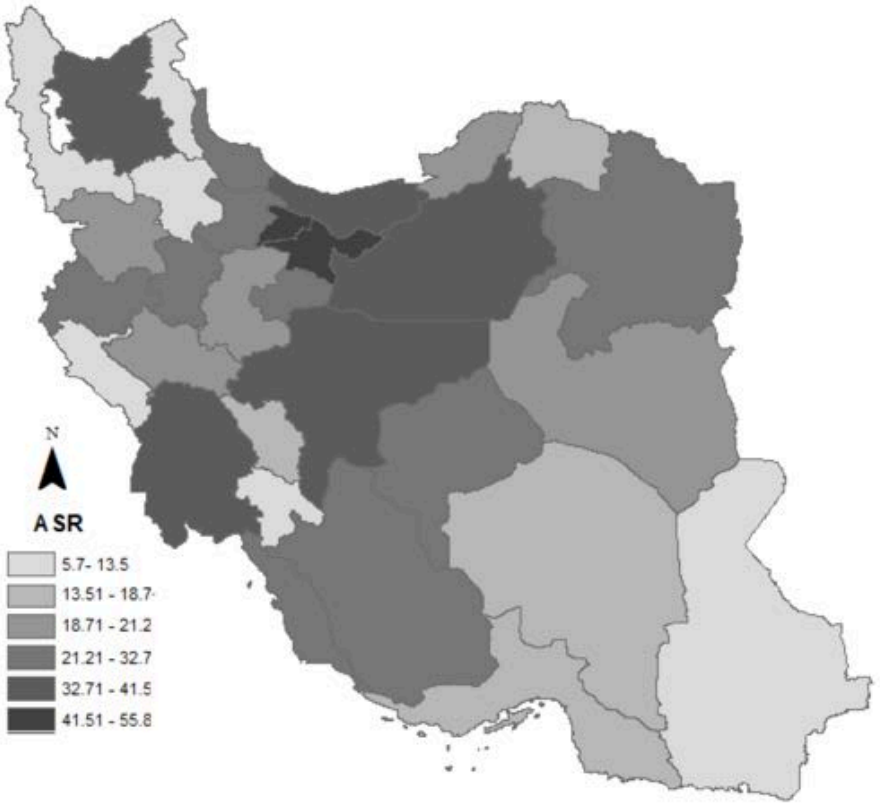

Figure 1. The geographic distribution of age specific rate (ASR) of breast cancer for females in Iran. A relatively variation in the ASR of breast cancer where surrounding province and along the edge of Iran have lower ASR and area near to center had higher ASR.

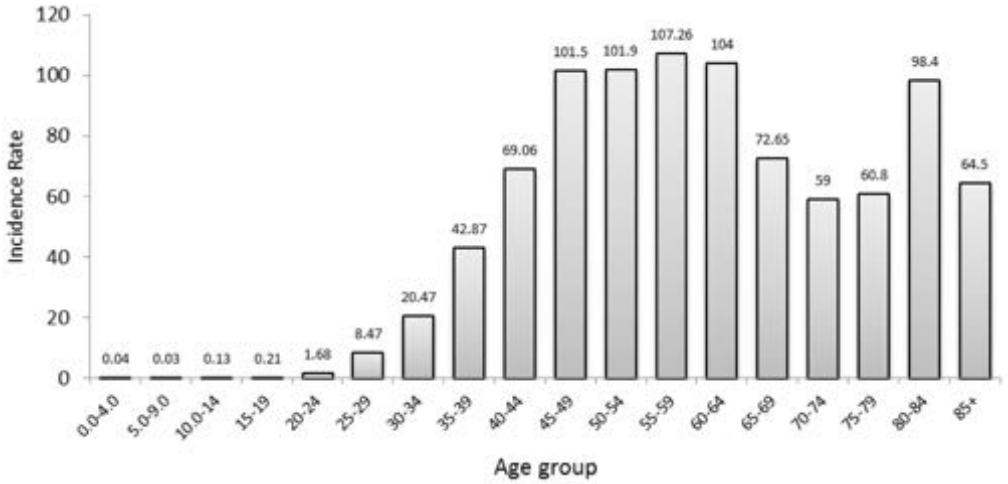

Figure 2. Incidence of breast cancer in females by age group in 2008; a relatively binomial distribution exists in age distribution of incidence of breast cancer.

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Table 1. The statistics of breast cancer according to gender and province

Province	Males				Females			
	Frequency	CR	ASR	%	Frequency	CR	ASR	%
Semnan	-	-	-	-	88	30.7	41.5	23.3
Tehran	57	0.83	1.1	0.54	2624	40.4	55.8	33.4
East Azarbayejan	7	0.38	0.49	0.27	452	25.8	34.1	22.7
Zanjan	-	-	-	-	37	7.95	10.2	14.9
Kordestan	3	41	0.58	0.39	117	16.7	21.2	16.3
Yazd	3	0.59	0.86	0.46	112	23.3	31.4	19.4
Hamadan	2	0.23	0.34	0.19	153	18.5	24.6	22.9
Khozestan	12	0.55	0.7	0.45	643	31.1	41	26.7
Khorasan Razavi	15	0.51	0.65	0.43	598	21.4	28.8	21.5
Gilan	8	0.66	0.85	0.57	273	23.6	32.7	26.4
Mazandaran	10	0.67	0.98	0.56	423	30	39.8	28.4
Isfahan	12	0.51	0.77	0.45	607	27.1	37.3	27.7
Fars	11	0.5	0.69	0.46	489	23.5	32.2	24.8
Lorestan	6	0.7	0.88	0.62	125	15.1	20	17.6
Kermanshah	3	31	0.51	0.29	193	21.2	27.2	23.6
Ardebile	3	0.48	0.61	0.44	45	7.55	9.75	10.1
Ghazvin	3	0.52	0.74	0.56	115	20.9	27.8	26.7
Chaharmahal & Bakhtiari	1	0.23	0.27	0.25	60	14.4	18.7	21.7
West Azarbaijan	5	0.34	0.48	0.41	145	10.4	13.5	16.6
Markazi	2	0.29	0.48	0.35	96	14.6	19.9	24.9
Kerman	6	0.45	0.6	0.56	160	12.6	16.4	18.8
Ilam	1	0.36	0.49	0.47	25	9.4	12	17
Kohkiloyeh& Boyerahamad	-	-	-	-	19	6.2	7.2	10.7
Golestan	2	0.24	0.26	0.32	122	15.6	20.4	24
Qom	2	0.37	0.45	0.49	98	19.3	24.8	29
North Khorasan	1	0.24	0.46	0.33	50	12.7	15.8	20.1
South Khorasan	1	0.35	0.43	0.52	41	15.1	21	22
Bushehr	1	0.22	0.27	0.35	87	19.9	27	31.8

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Hormozgan	2	0.29	0.35	0.66	95	14.3	18.6	31.5
Sistan & Balochestan	2	0.16	0.22	0.58	53	4.5	5.7	17
Total	**192**	**0.53**	**0.72**	**0.45**	**8424**	**24.66**	**33.21**	**24.9**

Table 2. Morphological subtype of breast cancer in 2008

Gender	Neoplasm malignant N (%)	Lobular carcinoma N (%)	Basal Cell Carcinoma, NOS, N (%)
Females	324 (3.85)	460 (5.46)	6452 (76.59)
Males	8 (4.17)	8 (4.17)	136 (70.83)

CR: Crude Rate,
%: Percent from all cancers that attributed to this cancer

Discussion

Our study indicated that ASRs of BC in Central and Northern provinces of Iran were higher than elsewhere. We found ASR have a binomial distribution, in women age 40 and over is increased, first and second peak is occurred in 55-59 and 80-84 years respectively.

In Iran ASRs of BC in various geographical regions were different, and the highest rate of ASR was observed in metropolitan provinces. BC is a multifactorial disease and there are several risk factors recognized for that, such as fertility rates (Ruddy et al., 2014), the first pregnancy in older age, infertility (Horn et al., 2013), socio economic status, educational level (Hogan et al., 2007), hormone use (Jung et al., 2013), alcohol consumption and smoking (Nelson et al., 2012) which have different patterns among various regions and states. Our results found Tehran and Sistan & Balochestan had highest and lowest incidence of BC, Tehran has a high SES and educational level, and Women of this city would rather have a Job outside the Home, besides, marriage and child bearing in Tehran occurs in older ages. On the contrary, Sistan & Balochestan has a low SES and educational level, and marriage and childbearing in this city occur in earlier ages.

The difference in incidence rates among various geographical regions was pointed out in several studies (de Grubb et al., 2013; Reynolds et al., 2005). Iran's Population consists of various ethnic groups so that this variety may affect the risk factors associated with incidence of BC.

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Industrialization, social and economic status is also brought into account as risk factors of BC. With the rapid rate of industrialization of Nigeria, BC incidence rate among Nigerian has significantly increased over the past three decades (Alatise and Schrauzer, 2010). These results, being consistent with our findings, suggest that the regions with high ASR, have been undergoing social and economic transitions which lead to change in life style, rise in age of marriage, and increase rate of the first pregnancy at older age.

Others studies suggested that environmental factors (e.g. air pollution) influence BC risk (Brody and Rudel, 2003) and may be cause of disease clustering in some geographical regions. Several studies have reported that environmental pollutants may contribute to BC risk through destroying DNA, accelerating tumor growth rate, or increasing susceptibility to the BC (Rudel et al., 2007). In this study; Provinces with high ASR, mainly located in the central regions of Iran, in many aspects are different from the other provinces. Most industrial factories located in central provinces. The number of vehicles in these provinces is very high and air pollution is considered as the main problem (especially in Tehran).

Moreover, the regional differences in incidence of BC could be due to the differences in the early detection and availability of mammography in various regions (Edwards et al., 2010). Another reason could be the centralization of diagnostic and therapeutic interventions in metropolitan areas, so that the cases that belong to other regional areas may be referred to these provinces, where they are registered.

Our findings indicated that the incidence rate of BC is increased with aging and it reaches its peak between the ages of 55 to 59 years. The second peak was observed between the ages of 80 and 84 years. The steroid hormones directly affects the development and function of breast in reproductive years for women (between menarche and menopause) (Cancer, 2012). This hormone fall rapidly in post menopause and risk of breast cancer is decreased. Women become menopause mostly between the ages of 45 and 54 years. Late menopause is a known risk factor for development of BC; the risk of this factor in premenopausal women is 40 percent higher than that of postmenopausal women with the same age (Reeves et al., 2006). Some studies have suggested a decreased in the incidence of breast cancer as a result of decline in the use of hormone. These studies have also shown that the hormone users have a higher risk of being diagnosed with BC (De et al., 2010; Jemal et al., 2007). These results are consistent with our study. The second peak may happen due to the exogenous use of hormone in postmenopausal.

We found that BC in under 40 year old individuals has low incidence. Other studies have estimated an incidence of approximately 7% (Anders et al., 2009) which is consistent with our results. BC in under 40 year old individuals may be due to familial and genetic history, causing BC in younger ages. Both cumulative exposure to risk factors and BC incidence for under 40 year old people are low.

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Our study was subjected to some limitations. We did not have access to data regarding cancer stage at diagnosis time in order to conduct a more detailed investigation. But this limitation have no high effect on geographical distribution at provincial level. Also we did not have data on age average of provinces. One of the most important advantages of this study is the use of routine data according to Population based on Cancer Registry with the coverage of 86.7 %.

Conclusion

This study determined the hot zones of breast cancer in Iran and can be considered as a guideline for policy maker in allocation of diagnostic and therapeutic interventions. Findings are demonstrated that the central and northern provinces need more attention. Increased access to screening for early detection is beneficial and cost-effective particularity in high incidence regions.

Abbreviations

ASR: Age specific rate
BC: Breast cancer
CR: Crude rate
NCR: National registry of cancer
SES: Socio-economic status

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Author contribution

FKS, EA and SK designed the study. KM, SMH and YM processed the data. EA, MS and YM performed the statistical analysis. FKS, SK and KM interpreted the results. FKS, KM, SK and EA wrote the first draft. FKS, SK and EA revised the final draft. All authors read and approved the final manuscript.

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Original Research

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Haloperidol induced Parkinson's disease mice model and motor-function modulation with Pyridine-3-carboxylic acid

Atif Saeed[1], Lubna Shakir[1], Mahtab A. Khan[2], Arsalan Ali[1], Muhammad Yousaf[1,*], Awais Ali Zaidi[2]

[1]Faculty of Pharmacy, Hajvery University, Lahore-Pakistan
[2]Faculty of Pharmacy, The University of Lahore, Lahore-Pakistan

Abstract

Introduction: Motor-function modulation through Pyridine-3-carboxylic acid was assessed against. Haloperidol induced Parkinson's disease (PD) in albino-mice. The objectives of this study were to test the effect of Haloperidol in development of PD, effectiveness of Pyridine-3-carboxylic acid in mice and evaluation of the motor-function changes in mice before and after treatment. **Methods**: The study was divided into 3 phases: During Phase-I (randomization), all the subjects were randomly divided into 4 groups and trained for wire-hanging, grip strength, vertical rod and swim tests for 1 week. During Phase-II (disease induction), PD was induced by intra-peritoneal (ip) administration of Haloperidol (HP) in a dose of 1 mg/kg/d for 7 days. Group-A comprised of healthy controls, Group-B (Diseased), Group-C (HP+Pyridine-3-carboxylic acid 7.15 mg/kg/d) and Group-D (HP+Pyridine-3-carboxylic acid15 mg/kg/d). **Results**: Pyridine-3-carboxylic acid treatment continued for 5 weeks. During Phase-III the above mention tests were performed and the effects of Pyridine-3-carboxylic acid were recorded. However, in swim test Group D is statistically insignificant as compared to Group B ($p=0.284$). In recent study, haloperidol is found to be effective in inducing motor function anomalies likewise in Parkinson's disease including movement slowness, difficulties with gait and balance. **Conclusion**: oral administration of Pyridine-3-carboxylic acid improved Motor-function changes in diseased mice. Therefore, it is concluded that Pyridine-3-carboxylic acid may help to alleviate PD symptoms.

Keywords

Pyridine-3-carboxylic acid, Haloperidol, motor-function modulation, Parkinson's disease, Mice

***For correspondence:**

dr.yousaf_muhammad@yahoo.com

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Introduction

Parkinson's disease (PD) was first illustrated by James Parkinson in 1817 in "An Essay on the Shaking Palsy" (Kempster et al., 2007). The major symptoms of PD are tremors, bradykinesia, postural instability and rigidity, postural abnormalities, akinesia and festinating gait. These symptoms are led by psychological symptoms such as depression and more general non-motor symptoms such as olfactory dysfunction constipation, and sleep disturbances (Klockgether, 2004). The incidence of Parkinson's disease (PD) is nearly about 1% at the age of 65 years which further increased to 5% with the age of 85 years (Hirtz et al., 2007). Haloperidol is a typical neuroleptic drug and shows effect by blocking the postsynaptic dopamine D_2 receptors in the mesolimbic system and cause an escalation of dopamine turnover by blockage of the D_2 receptors (Zaidi et al., 2016a). Anticholinergic and β-adrenergic receptor blocking effects of haloperidol is quite weak. Parkinson disease is characterized majorly by the loss of melanin containing dopaminergic neurons in zona compacta of the substantia nigra (Bernheimer et al., 1973). Haloperidol causes a decrease in dopamine neurotransmission (Naidu et al., 2003). Haloperidol exerts its antipsychotic effect most likely through potent blockade of central dopamine receptors and marked rigidity linked with haloperidol administration (Zaidi et al., 2016b). In animal studies, neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP) and 6-OHDA and haloperidol are used commonly to create experimental model of PD (Fernagut et al., 2002; Sheidaei, 2010) by which certain aspects of the disease such as motor abnormalities and slowing and of movement and catalepsy can be modeled (Scholtissen et al., 2006; Wang et al., 2005). Clinical symptoms appear only when dopaminergic neuronal death exceeds a critical threshold 70-80% of striatal nerve terminals.

Vitamin B_3 also known as Pyridine-3-carboxylic acid and found in foods including certain types of meat and organ meat, tuna fish, seeds, mushrooms and others. Vitamin B_3, which is usually medically referred to as Pyridine-3-carboxylic acid, comes in 3 forms nicotinic acid, Pyridine-3-carboxylic acid amide and Inositol HexaPyridine-3-carboxylic acidate. Vitamin B_3 Pyridine-3-carboxylic acid has been studied extensively to treat of many commonly occurring health problems. Pyridine-3-carboxylic acid is an important vitamin for maintaining healthy brain function and healthy cardiovascular system and metabolism, especially balancing blood cholesterol levels (Gurakar et al., 1985; Morris et al., 2004). Nicotinic acid (Pyridine-3-carboxylic acid) has shown neuro-protective role in mice-stroke model by promoting the monocyte polarization into protective phenotype in brain (Rahman et al., 2014). Absorbed niacin is used in the synthesis of nicotinamide adenine dinucleotide (NAD) in the body, and in the metabolic process NAD releases nicotinamide by poly ADP-ribosylation, the activation of which has been reported to mediate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease (Molina et al., 1996).

The objectives of this study were to evaluate the effect of Haloperidol in development of PD, effectiveness of Pyridine-3-carboxylic acid to modulate

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Motor-function activities in mice model PD and record the Motor-function changes in mice before & after the treatment of PD with Pyridine-3-carboxylic acid.

Materials - Methods

Study design

Albino mice (20-25g) were taken from animal house of UVAS (University of veterinary sciences, Lahore-Pakistan). The animals were acclimatized and kept under specified temperature (22±2°C) and humidity (60±2%) under 12-hours light/dark cycles with food and water ad libitum. Experimental procedures and animal handling were approved by Institutional Committee of Research Ethics, Hajvery University (Ethical no. 720EN-2016) Lahore, Pakistan.

The study was divided into three phases.

Table 1. Experimental layout of this study.

Group	Count	Haloperidol	Pyridine-3-carboxylic acid	Normal saline
Group A	n=5	(-)	(-)	(+)
Group B	n=5	(+)	(-)	(-)
Group C	n=5	(+)	(+)	(-)
Group D	n=5	(+)	(+)(+)	(-)

Phase-I

During Phase-I, the motor-function modeling of mice was performed using wire hanging test, grip strength test, vertical rod test, and swim test. All subjects were trained and divided into four groups comprising of 7 mice in each.

Wire Hanging Test

The wire hanging test was used to assess muscle function and coordination over time. The test is based on the latency of a mouse to fall off a metal wire upon exhaustion. The wire hanging test is performed in order to demonstrate a motor neuromuscular impairment and motor coordination. This test was also used for evaluating the neuromuscular tone. A 55 cm wide 2 mm thick wire is secured to two vertical stands. The wire must be tightly attached to the frame to avoid vibration or unwanted displacement of the wire while the investigator is handling the animals or during the measurements, since these unwanted effects would

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interfere with the animal's performance (Aartsma-Rus and van Putten, 2014; Klein and Lewis, 2012).

Grip Strength Test

The grip strength test is a modest non-intrusive method intended to assess mouse muscle power in vivo, by taking benefit of the animal's affinity to grip a flat metal bar or framework while suspended by its tail or each of the four appendages. This is a pure test of strength, although as for any test motivational factors could potentially play a role. The inverted screen is a 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire. We place the mouse in the center of the wire mesh screen, start a stop clock, and rotate the screen to an inverted position over 2 sec, with the mouse's head declining first. We hold the screen steadily 40-50 cm above a padded surface.

Vertical Pole Test

Vertical pole test is used to measure the sensorimotor function of mice. PD motor tests provide a good read-out of neurological function.. Each mouse is placed head upwards at the centre of a round pole that is inclined at 90° and performance is determined by the latency(s) of the mouse to turn downwards and completely descend the pole. In a habituation period one day prior to testing, each mouse is allowed to attempt to descend the pole. Each testing session lasts for a maximum of 180 seconds.

Swim Test

The forced swim test, also known as the behavioral despair test, is used to test for depression-like behavior in both mice and rats. The test includes placing a rat or mouse inside a cylinder filled with water. The mobility of the animal is measured. Traditionally, 'floating behavior' (the animal remains almost immobile and with its head above water). The forced swim test is a mice Motor-function test utilized for assessment of stimulant medications, upper adequacy of new mixes, and trial controls that are gone for rendering or anticipating depressive-like states

Phase-II

During Phase-II, PD was induced by administering Haloperidol of 1mg / kg per day (ip) for 7 days. All animals were observed for 30 minutes post injection and then hourly intervals for next 3 hours. At the end of the 7th day, PD was assessed by hind limb movements and behavior (Manikandaselvi et al., 2012). After induction of disease, subjects were divided into four groups. Group A (Normal) containing normal mice with (i.p) injection of saline served as control. Group B served as diseased group received (i.p) injection of haloperidol (1 mg/kg per day) for seven days. Group C and D were administered Pyridine-3-carboxylic acid orally, 7.15 mg and 15 mg per kg of body weight orally for 36 days respectively.

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Phase-III

During Phase-III, following four tests were performed on daily basis to check the difference in behavior before induction of PD and after treatment. These tests include wire hanging test, grip strength test, Vertical rod test, and swim test (Zaidi et al., 2016a).

Statistical Analysis

Data was statistically analyzed on SPSS version 22.0 using ANOVA with a $p < 0.05$ considered as significant.

Results

The present study was conducted to assess the neuro-protective and motor-function modulation activity of Pyridine-3-carboxylic acid in HP induced PD animal model. Mice were acclimatized and their Motor-function modeling was done. Following four tests were applied to mice. These are swim test, vertical pole, grip strength, and wire hanging test. During these tests, physical and social activities of the mice were monitored.

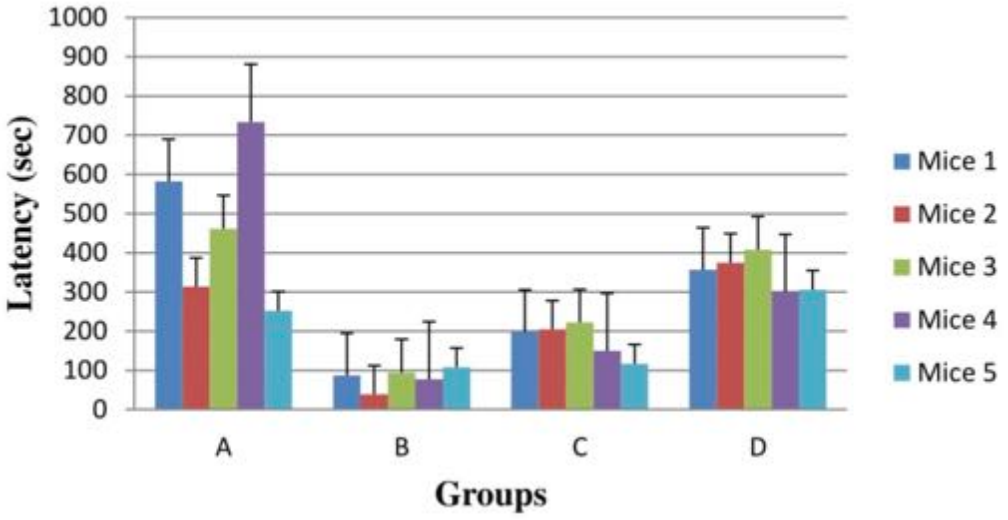

Figure 1. Wire hanging test. It was observed during experimentation that Group D showed more neuromuscular strength than Group C as shown in However, results of group D were not statistically much significant (P=0.082) in comparison with Group C.

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Wire Hanging Test

During motor-function modulation, an important parameter for treatment effectiveness is neuromuscular strength. Wire hanging test was performed to assess the neuromuscular ability of mice. While performing this test, animal were suspended by its fore paws with a 2 mm wire 30 cm above the ground. By performing this test, the measure of motor coordination and animal's ability to take on its hind limbs and tail with a specific end goal to grasp wire was observed. Latency to fall was measured from the time a mouse hanged by its forepaws till it falls. The test was performed five times for each mouse and a mean value was considered and analyzed by one way ANOVA. It was observed during experimentation that Group D showed more neuromuscular strength than Group C as shown in Fig. 1. However, results of group D were not statistically much significant (P=0.082) in comparison with Group C.

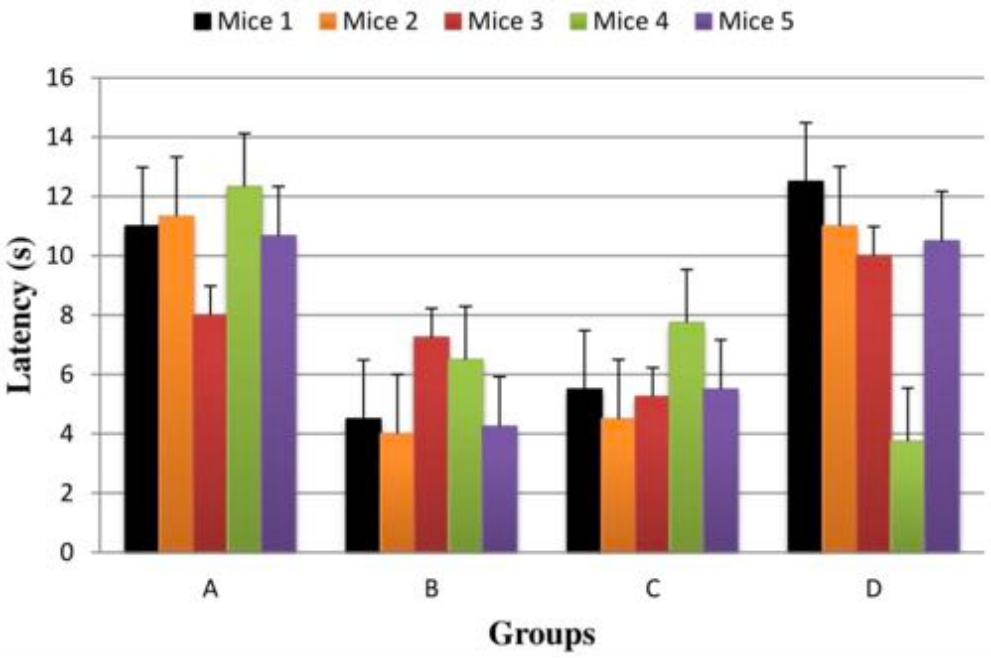

Figure 2. Grip strength test. It showed that Group D mice had strong grip as compared to Group C and Group B mice as shown in Results were found statistically significant for Group D in comparison with Group C (P=0.047). It was also observed during study that Group B had shown least strength of grip than Group A (P=0.005).

Grip Strength Test

The modified screen is a 43 cm square of wire cross section comprising of 12 mm squares of 1 mm measurement wire. It is encompassed by a 4 cm profound

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wooden beading which keeps the infrequent mouse which endeavors to from hopping on to the next side. It is a test of muscle quality utilizing each of the four appendages. Most ordinary mice effortlessly score greatest on this undertaking; it is a brisk yet heartless gross screen. A common error with commercial strength meters is that the bar or other grip feature is not thin enough for mice to exert a maximum grip. As a general rule, the thinner the wire or bar, the better a mouse can grip with its small claws.

Another key feature for treatment effectiveness is neuromuscular strength. This is an immaculate test of quality, despite the fact that concerning any test motivational variables could conceivably assume a part. Grip strength test showed that Group D mice had strong grip as compared to Group C and Group B mice as shown in **Fig. 2**. Results were found statistically significant for Group D in comparison with Group C (P=0.047). It was also observed during study that Group B had shown least strength of grip than Group A (P=0.005) as demonstrated in **Fig. 2**.

Vertical Pole Test

This test is used to measure the sensorimotor function of mice. PD motor tests provide a good read-out of neurological function. In case of Group D, it was seen that this group was statistically insignificant than Group B (P=0.409) while it was also noted that this group showed better neurological function as compared to Group C. However, results were also found insignificant for Group C as compared to Group B (P=1.00).

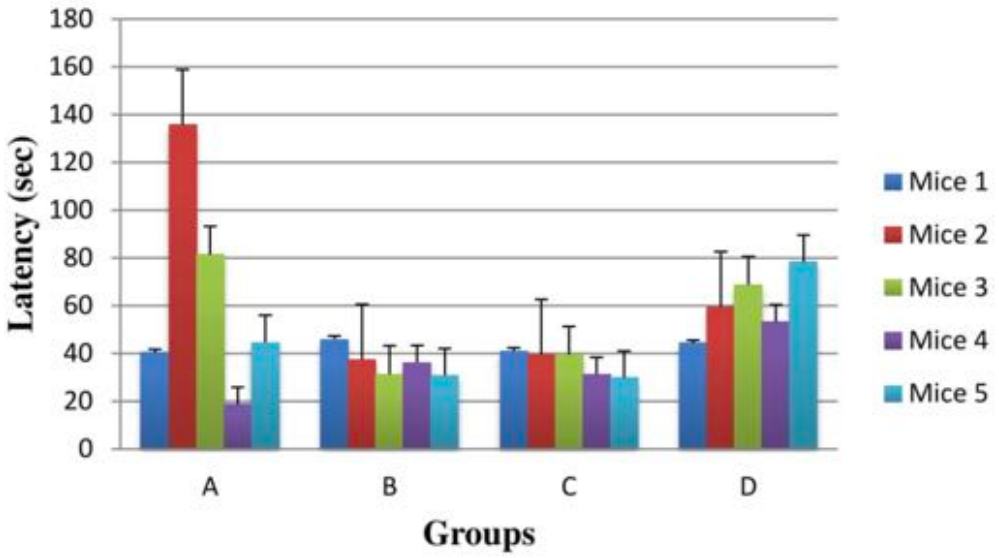

Figure 3. Vertical pole test. In case of Group D, it was seen that this group was statistically insignificant than Group B (P=0.409) while it was also noted that this group showed better neurological function as compared to Group C.

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Swim Test

The forced swim test is a rat Motor-function test utilized for assessment of stimulant medications, upper adequacy of new mixes, and trial controls that are gone for rendering or anticipating depressive-like states.

In case of Swim Test, Group D is statistically insignificant in association with Group C (P=0.284) and Group B (P=0.221). Therefore, it was observed that results were not statistically significant among all four groups in case of swim test as demonstrated in Fig. 4. Consequently in our study, it was observed that Pyridine-3-carboxylic acid 15 mg/kg/day PO dose is found effective as compared to Pyridine-3-carboxylic acid 7.15 mg/kg/day PO dose.

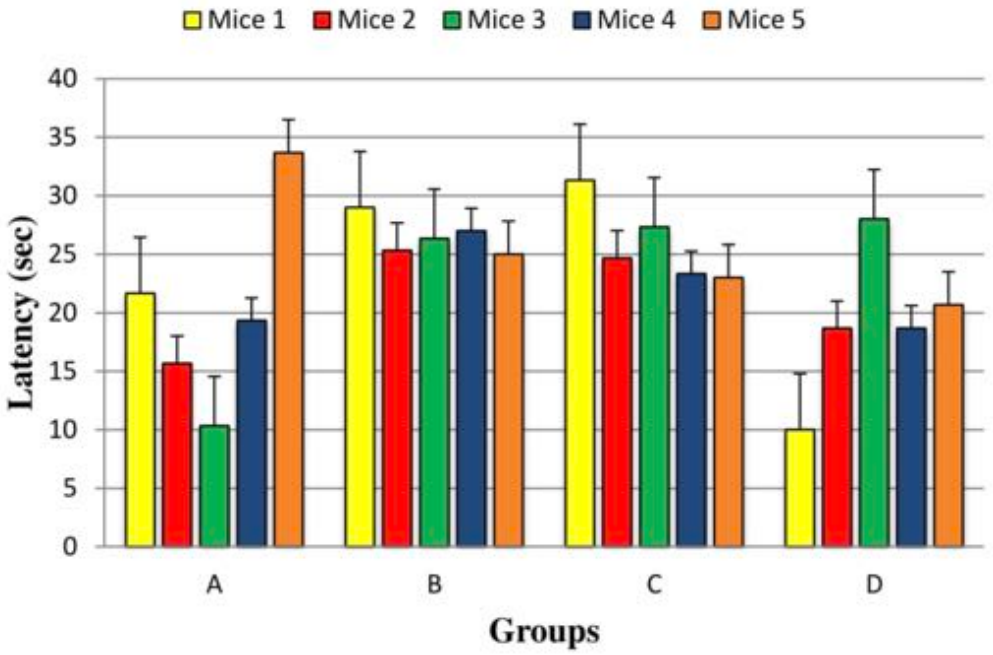

Figure 4. Swim test. In case of Swim Test, Group D is statistically insignificant in association with Group C (P=0.284) and Group B (P=0.221). Therefore, it was observed that results were not statistically significant among all four groups.

Discussion

The principle objective of exploration study displayed in this postulation is to describe the impacts of HP in the improvement of PD models and Motor-function undertakings some time recently, amid, and after treatment with Pyridine-3-carboxylic acid. In the present study, HP treatment altogether expanded vacuous biting development and tardive dyskinesia when contrasted with control mice. Neuroleptics act by blocking dopamine receptors (Creese et

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al., 1976). Such blockage results in expanded dopamine turnover, which thus prompts expanded generation of hydrogen peroxide, bringing about oxidative anxiety (Chauhan et al., 2004; Elkashef and Wyatt, 1999). Existing proof shows that inordinate creation of free radicals is connected with interminable neuroleptic utilize and may add to the onset of tardive dyskinesia and other development issue, for example, dystonias and Parkinsonism (Burger et al., 2005).

During motor-function modulation, an important parameter for treatment effectiveness is neuromuscular strength. Wire hanging test was performed to assess the neuromuscular ability of mice. It was observed during experimentation that Group D showed more neuromuscular strength than Group C as shown in **Fig. 1**. However, results of group D were not statistically much significant (P=0.082) in comparison with Group C. In one of the study where Effect of hypericum hookeranium on HP induced neuromuscular weakness was tested by wire hang test. Less latency to fall by releasing the wire soon indicates the apathetic state in the induced animal. The latency in falling represent the improved neuromuscular strength in 400 mg/kg EEHH treated animals with the same effect as that of the standard drug scopolamine. Before treatment animals had excellent neuromuscular activity, reduced by the treatment of Haloperidol. hypericum hookeranium at the dose 400mg/kg significantly (Pongiya et al., 2014). The effect of low doses of Pyridine-3-carboxylic acid show more promising effects as compared to hypericum hookeranium 400mg/kg in this study.

Grip strength test is a test of muscle strength using all fore limbs. However, in our research study, Grip strength test showed that Group D mice had strong grip as compared to Group C and Group B mice as shown in **Fig. 2**. Results were found statistically significant for Group D in comparison with Group C (P=0.047). It was also observed during study that Group B had shown least strength of grip than Group A (P=0.005) as demonstrated in **Fig. 2**. The dose of 15mg/kg on HP induced mice model of PD show more promising effects during grip strength test as compared to 7.15 mg/kg. In one of the study, same tests were applied on mice model to check the impacts of HP on hold quality test are outlined at the lower left partition. There was a measurably huge general treatment impact and a pattern toward expanded grasp quality in the haloperidol-induced mice when subjected to motor integration tests. Grip strength test showed a decrease in muscle coordination which could be due to a loss of muscular strength. Treatment with MECD showed a significant improvement in the muscle coordination as there is an increase in retention time and fall-off time in grip strength test respectively. Locomotor activity was also studied using actophotometer in which MECD improved the photocells count which was significantly less in HP treated group (Pavan et al., 2015).

Vertical pole test is used to measure the sensorimotor function of mice. PD motor tests provide a good read-out of neurological function. The vertical pole test provides the information about the level of catalepsy generated by HP in

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mice model of PD and its effects on mice during and after the treatment on vertical pole test. In one of the study, HP created the prolongation of T-turn and TLA as a marker of bradykinesia in mice and the prolongation kept going no less than 7 hr after HP treatment. Intraperitoneal co-pretreatment with L-DOPA (400 mg/kg) + carbidopa (10 mg/kg) in mice diminished the catalepsy instigated by HP at a measurements of 0.125 mg/kg, while co-pretreatment with L-DOPA (200 and 400 mg/kg) + carbidopa (10 mg/kg) dosage conditionally diminished the HP (0.125 mg/kg) actuated bradykinesia. The impact of LDOPA + carbidopa in post-test was more purported than that in catalepsy test (Kobayashi et al., 1997). The treatment with Pyridine-3-carboxylic acid in our research study show significant results in mice model of PD. In case of Group D, it was seen that this group was statistically insignificant than Group B (P=0.409) while it was also noted that this group showed better neurological function as compared to Group C. However, results were also found insignificant for Group C as compared to Group B (P=1.000). The data show that the both doses of Pyridine-3-carboxylic acid did not put much influence in this analytical parameter.

The forced swim test were utilized for assessment of upper medications, energizer viability of new mixes, and trial controls that are gone for rendering or counteracting depressive-like states. In one of the research study, swim test was used to evaluate the effectiveness of S-acetylcysteine on HP induced mice model of PD. In case of Swim Test, Group D is statistically insignificant in association with Group C (P=0.284) and Group B (P=0.221). Therefore, it was observed that results were not statistically significant among all four groups in case of swim test as demonstrated in figure 4. Consequently, in our study, it was observed that Pyridine-3-carboxylic acid15mg/kg/day PO dose is found effective as compared to Pyridine-3-carboxylic acid7.15mg/kg/day PO dose..

Conclusion

In the present study, the activities of mice get reduced after the administration of haloperidol due to its effect on dopamine blockade and development of symptoms of PD and Motor-function changes. The findings of the present study suggested that the use of Pyridine-3-carboxylic acid may be helpful as an adjunct therapy with standard therapy of Levodopa/Carbidopa in PD patients and can also decrease the level of association of free radicals in the improvement of neuroleptic prompted PD.

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Abbreviations

EEHH: ethanolic extract of hypericum hookeranium
HP: haloperidol
I.P: intraperitoneal
L-DOPA: levo dopa
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine
NAD: nicotinamide adenine dinucleotide
PD: Parkinson's disease
PO: per oral

Author contribution

Atif Saeed, Arsalan Ali and Muhammad Yousaf performed lab work and behavioral testing. Awais Ali Zaidi give the concept, designed experiment and supervised the project. Lubna Shakir helped in data acquisition and experiment conditions optimization Mahtab Ahmad Khan analyzed data and gave final approval of the project

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Original Research

Blood lactate level in Elite boy swimmers after lactate tolerance exercise test

Asghar Nikseresht[1], Iman Yabande[2], Karamatollah Rahmanian[2,*], Abdolreza Sotoodeh Jahromi[3]

[1]Department of Sport Physiology, Islamic Azad University, Jahrom Branch, Jahrom, Iran
[2]Research Center for Social Determinants of Health, Jahrom University of Medical Sciences, Jahrom, Iran
[3]Research Center for Non-Communicable Diseases, Jahrom University of Medical Sciences, Jahrom, Iran

***For correspondence:**

rahmaniank47@yahoo.com

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Abstract

Introduction: To avoid injuries during high-intensity sports training, it is important to recognize conditions of bodily consumption and production of adequate energy; exercise increases the concentration of the blood lactate. This paper is an attempt to compare pre and post lactate tolerance exercise test - blood lactate concentrations - of elite boy swimmers. **Methods:** Blood lactates are measured by an enzymatic method on 12 subjects 30 minutes before and adjust and 24 hours after the test. **Results:** The mean lactate concentration of 30.35±12.16 mg/dl is observed in swimmers 30 minutes before the test. Swimmers adjust after the test show mean blood lactate concentration of 108.52±18.17 mg/dl that is significantly higher than 30 minutes before the test ($p<0.001$). Then blood lactate level decreases below baseline level at 24 hours after the test. **Conclusion:** Blood lactate increases with the test and decreases below baseline within 24 hours after the test.

Keywords

Lactate Tolerance Test, Swimmer, Blood lactate, Male

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Introduction

During high-intensity sports training, it is important to recognize in what conditions the body consumes and produces adequate energy to avoid injuries. Exercise increases the concentration of the blood lactate. Additionally, blood lactate is related to intensity (Rogatzki et al., 2014; Vescovi et al., 2011) and distance of exercise (Keskinen et al., 2007; Vescovi et al., 2011). Issurin et al (Issurin et al., 2001) has found that the highest level of lactate concentrations is formed during butterfly swimming, followed by other styles including breaststroke, backstroke and freestyle swimming indicated by three different tests with 22 highly trained swimmers (14 male, 8 females) as subjects. The blood lactate significantly increased with the gradually enhanced achievement (LI, 2010). Conversely, Halfslder et al reported that the mean blood lactate concentration decreased in longer distance of swimming (Holfelder et al., 2013). In another study conducted by Sawka et al (Sawka et al., 1979), swimmers in the 200-yd butterfly, back, breast and freestyle races had similar mean blood lactate concentrations (ranging from 16.4-20.6 mm/l).

Blood lactate level was significantly increased immediately after 3 exercises and returned to the basic level within the next 3 hours (Zaree and Yarahmadi, 2013). The results showed that the plasma lactate concentration increased immediately after the exercise in comparison to former results - 24 hours after exercise - at all levels of intensity (Sholi et al., 2015). Rogatzki et al reported that blood ammonium and lactate seemed to accumulate in response to an increasing number of repetitions with decreasing rest time between sets (Rogatzki et al., 2014). Bonifazi et al showed that the post-competition blood lactate concentrations were higher in the main competitions than in the preparatory competitions (Bonifazi et al., 2000). Altimari (Altimari et al., 2010) suggested that increasing the swimming distance significantly decreased the mean speed ($p<0.01$). The mean blood lactate concentration 7 min after the induction of acidosis during the lactate minimum test was 10.79±1.65 mm/l (Altimari et al., 2010). Gorostiaga et al (Gorostiaga et al., 2014) used leg press exercise to examine blood lactate and ammonia and muscle lactate. Thirteen men participated and 1 repetition maximum leg press strength 199 ± 33 kg performed either 5 sets of 10 repetitions to failure (5×10RF), or 10 sets of 5 repetitions not to failure (10×5RNF) with the same initial load (10RM) and inter-set rests (2 minutes) on 2 separate sessions in random order. The 5×10RF resulted in significant high levels of muscle lactate (25.0 ± 8.1 mmol/kg wet weight), blood lactate (10.3 ± 2.6 mmol/L), and blood ammonia (91.6 ± 40.5 μmol/L). During 10×5RNF no or minimal changes were observed (Gorostiaga et al., 2014).

Lactic acid was not a waste material after anaerobic exercise, on the other hand it was such a vital, energetic substrate for oxidization (Hashimoto and Brooks, 2008). Thus, a high glycolytic capacity might mean that a player could produce more lactate to perform high-intensity exercise and could use the lactate for

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oxidation by continuing to exercise at a high level of intensity. Muscle cells had 2 major functions including the production and clearance of lactate, and blood lactate levels changed as a result of a balance between production and clearance.

Blood lactate measurement was a classical method for many decades (Beneke et al., 2011; Devlin et al., 2014). Generally, blood lactate was increased with exercise intensity (Aguiar et al., 2015; Benelli et al., 2007) which showed a clear transition from aerobic activity to anaerobic activity (Aguiar et al., 2015). In details, blood lactate was increased slowly at the beginning and then elicited an exponential rise during graded incremental exercise. For now, lactate accumulated as it was produced much faster than its decomposition. Baron et al in a 2 hour swimming test showed that capillary lactate concentration decreased significantly between 10th to 20th minute after the test (Baron et al., 2005).

The aim of present study was to compare blood lactate thirty minutes before and just and twenty-four hours after the lactate tolerance exercise test.

Materials - Methods

Twelve male swimmers who were members of the Fars Province team with at least 5-10 years of experience, agreed to participate in this study. They were between 14 and 18 years old. Swimmers were informed about the experimental procedure and of the potential risks and benefits of the study. Swimming players signed written consent for participation. The present study was approved by the Human Ethics Committee of the Jahrom University of Medical Sciences (JUMS.REC.1393.016).

After a resting of 24 hours, a self-selected warm-up swim was done. Then swimmers performed Lactate Tolerance Exercise Test (LTET) which included an 800 m swim with higher than threshold intensity during eight times using a normal diving start at 1-min intervals. The test was performed in a 50-m indoor pool.

Blood samples were collected from the subjects 3 times in total; that is, 30 minutes before and just and 24 hours after trial set. Blood lactate levels were analyzed by ELIZA and auto-analyzer instrument.

The Kolmogorov–Smirnov test was applied for testing each variable's normality. To compare the study variables (blood lactate before and after LTET), we used Pair t test to compare blood lactate before test with adjust and with 24 hours after the test. Mean and SD values were obtained for all descriptive variables. A p value of <0.05 was considered significant.

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Results

According to Table 1, the analysis showed that there was a significant difference between blood lactate 30 minutes and just after the LTET ($p \leq 0.001$) and between adjust and 24 hours after the LTET ($p \leq 0.001$). Blood lactate concentration decreased after interruption of the test and approximately returned to below the baseline level at 24 hours after the test.

Table 1. Blood Lactate concentration before and after Lactate Tolerance Swimming Test in elite boy swimmers

	30 minutes before competition		Just after competition		24 hour after competition	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Blood lactate (mg/dl)	30.35	12.16	108.52	18.17	26.51	8.57

Table 2. Blood Lactate levels (mg/dl) in individual boy elite swimmers

Record	30 minutes before lactate tolerance exercise test (1)	Just after lactate tolerance exercise test (2)	24 hour after lactate tolerance exercise test (3)	Different between (1) and (2)
1	26.44	136.63	24.85	110.19
2	18.91	98.61	24.00	79.70
3	32.9	86.88	21.20	53.98
4	15.53	97.12	24.55	81.59
5	35.44	128.53	23.05	93.09
6	40.08	84.99	44.05	44.91
7	26.43	109.19	19.15	82.76
8	62.64	108.70	41.07	46.06
9	27.06	88.90	18.75	61.84
10	27.90	106.14	24.66	78.24
11	23.15	125.35	18.78	102.20
12	27.72	131.24	34.05	103.52

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The highest blood lactate levels were observed in swimmer number 8 and the lowest in swimmer number 4 who participated 30 minutes before lactate tolerance test (**Table 2**). Just after the test, the swimmer number 1 had the highest and number 3 showed the lowest blood lactate level. The swimmer number 8 and 11 had respectively the highest and the lowest blood lactate level at 24 hours after the test. The maximum different blood lactate level was observed for swimmer number 1 in the span of time of before and just after the test. On the contrary, the athlete number 6 had lowermost blood lactate different.

Discussion

During sports activities and physical exercise, sequences of physiological and biochemical changes happen, which reflect the stress of body under the load of training. For coaches and athletes, determining the load and duration of training has been scientifically a difficult problem for many years. When the training load and duration is larger than athletic abilities, it always causes injuries for athletes. On the contrary, too small loads fail to improve sports abilities and level effectively.

The production of lactate is believed to be augmented during exercise. This substrate diffuses from the muscle and accumulates in the blood. If the blood lactate is measured, it can serve as an indicator of activated processes during a workout. In light to moderate practices, the accumulation of lactate in the blood does not exist or is low. As the workload increases, blood lactate is also increased. However, blood PH is decreased when the level of lactate in muscles and blood goes up. Increment of blood PH may interfere with enzymatic activity of several glycolytic enzymes and actin-myosin interaction with the contractile process that may serve as a limiting function in exercises. Lactate has often used as an indicator of the intensity of exercise as well as the recovery from it.

We found that blood lactate level was significantly increased during LTET test. This result was similar to previous studies. Ikeda suggested that the twelve swims rushes with approximately 1 minute interval induced a significant increase in serum lactate (Ikeda, 2002). Mean blood lactate levels rose suddenly from an initial level of 1.34±0.35 mm before the first set to 12.28±1.55 mm after the first set. Then, lactate levels fell before rising again after the sets. Takagi et al suggested that the highest blood lactate levels were observed after the first trial set, they decreased significantly toward the second trial set, and were maintained at the same level after the third trial set (Takagi et al., 2013). Also, Kantanista et al (Kantanista et al., 2016) found that exercise increases blood lactate.

Contrary to our results, Melchorrim et al reported that mean blood lactate level was 7.7±1.0 mm, and that blood lactate levels were 7.7±1.2, 7.8±0.6, 7.5±0.9,

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and 7.2±1.6 mm during the first, second, third, and fourth quarters, respectively (Melchiorri et al., 2010). Also, Vicente et al suggested that lactate concentration remained stable during half squat exercise at the lactate threshold among 13 healthy subjects (Garnacho-Castaño et al., 2015).

In our study the concentration of lactate in the blood achieved its below resting value after 24 hours of recovery. This data was in line with the findings of Degoutte et al (Degoutte et al., 2003), who observed that lactate concentrations in blood were appeared to return to the baseline level within 24 hours. Also, in study conducted by Kantanista blood lactate decreased after the interruption of exercise (Kantanista et al., 2016). Adversely, Jafari et al suggested that the blood lactate significantly increased 24 hours after 1600 meter running among 27 non athlete boys (Jafari et al., 2016). Other research (Ament et al., 1999) indicated that lactate still elevated after 30 minutes of recovery in healthy volunteers.

The investigators suggested a positive correlation between lactate production and average speed or distance of exercise (Avlonitou, 1996). Also, Benelli et al in 52 male swimmers aged 40-79 years suggested that the blood lactate is dependent to the intensity and distance of competition (Benelli et al., 2007). They stated that peaks of lactate were observed in distance of 100 m. Also, Kantanista reported that by increasing the speed of the treadmill, blood lactate was increased (Kantanista et al., 2016).

Diet and nutritional status may stimuli strength and power adaptation (Crewther et al., 2006; Duke et al., 2011). Short-term diet modification has Influence on the blood lactate to rating of perceived exertion (Arshadi et al., 2017). The effect of sleep and sleep deprivation on cortisol and testosterone responses, anaerobic performance indices and blood lactate have been approved in active men (Sholi et al., 2015). We didn't evaluate these factors in present study. This can be seen as a limitation of our study. But blood sampling was drawn for participants 30 minutes and adjust after exertion and due to individual self-control, effect of these variables become disinterested.

Conclusion

We conducted an experiment to estimate lactate production during a lactate tolerance swimming test in swimming players. Our results showed a significant increase in blood lactate just after the test; and decreased to below of baseline 24 hours after the test.

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Abbreviations

ELIZA: Enzyme-Linked Immunosorbent Assay
L: Litter
LET: Lactate Tolerance Exercise Test
Mg/dl: milligram/deciliter
mmol: millimole
REC: RECord
RF: Repetitions to Failure
RM: Repetition Max
RNF: Repetitions Not to Failure
SD: Standard Deviation
µmol: micromole

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Author contribution

Asghar Nikseresht: Design of study, proposal of study writing, final content of study
Imman Yabande: proposal of study writing, enrolled data, manuscript writing
Karamatollah Rahmanian: Design of study, proposal of study writing, analysis, interpretation, manuscript writing, final content of study
Abdolreza Sotoode Jahromi: proposal of study writing, interpretation, final content of study

ISSN: 2198-4093
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Original Research

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Comparison of the predictive value of prooxidant-antioxidant balance and heat shock proteins in the diagnosis of neonatal asphyxia

Hassan Boskabadi[1], Gholamali Maamouri[1], Majid Ghayour-Mobarhan[2], Fatemeh Bagheri[3], Maryam Zakerihamidi[4], Maryam Kalate Mollaey[5,*], Elahe abbasi[6], Afsaneh zareh[6], Akram Tamannanlo[6]

[1]Department of Pediatrics, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran

[2]Biochemistry of Nutrition Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[3]School of Nursing and Midwifery, Mashhad Branch, Islamic Azad University, Mashhad, Iran

[4]Department of Midwifery, School of Medicine, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran

[5]Master of Science in Neonatal Intensive Care, Mashhad Medical University, Iran

[6]Bachelor of Science in Nursing, Mashhad Medical University, Iran

***For correspondence:**

kalatemm2@mums.ac.ir

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Abstract

Introduction: Asphyxia is one of the important cause of infants' mortality. Accurate and early diagnosis of asphyxia has an important role in performing appropriate protective treatment protocol; therefore, we compared the diagnostic value of two methods of Prooxidant-Antioxidant Balance (PAB) and Heat shock proteins 70 (HSP70) among healthy term infants and neonates with asphyxia. **Methods:** In this prospective case-control study, we compared the diagnostic value of two methods of PAB and HSP70 in healthy term infants (N=38) and Neonates with asphyxia (N=30) in Mashhad Ghaem hospital from 2011 to 2015. The diagnostic value of HSP70 and PAB was compared with statistical tests of Chi-square, T-Test, Man-Whitney, Roc curve and regression models. **Results:** The newborns in two groups were significantly different in terms of the first (P=0.000) and fifth minute Apgar score (P=0.000), HSP70 (P=0.000), PAB (P=0.000), PH (P=0.000), BE (P=0.000) and HCO3 (P=0.015). HSP>0.218 ng/dl has 60% sensitivity and 76% specificity for the diagnosis of asphyxia while PAB>11.3 HK has 84% sensitivity and 92% specificity for the diagnosis of asphyxia.

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Conclusion: According to the results of this study, HSP70>0.22 ng/dl and PAB>11.3 HK Unite can be used as biochemical markers for the diagnosis of perinatal asphyxia (P=0.001). The sensitivity and specificity of PAB in the diagnosis of asphyxia is higher than HSP70 and simultaneous measurement of these two markers can correctly diagnose 84% of asphyxia cases.

Keywords

Asphyxia, Heat shock proteins (HSPs), Neonate, Prooxidant-Antioxidant Balance (PAB)

Introduction

The term asphyxia, is used to describe the interrupted supply of oxygen through umbilical cord to the fetus (Boskabadi et al., 2015). It is estimated that 2 to 4 of every 1,000 term newborns suffer from birth asphyxia. Approximately, 15 to 33% of infants with asphyxia who show the symptoms of hypoxic ischemic encephalopathy (HIE) die during the neonatal period. 25% of survivors suffer from permanent neurological and psychological damages (Ceccon, 2003; Volpe, 1995). Asphyxia is defined as the signs of neonatal encephalopathy (hypotonia, reduced reflexes, the pupils' status, and seizure), first minute Apgar score of less than 4 and the fifth minute Apgar score of less than 7 and or umbilical pH of less than 7. Neonatal asphyxia is a serious and prevalent problem in prenatal cares (Ceccon, 2003; Kliegman, 2011; Menkes H., 2006; Volpe, 1995).

Asphyxia affects many major organs of the infant, but one of the irreversible and serious effects is its impact on the central nervous system. It leads to hypoxic ischemic encephalopathy, cerebral palsy, seizure and loss of learning (Ashrafganjuee; Waldemar, 2011). In a study in Iran, asphyxia was reported the reason of 31% of infant mortality that means asphyxia is the second cause of neonatal death after severe prematurity (Boskabadi H., 2010).

Although recognition of causing mechanisms and complications of asphyxia developed lately but the early diagnosis of brain damage following hypoxic-ischemic events is one of the most difficult problems in neonatal care yet (Marlow, 2012; Perlman, 1999; Volpe, 1995). There is no specific reliable marker which is correlated with the extent of the damage of intrauterine hypoxia, hereupon diagnosis is usually based on non-specific clinical criteria. In the examination of neonates with asphyxia, usually no specific finding is obtained and it is suggested in differential diagnosis of other diseases such as sepsis, metabolic disorders, congenital metabolic defects, and so on (Kliegman, 2011; Low, 1997; Siciarz et al., 2001). Parameters that are currently used for predicting

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or determining of perinatal asphyxia are as following: Apgar score, excessive umbilical arterial acidemia, and fetal electrical monitoring in the scalp with the presence of meconium. These findings are mostly non-specific and may have abnormal results when there is no major brain damage (Aly et al., 2009; Ashrafganjuee, 2004; Boskabadi H., 2010; Ghosh et al., 2003; Kliegman, 2011; Low, 1997; Marlow, 2012; Perlman, 1999; Siciarz et al., 2001; Waldemar, 2011).

Some researchers have applied biochemical (increased lactate, LDH: Lactate dehydrogenase, creatine kinase in the plasma, protein S100B, the retinol binding protein) and hematologic markers (NRBC count in umbilical vein blood) for the early detection of this damage (Banupriya et al., 2008; Basu et al., 2008; Boskabadi et al., 2010; Chen et al., 2000; Ghosh et al., 2003).

Definite diagnosis, leading to differentiate asphyxia from more treatable problems and improve prognosis of clinical and neurological status of the newborn. In addition to the above, cerebral hypothermia and antioxidant treatments are suggesting to limit the nerve complications caused by ischemic-hypoxic damage (Cheng et al., 1997; Gunn et al., 2005; Kliegman, 2011). These treatments have most effectiveness when used in early stages.

Heat shock proteins (HSP) are present in all organisms and cell types. In stable condition, HSP serum levels are very low. In response to stress such as high temperature, free radicals, fast tension and toxins, cells release HSP family (24). Some researches revealed that HSP70 is an important factor in the correct and timely diagnosis of ischemic-hypoxic events (Child et al., 2006; Fiedorowicz et al., 2008; Jiang et al., 2004; Ozer et al., 2002).

Lack of reliable and accurate method in determining the balance between oxidants and antioxidants of the patients is a major limitation. Recently, with simple and inexpensive method 3, 3', 5, 5'-tetramethylbenzidine (TMB), this problem is partially solved (Alamdari et al., 2007; Boskabadi et al., 2014). Regarding to this fact that was not find any study which compared diagnostic value of the two methods of Prooxidant-Antioxidant Balance (PAB) and Heat shock proteins (HSPs) in term newborns with and without perinatal asphyxia and due to the complications caused by asphyxia and early diagnosis importance, the researchers decided to perform this study to achieve a reliable and accurate method in proper diagnosis of asphyxia, and evaluate the diagnostic value of two new biochemical criteria too.

Materials - Methods

This prospective study was performed from December 2010 to April 2015 in Ghaem hospital, Mashhad, Iran. Among 80 evaluated neonates, 68 eligible infants enrolled to the study, and HSP70 and PAB were measured for them. This

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study was approved by Mashhad University of Medical Sciences Ethics Committee (900514 and 900660).

In an analytical-observational study, the researchers evaluated HSP70 and PAB in term infants with perinatal asphyxia and compared it with healthy term infants. The infants in case group had at least two of the following symptoms:

1. Signs of fetal distress (FHR <100, no heart rate variability, Late deceleration).
2. Thick meconium in addition to hypotonia or bradycardia or respiratory depression.
3. Apgar score less than 4 in first minute and less than 7 in fifth minutes.
4. The need to CPR more than one minute with IPPV and oxygen.
5. PH <7.2 or BE <-12 during the first 6 hours after birth in newborn.

The control group included term newborns who had normal pregnancy and vaginal delivery and had stable clinical status during the first week of birth. Exclusion criteria in the case group included the infants with congenital abnormality and infection, sepsis, hypothermia, hypoglycemia, congenital heart disease and primary neurologic disorder. In control group neonate hospitalization during the first week and maternal problems during pregnancy or delivery were exclusion criteria.

The required data were collected through a researcher-made questionnaire covering information related to mothers (age, parity and method of delivery) and babies (gestational age, sex, birth weight, length, head circumference, length of stay, Apgar score).

The infants who were recruited to the study during the first day were completely examined by a neonatologist. In the third and seventh days, examination was repeated and the checklist was filled. The criteria to determine the severity of asphyxia was based on Sarnat clinical staging. In the examination, the infant's neurological function in the first, third and seventh days was evaluated by the examiner. This evaluation was as follows: Consciousness status, function of cranial nerve and movement and sensory systems. In the movement system examination, muscular tone and spontaneous movements of the infants were evaluated. Posture and muscles' strength vs. passive movements were examined to evaluate active tone.

In the infants of case group, the severity of HIE was determined based on Sarnat clinical staging. Mild HIE or HIE grade 1 defined as excessive vigilance, irritability and hyper reflex and no seizures for at least 24 hours after birth. The case of being lethargic, hypotonia and decreased reflexes, miotic pupils and seizures was considered as moderate HIE or HIE grade 2 and the case of apnea,

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stuporous, flaccid without primitive reflexes, severe convulsions or coma was considered as sever HIE or HIE grade 3.

Asphyxia complications such as breathing, cerebral, cardiac, gastrointestinal and kidney problems were monitored and recorded during the study. Evaluation of the patients was performed based on clinical examination, and laboratory required evaluation, and if there were medical indications, it was performed by imaging methods such as CX Ray, abdominal sonography and Brain CT Scan.

In both of the control and experimental group after obtaining the written consent of the parents 2 ML of umbilical cord blood was collected and delivered in sterile tubes. After being allowed to clot, the tubes were centrifuged at 1000 rpm at room temperature to obtain serum. Hemolytic samples were excluded from analysis. Serum was stored at -70°C prior to analysis.Quantitative analysis (HSP70) and measurement of oxidant-antioxidant balance was performed in Department of Biochemistry of Bouali Research Institute.

Serum Hsp 70 antigen concentrations were determined using a sandwich ELISA (The enzyme-linked immunosorbent assay) in-house. After overnight incubation 100 µL monoclonal Hsp70 antibody at 4°C, the plate was washed and non-specific binding sites blocked by incubation with 0.1% BSA. Plates were washed and 100 µL of standards, 5000 ng/mL of recombinant Hsp70, and undiluted serum were incubated for 2 hours at 37°C. After adding 100 µL of rabbit polyclonal anti-Hsp70, 100 µL of an anti-rabbit immunoglobulin peroxidase conjugate was added to the plate for 1 hour at 37°C. After adding 100 µl of TMB substrate, the reaction was stopped after 20 minutes with 2 M HCl and the absorbance read at 450 nm. The sensitivity of the assay was 39 ng/mL, and the inter- and intra-assay coefficient of variation was 9% and 6% respectively.

Finally, a suitable substrate such as tetramethyl benzidine creates blue color that changes to yellow color by the two normal chloridric acid which has absorption at wavelength of 450 nm. The different concentrations of the antigen is a result of the rate of absorption.

TMB determined Oxidant-antioxidant balance in two different reactions: in enzyme reaction chromogen is oxidized by peroxides (in this test, H2O2) to TMB containing cation. In latter reaction TMB containing cation is resuscitated by antioxidants (in this test, uric acid). During six stages with certain concentrations, a standard curve is obtained. This curve determines concentration of serum samples with detectable absorptions at 450 nm wavelength and calculate the oxidant-antioxidant balance. The standard solutions were prepared by mixing varying proportions (0–100 %) of 250 µM hydrogen peroxide with 3 mM uric acid (in 10 mM NaOH). For the preparation of the TMB cation, 60 mg TMB powder was dissolved in 10 mL DMSO; then 400 µL of TMB/DMSO was added in 20 mL of acetate buffer (0.05 M buffer, pH 4.5), and then 70 µL of fresh chloramine T (100 mM) solution in distilled water was added into this 20 mL, mixed well, incubated for 2 h at room temperature in a dark place; 25 units of peroxidase

ISSN: 2198-4093
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enzyme solution was added into 20 mL TMB cation, dispensed in 1 mL and stored at −20 °C. In order to prepare the TMB solution, 200 µL of TMB/DMSO was added into 10 mL of acetate buffer (0.05 M buffer, pH 5.8); the working solution was prepared by mixing 1 mL TMB cation with 10 mL of TMB solution, incubated for 2 min at room temperature in a dark place and immediately used. Ten microliters of each sample, standard or blank (distilled water) were mixed with 200 µL of working solution, in each well of a 96 well plate, which was then incubated in a dark place at 37 °C for 12 min; at the end of the incubation time, 100 µL of 2 N HCl was added to each well; and measured in an ELISA reader at 450 nm with a reference wavelength of 620 nm. A standard curve was constructed from the values derived using standard samples. The values of the PAB were expressed in arbitrary HK units, which represent the percentage of hydrogen peroxide in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the standard curve.

Data analysis and statistical analysis

All statistical analyses were performed with Statistical Package for the Social Sciences 15 (SPSS Science, Apache Software Foundation, and Chicago, IL, USA). Values were expressed as mean ± SD. Student t test, Kruskal-Wallis test and Mann–Whitney test were used as appropriate. Parametric and non-parametric correlations were assessed using Pearson correlation coefficients and Spearman correlation coefficients, respectively. $P<0.05$ was considered significant. Roc curve to compare the diagnostic value of HSP70 and PAB in the diagnosis of asphyxia it was plotted to calculate sensitivity and specificity of the test and comparing with conventional methods, after that regression methods to process the appropriate model of comparison of HSP70 and PAB in neonatal asphyxia.

Results

Thirty neonates in the case group and 38 neonates in the control group were studied. Forty percent of cases were female and 60 % were male. 42% of controls were female and 58% were male (P=0.861).

Sex, height, gestational age and maternal age in two groups are homogeneous ($P>0.05$, **Table 1**). First and fifth minute Apgar score, HSP70, PAB, PH, BE and HCO3 in two groups had significant statistical difference ($P<0.05$, **Table 1**).

In case group, 9 infants had seizures, 4 cases died and 26 have been survived. In terms of grading HIE in case group, 21 infants catch Ischemic-Hypoxic Encephalopathy grade 1, 8 cases of grade 2 and 1 case of grade 3 HIE.

Mean amount of PAB in normal infant, hypoxic-ischemic grade 1 and grade 2 include 7.03 HK, 19.4 HK and 21.6 HK, respectively (P=0.000). Mean of HSP70 in normal infant was 0.25 ng/dl, and in hypoxic-ischemic grade 1 was 0.38 ng/dl,

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and in grade 2 was 0.45 ng/dl (P = 0.005). Significant correlation was between HSP70 and PAB at the 0.01 level (Pearson correlation=0.392) .

Table 1. Clinical and demographic characteristics of mothers and newborns in case and control groups

Variables	Mean ± SD		p-value
	Control	Case	
Neonatal weight (g)	3300.52±341.72	3113.33±402.36	0.042
Neonatal hight (cm)	50.02±1.72	49.66±1.62	0.347
Maternal age	28.39±5.88	28.06±5.48	0.815
First minute Apgar	8.92±0.27	4.26±1.11	0.000
Fifth minute Apgar	9.00±0.00	6.26±1.28	0.000
White blood cells	15600 ±8536	21615 ±3237	0.005
Primary PH	7.34± 0.06	7.17±0.050	0.000
BE	−3.71±3.41	11.7±3.6	0.000
HCO3	18.85±1.57	16±2.1	0.015
ag 70	0.25±0.11	0.39±0.21	0.000
PAB	7.03±5.62	19.68±7.72	0.000

In case group, 90% of infants had PAB>11.01 that this rate was 13.2% in the control group, and two groups showed a significant difference in terms of PAB levels (P=0.000).

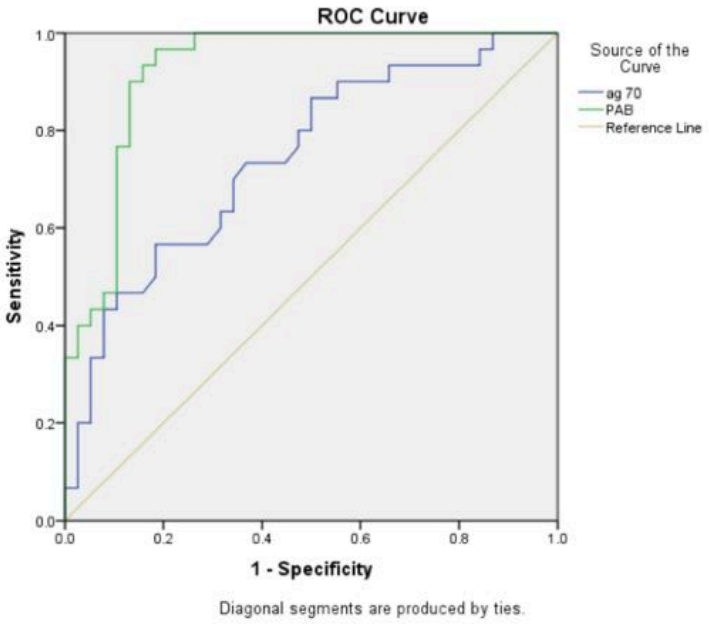

Figure 1. The sensitivity and specificity of PAB and ag70 for the diagnosis of asphyxia in case and control groups.

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Fig.1 illustrates the sensitivity and specificity of the two criteria of PAB and ag70 for the diagnosis of asphyxia. The amount of HSP>0.218 has 60% sensitivity and 76% specificity for the diagnosis of asphyxia, while PAB>11.3 has 84% sensitivity and 92% specificity for the diagnosis of asphyxia. The values of PAB and ag70 have significant statistical difference between case and control groups (P=0.001). It seems that specificity and sensitivity of PAB is more than ag70 for the asphyxia diagnosis (Fig. 1).

Table 2 exhibits predictive values of HSP70 and PAB alone or together. As seen in the table, considering of HSP70 and PAB can distinct 83.8% cases of asphyxia correctly.

Table 2. Predictive values of HSP70 and PAB alone or together

Diagnostic methods	-2 log likehood	Cox & Snell R Square	Nagelkerke R Square	Predicted Percentage Correct	Hosmer & Lemeshow Test (sig)
PAB +HSP	45.52	0.505	0.676	83.8	0.891
PAB	51.655	0.458	0.614	82.4	0. 651
HSP	80.203	0.175	0.235	67.6	0.853

Discussion

According to the results of this study, although measurements of both laboratory markers of ag70 and PAB is effective in the diagnosis of neonatal asphyxia, but the value of PAB has more specificity and sensitivity than the value of HSP70, and measurement of both of these markers in the same time can detect 84% cases of asphyxia correctly.

In the current study, the first and fifth minute Apgar had significant statistically difference in the two groups. In the Bijari study, the neonatal parameters such as fifth minute Apgar score, PH and bicarbonate were less in the infants with asphyxia than healthy infants (Bahman Bijari et al., 2010). In the Hogan study , all formal criteria are fulfilled in 10% of asphyxia, and they have observed Apgar 4-6 in prediction of half of the asphyxia (Hogan et al., 2007). Conventional Apgar score cannot predict the status of the infant in asphyxia. It neglects prematurity characteristics and the course of resuscitation measures. Indeed fifth Apgar scores and even 10 minute of undergoing resuscitation or ventilated neonates have no value for evaluating asphyxia and predicting other problems associated with it. Regarding above Rüdiger and his colleagues in one of their studies reported that the value of Apgar is limited for the infants who are premature and

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survived (Rudiger, 2008). Low Apgar score alone is inappropriate to assess asphyxia" Lopriore et al"said (Lopriore et al., 2004).

According to the results of this study control and patient groups have significant difference in terms of the amount of HSP70 antigen (P=0.001). The HSP70 antigen rate is much higher in the patient group. Against environmental stress, cells function such as the synthesis of DNA, RNA and protein is decreased or completely impaired. In such circumstances, certain proteins usually appear in the cells which are called protein stress. HSP70 is one of stress proteins that play an important role in protecting cells against stress and injuries. These proteins identify damaged molecules and divide them into two categories of repairable and unrepairable. After that it bind to damaged and repairable proteins hold them steady until the cell is repaired and obtain the energy to construction. HSP70 inhibits apoptosis and cell death in a complex mechanism. Asphyxia is a type of environmental stress that in different animal studies its relation with HSP70 has been examined. Dr. Ozer express after the hypoxic stress, HSP70 increases more in 12 days mice (equivalent to term infant) than 7 days mice (human fetus with 32 to 34 weeks gestational age) (P=0.003). Above results frame an important role for HSP70 in response to hypoxic stress in older newborns (Ozer et al., 2002).

Chen study was about the regulation effects of memantine on the synthesis and expression of HSP70 gene following hypoxia and ischemia in rats' infants. He exposed to discussion that the synthesis and expression of HSP70 gene is increased in hypoxia and ischemia and can be a sensitive marker in this relation (Chen et al., 2003). Also, levels of HSP70 and then HSP27 are increased following hypoxia and ischemia in the cortex and hippocampus of mice significantly (P<0.05) (Jiang et al., 2004). In the Cheng study on 24 pigs at third day of birth, it was found that after hypoxic-ischemic encephalopathy, expression of HSP70 after the third hours began to increase and reaches its maximum at 6th hour (P<0.01) (Cheng et al., 2005). Despite these animal studies, the researcher only found just a human study on the role of HSP 70 in the neonatal asphyxia. In a study in 2015 that has compared the amount of HSP70 in 51 neonates with asphyxia and 50 healthy infants showed that mean level of HSP 70 in neonates with asphyxia was 0.36 ng/mL and in healthy infants was 0.24 ng/mL (P=0.001) (Boskabadi et al., 2015).

The results of current study show that the HSP> 0.218 ng/dl has 60% sensitivity and 76% specificity for the diagnosis of asphyxia. In another study, this antigen had 58% sensitivity and 73% specificity for the diagnosis of asphyxia, which means that if it was positive, it is highly suggestive of the diagnosis of asphyxia, but if negative, the diagnosis of asphyxia cannot be denied base on it (Boskabadi et al., 2015). Therefore, it seems that despite helping to diagnose asphyxia, it cannot be used for definite diagnosis alone, and it is better to use it along with other criteria.

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We have found that PAB values were significantly higher in neonates with perinatal asphyxia. According to the results of our study, the PAB in neonates with asphyxia was about three times more than normal infants (P=0.000). In a study conducted by Ashok Kumar and colleagues, the level of oxidative stress in perinatal asphyxia has been studied. Plasma malondialdehyde levels and malondialdehyde of cerebrospinal fluid and ratio of plasma / cerebrospinal fluid of malondialdehyde were significantly higher in the infants with asphyxia in comparison with the infants in control group (62.5 vs. 88.2 mmol/L in plasma). Excessive production of free radicals of oxygen and lipid peroxidation is actors who play important roles in perinatal asphyxia (Kumar et al., 2008). In another study, the value of PAB was significantly higher in the infants with asphyxia than healthy infants that is consistent with the present study (Boskabadi et al., 2014). There is a critical balance in cells between the formation of free radicals and antioxidant defense and restorative systems; it means that in physiological conditions, there is a balance between antioxidants and peroxidants. In normal condition free radicals are neutralized by antioxidant system. Hypoxic conditions like as birth asphyxia increase the production of free radicals in the blood and cells, thus balance of peroxide-antioxidant is impaired (Boskabadi et al., 2014). Stressful conditions such as hypoxia stimulates the production of free radicals by reduction in oxidation and phosphorylation pairing in the mitochondria it leads to increased leakage of electrons and excessive production of superoxide radicals. When the production of free radicals was more than the capacity of antioxidant system for the neutralization, lipid peroxidation damage to the unsaturated lipids in the cell membrane, amino acids in proteins and nucleotides in the DNA.

As a result, the integrity of cell and membrane is impaired. This status will be more severe by reduced the efficiency of the immune system and unfavorable changes in the cardiovascular system, brain and nervous system and muscular system through increased lipid oxidation; therefore, many of the symptoms and complications of asphyxia may occur following the imbalances of the antioxidant-oxidant balance (Boskabadi et al., 2014; Surai, 2007).

According to the results of this study, PAB>11.3 HK Unite had sensitivity of 84% and specificity of 92% for the diagnosis of asphyxia that revealed it as a suitable factor for the diagnosis of asphyxia and has better sensitivity and a higher specificity compared to HSP70.

Finally, based on the results of current study, levels of HSP70>0.22 ng/dl and PAB >11.3 HK Unite can be suitable biochemical markers for the diagnosis of perinatal asphyxia (P=0.001). Although PAB has a greater value than HSP70 in the diagnosis of perinatal asphyxia and has higher sensitivity and specificity, but simultaneous use of these two factors at the same time can diagnose more than 84% of asphyxia cases properly.

Low cooperative parents and small sample subgroup of HIE was the major restriction in the current study. Further studies are needed to confirm the

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emerging data and value of PAB and HSP assay for identification of asphyxiated infants.

Conclusion

The PAB and HSP70 methods are rapid tests that may be useful for risk prediction in perinatal asphyxia when used with other forms of assessments e.g to consider first minute and fifth minute Apgar scores in judgment can distinguish among healthy and asphyxiated infants. The authors admit that in this study PAB shows high sensitivity and specificity in comparison HSP70.In addition above gasometrical parameters such as HCO3, BE and Primary PH were statistically significant so they can help in the diagnosis. However, further clinical research is required on larger populations, as well as on various physiological and pathological correlates of oxidative stress and parameters of asphyxia.

Abbreviations

APX: asphyxia

CPR: Cardiopulmonary resuscitation

CT: Computed tomography

ELISA: The enzyme-linked immunosorbent assay

FHR: fetal heart rate

HCl: hydrogen chloride

HIE: hypoxic ischemic encephalopathy

HSPs: heat shock proteins

IPPV: intermittent positive pressure ventilation

LDH: Lactate dehydrogenase

mL, ml, or mℓ: Milliliter

PAB: prooxidant-antioxidant balance

pH: potential of hydrogen

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Biomedical
Research & Therapy

ISSN: 2198-4093
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Author contribution

Hassan Boskabadi conducted the research, Gholamali Maamouri performed data analysis, Maryam Kalate Mollaey drafted the manuscript, Majid Ghayour-Mobarhan reviewed the manuscript, Maryam Zakerihamidi supervised the study, and Fatemeh Bagheri, Elahe abbasi, Afsaneh zareh, Akram Tamannanlo sampling and collecting and recording data in statistical software.

ISSN: 2198-4093
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Method

Quantification of angiogenic characteristics of Naproxen sodium through chorioallantoic membrane assay

Hafiz Muhammad Shoaib Zahid[1], Tanveer Ahmed Khan[1*], Muhammad Ijaz-ul-Haq[1], Humayun Riaz[2], Syed Atif Raza[3], Zia Mohy-ud-din Khan[2]

[1]Faculty of Pharmacy, Hajvery University, Lahore-Pakistan
[2]Rashid Latif College of Pharmacy, Lahore-Pakistan
[3]University College of Pharmacy, University of the Punjab, Lahore-Pakistan

***For correspondence:**

tanveerahmedkhan754@gmail.com

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Abstract

Introduction: The aim of the current study was to determine the angiogenic/anti-angiogenic effect of Naproxen Sodium and to determine the effective dose of NapS for angiogenesis. **Method**: Fertilized eggs (5days old) of chicken were acquired from a local hatchery. They were incubated at 37$_o$C with humidity of 55-60%. Then, on the 5th day of incubation, a window about 2cm in diameter was created by removing the shell and then the inner shell membrane. It was done under aseptic environment. There were 5 groups designed. Group A was kept as control and was given 0.2ml PBS, group B, C, D, and E were given 0.1ml of 0.086g/100µl, 0.042g/100µl, 0.021g/100µl and 0.011g/100µl of Naproxen Sodium (NapS) respectively. On the sixth day, prepared concentrations of NapS were administered and eggs were again sealed with paraffin film under aseptic conditions. These were placed back in the incubator for next 24 hours. After 24 hours, eggs were taken out from the incubator and images of all groups were made using a DSL camera. The development of blood vessels and other features were observed using Adobe Photoshop version 7.0, then these images were transferred to scan probing image processing (SPIP) software 6.6.2. The diameters of different blood vessels were measured by using expert design software. The parameters include: the diameter and branching system of blood vessels measured as per mm, and categories of blood vessels; i.e., primary, secondary and tertiary blood vessels. Data were analyzed on SPSS statistical software version 22.0 using ANOVA. Dunnett's test was applied considering $P < 0.05$ as significant. **Result**: Application of Naproxen sodium on chorioallantoic membrane of day six of incubation showed angiogenic effects in high concentration and anti-angiogenic effect in low concentration.

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Outcomes indicated significant changes in CAM's design, thinning of primary, secondary and tertiary blood vessels, reduction in surface roughness parameters, and decrease in Abbott curve. **Conclusion**: The substantial quantities of Naproxen sodium use locally may exhibit anti-angiogenic activity in the same manner those seen in vitro and explain its clinical efficacy.

Keywords

Angiogenesis, Chorioallantoic membrane, In vivo, Naproxen sodium

Introduction

The development of new blood vessels from pre-existing capillaries is termed as angiogenesis (Griffioen and Molema, 2000). This term angiogenesis (AG) was used in 1935 to define the development of new blood vessels in the placenta (Hertig, 1935). All cells and tissues survival are solely dependent on AG. Therefore, AG is extremely meaningful to different physiological functions like wound healing, menstruation, development of embryo and normal tissue growth (Griffioen and Molema, 2000). Physiologic AG involves many different pro- and anti-angiogenic stimuli including various environmental and growth factors (Griffioen and Molema, 2000). The AG switch is also observed as a distinct stage of cancer development, that occurs at any phase of tumor development and its micro-environment (Bergers and Benjamin, 2003). The use of anti-AG drugs for the management of AG established conditions have presented a lot of therapeutic potential. Numerous anti-AG medications are presently under development, involved in clinical trials or are being used in the treatment of AG complaints, mostly cancer (Abdollahi and Folkman, 2010). CAM model is used in the experiments in this study in order to test anti-angiogenic agents alone and in combination (Tufan and Satiroglu-Tufan, 2005). This model delivers a usual, *in vivo* setting of angiogenic blood vessels with all the multifaceted swarm communications on which angiogenic combinations can be verified (Tufan and Satiroglu-Tufan, 2005).

Naproxen sodium is a non-steroidal anti-inflammatory drug (NSAID). It functions by decreasing hormones that provoke agony and soreness in the body. The aim of current research was to determine the angiogenic/anti-angiogenic effect of NapS and to determine the effective dose of NapS for angiogenesis.

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Materials - Methods

Materials and Chemicals

This study is based on the investigation of naproxen sodium. Naproxen sodium is purchased from Sigma-Aldrich (MO, Louis St, CA). Visualization of blood vessels are achieved through DSLR Camera. 0.9% NaCl solution, which is purchased from Searl Pakistan (Pvt.) Limited, is used as a solvent to make dilutions for drug or alone as a control. Injections into the CAM's vasculature are achieved using a Microliter™ syringes fortified with 33-G metal (N) needles.

Fertilized eggs (5 days old) of chicken were purchased from a local hatchery. They were incubated at 37°C with humidity of 55-60%. On the day five of incubation, an opening about 2cm in diameter was produced by eliminating the shell and then the innermost shell membrane. It was done under aseptic conditions. Approximately 4-5ml of albumin was detached and opening was airtight with paraffin film tape and was positioned back in the incubator.

There were 5 groups designed. Group A was kept as control and was given 0.2ml phosphate buffer solution (PBS), group B, C, D, and E were given 0.1ml of 0.086g/100µl (220 mg which is least effective dose in humans), 0.042 g/100µl, 0.021 g/100µl and 0.011 g/100µl of Naproxen Sodium (NapS), respectively. At that point, pH of every one of these arrangements was checked with a pH meter and was acclimated to 6-7.4. Keeping in mind the end goal to decline the danger of tainting, all the readied weakenings were separated through 0.2µm syringe channels. On the 6th day, the readied centralizations of NapS were directed and eggs were again fixed with paraffin film under aseptic conditions. These were set back in the hatchery for next 24 hours. Following 24 hours, eggs were taken out of hatchery and pictures of all groups i.e. control and also those treated with various convergences of NapS were made utilizing a DSL camera. The improvement of veins and different elements were watched utilizing Adobe Photoshop adaptation 7.0, then, these pictures were exchanged to filter testing picture handling programming SPIP 6.6.2. Individual x, y and z measurements of every picture were stacked to decide diverse parameters to evaluate angiogenesis. The widths of different veins were measured by utilizing adjustment and estimation command.

Image-Processing Quantification (IPQ) Method

IPQ is executed on images obtain from the DSL cameras using a macro written for SPIP (version 6.6.2) and existing plug-ins.

The parameters reported describing the degree of inhibition of the naturally developing CAM include the diameter and branching system of blood vessels measured as per mm, and categories of blood vessels; i.e., primary, secondary and tertiary blood vessels.

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Statistical Analysis

Data was assessed on SPSS statistical software version 22.0 using one way ANOVA. Dunnett's Post Hoc test was applied considering $P < 0.05$ as significant.

Results

Qualitative Analysis

The inhibition of the physiologically-developing CAM is examined with Naproxen sodium (NapS). Exposure of the CAM with anti-AG compounds during the developmental phase characterized by exponential vascular growth prevents the regular growth of capillaries and vessels and directs the anti-angiogenic properties of the agents applied. The effects of this drug are visualized in **Fig. 1**. It shows that NapS is angiogenic at higher concentration and anti-angiogenic at low concentrations.

Quantitative Analysis

Quantitative analysis was done under three heads namely imaging studies, roughness analysis and diameter of blood vessels. These are discussed as below.

Imaging studies

The NapS was applied on CAM and images were captured at regular time intervals. The images captured were then analyzed with the software. These were transformed into three dimensional views of 45° as showed in **Fig. 2**.

Roughness analysis

There are no worldwide gauges for roughness investigation in light of surface pictures yet, however the actualized calculations are in assertion suggestions from driving specialists and a large portion of the definitions are regular augmentations of the ISO norms for surface profiles. With respect to all other expository outcomes the roughness parameters are composed of records, which can be transported in by worksheet suites and utilized for factual purposes. We also performed this parameter to check the roughness of images. Roughness of control and treated groups is given in **Table 1**.

For more accuracy, the control group revealed more roughness values than treated CAM. It indicates that surface roughness of treated CAMs was significantly less than control CAMs. The Abbott curve, a graphical representation of roughness, was also performed to calculate even slight alterations in the blood vessels heights on the CAM's surface. The heights of the Abbott curve for control and treated CAMs were observed as showed in **Fig. 3**.

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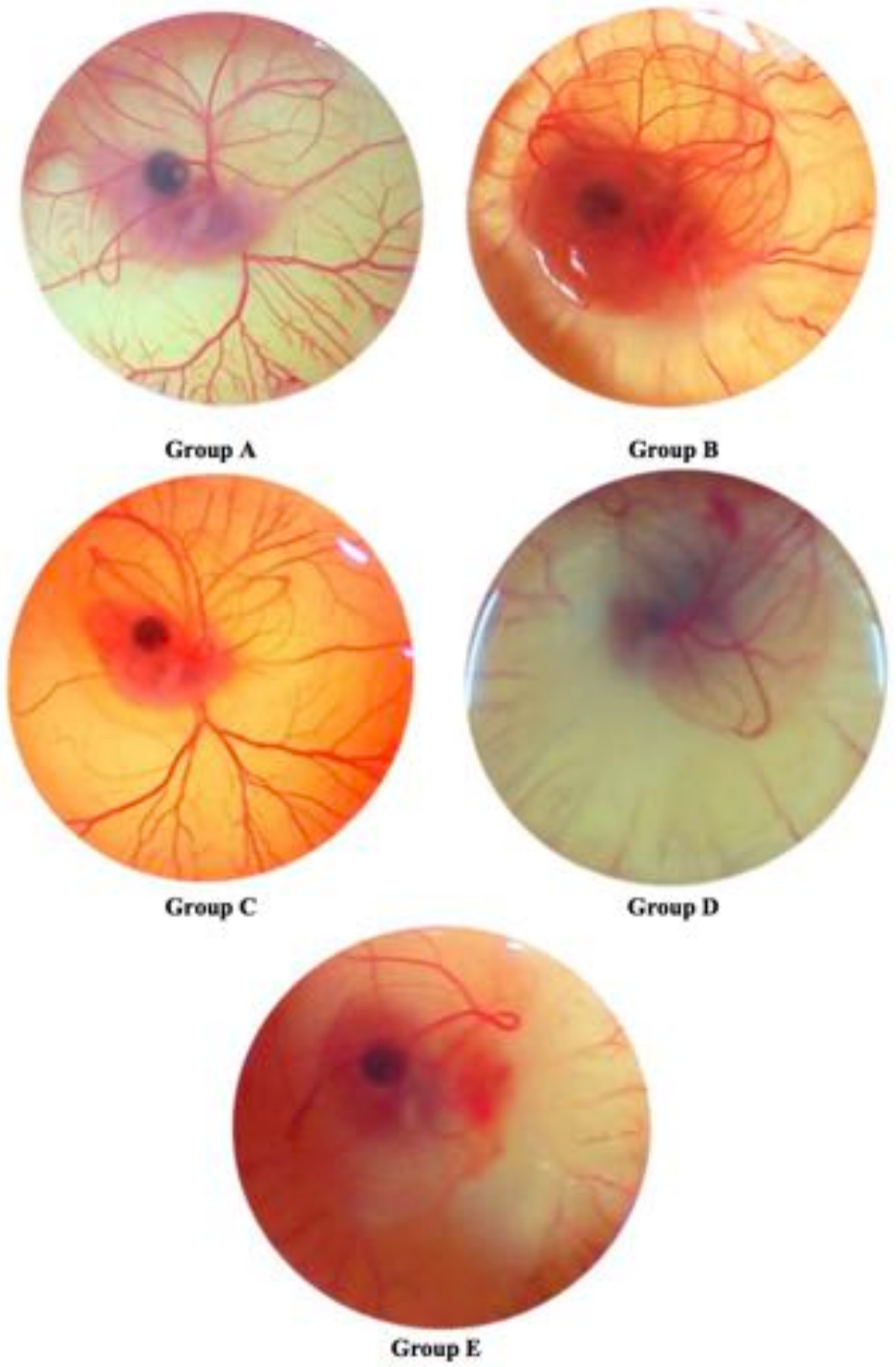

Figure 1. Angiogenesis with different concentration of NapS in all groups. Group A is controlled with full development of blood vessels. However, anti-angiogenic phenomena in group B,C and D. Death of embryo in group E.

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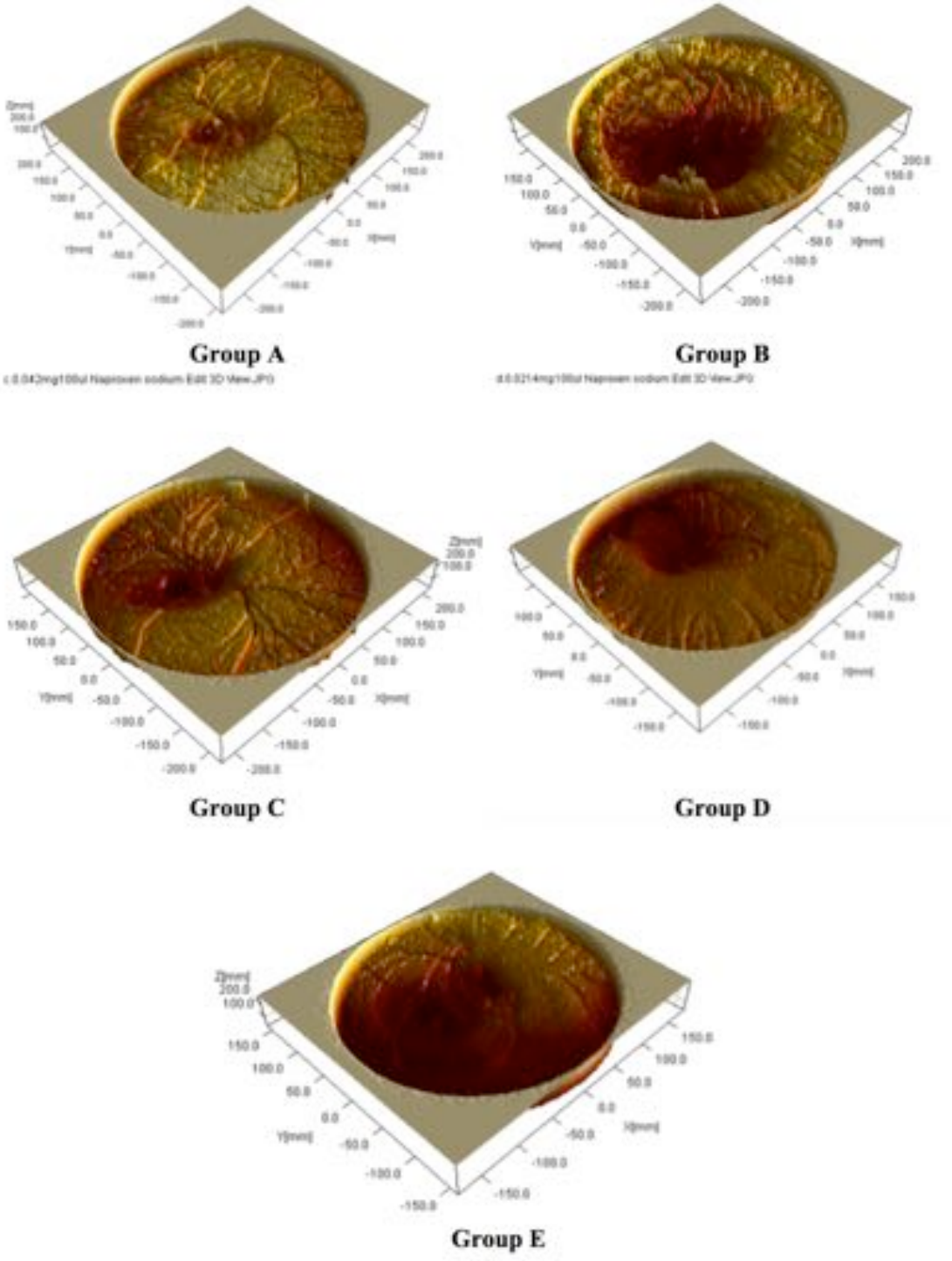

Figure 2. Angiogenesis phenomena in 3D view at 45°. Group A is controlled with full development of blood vessels. However, anti-angiogenic phenomena in group B,C and D. Death of embryo in group E.

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Table 1. Shows roughness values

Groups	Sa (nm)	Sq (nm)	Sz (nm)	Sv (nm)	Sp (nm)
A	4.00E+07	4.64E+07	2.43E+08	1.70E+08	7.34E+07
B	5.11E+07	6.28E+07	2.52E+08	1.30E+08	1.21E+08
C	4.64E+07	5.79E+07	2.34E+08	1.34E+08	1.00E+08
D	4.55E+07	5.53E+07	2.17E+08	1.02E+08	1.15E+08
E	6.57E+07	7.63E+07	2.61E+08	1.13E+08	1.48E+08

Sa: average roughness; Sq: root mean squaredeviation; Sz: maximum height of the surface; Sp: reduce summit height; Sv: reduce valley depth

Diameter of Blood Vessels

The captured images were evaluated with the software to determine the vessel diameters. Different segments in a selected area for measurement were identified. The respective diameters of each vessel portion located in different segments were identified using the software. Utilizing this procedure, the diameter of a specific vessel was determined automatically at each time point for the respective drug concentration. The distribution of vessel diameter of the CAM was observed to be normal. From the results obtained, it was obvious that the vessel diameters of the CAM did not change significantly over time. It was also noted that in Group A and B diameter increased which shows that NapS increases diameter in higher concentrations. However, there is no significant statistical difference between both groups as *P=0.010*. On the other hand, in low concentrations, NapS worked as an anti-angiogenic agent and diameter of blood vessels decreased with a significant difference among control and treated groups (*P=0.000*) as showed in **Fig. 4**.

All the diameters of blood vessels in all treated and control groups with respect to their branching are summarized in **Table 2**.

Table 2. Summarizes the diameter s of blood vessels in all groups

Sr. No.	Groups	Primary (mm)	Secondary (mm)	Tertiary (mm)
1	A (Control)	9.000	7.071	5.000
2	B (0.086mg/100ul Naproxen sodium)	10.000	8.062	5.000
3	C (0.042mg/100ul Naproxen sodium)	9.899	8.062	0.000
4	D (0.021mg/100ul Naproxen sodium)	9.055	0.000	0.000
5	E (0.011mg/100ul Naproxen sodium)	7.071	0.000	0.000

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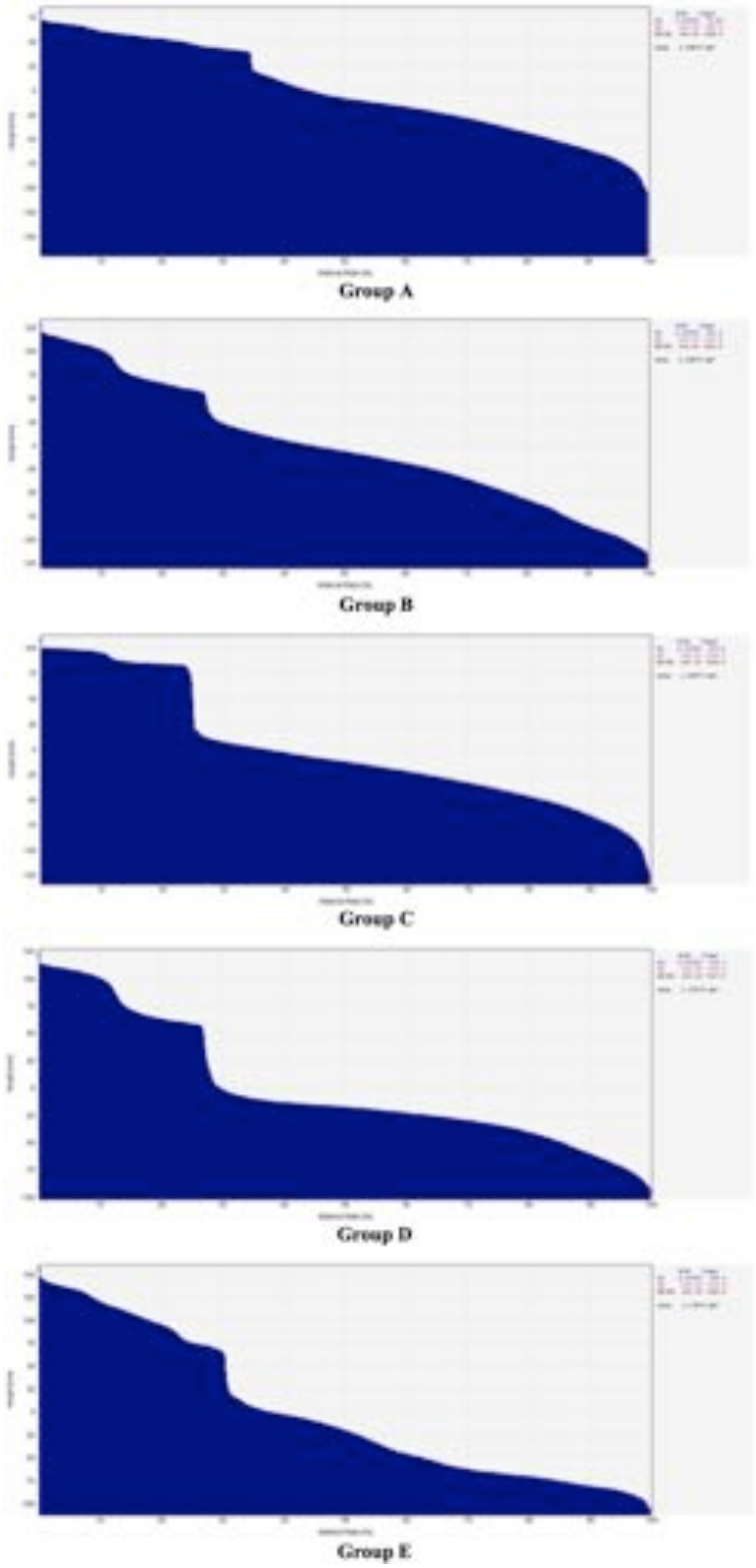

Figure 3. Abbott curves for roughness analysis. Control group A indicating more roughness and it is decreasing down from group B, C, D to E due to anti-angiogenic phenomena.

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Branching Pattern of Blood Vessels

The present study was also performed to investigate the development of blood vessels by the selection of a capillary plexus. To recognize this developmental process of blood vessels transformation, a series of photographs was recorded with a computer simulation of the process of *in vivo* vascularization. The simulation established that a positive feedback system contributed in the development of a branching pattern. As the embryo grew, it was witnessed that in Groups A and B, there was a fork branching pattern while in group C, D and E, there was polygonal and/or tree capillary networks as showed in **Fig. 5**. An area where progress was rapidly received much blood flow and produced finer networks of capillaries.

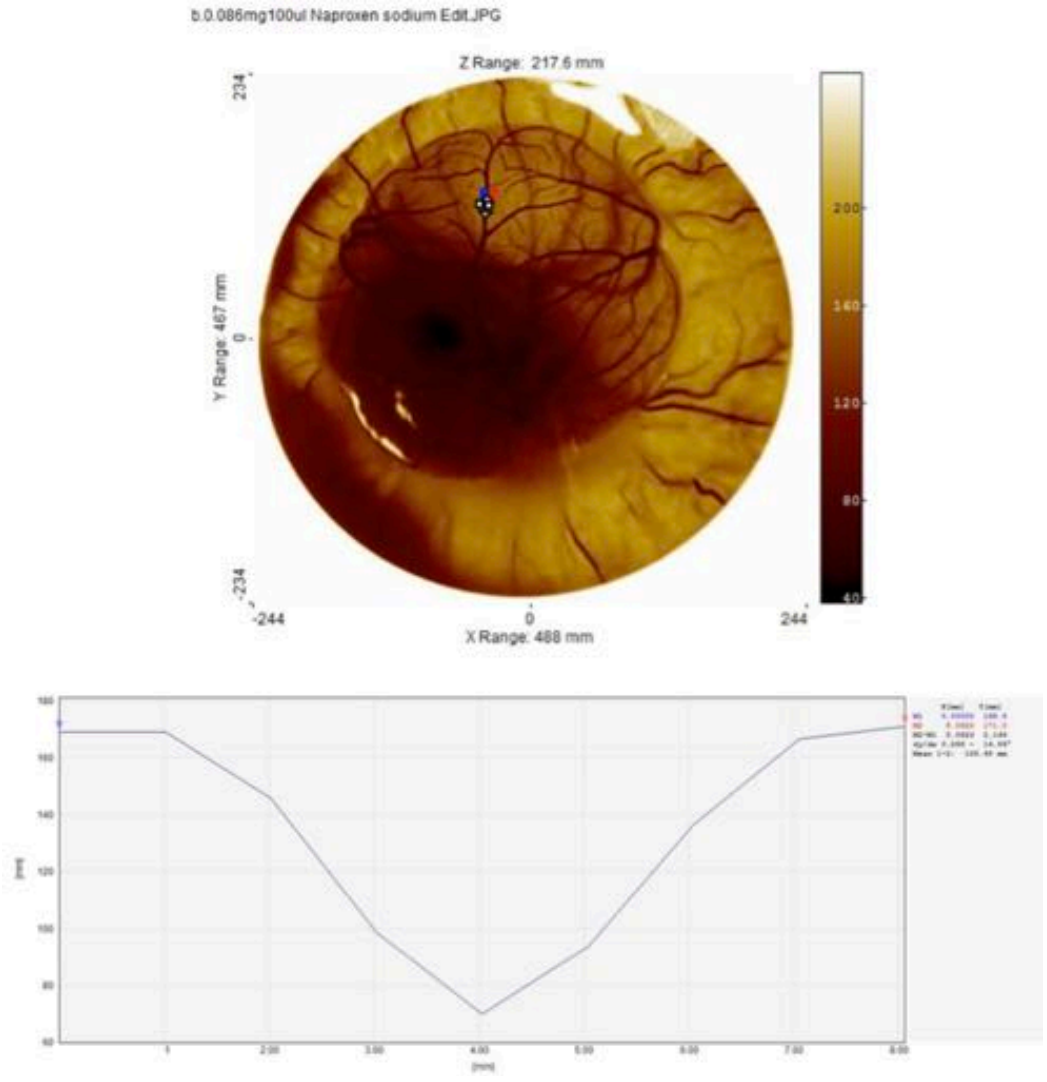

Figure 4. Diameter of group B (secondary blood vessel). Upper figure indicates the diameter of blood vessels in mm. Lower figure (graph) indicates the diameter of secondary blood vessel. i.e., 8mm.

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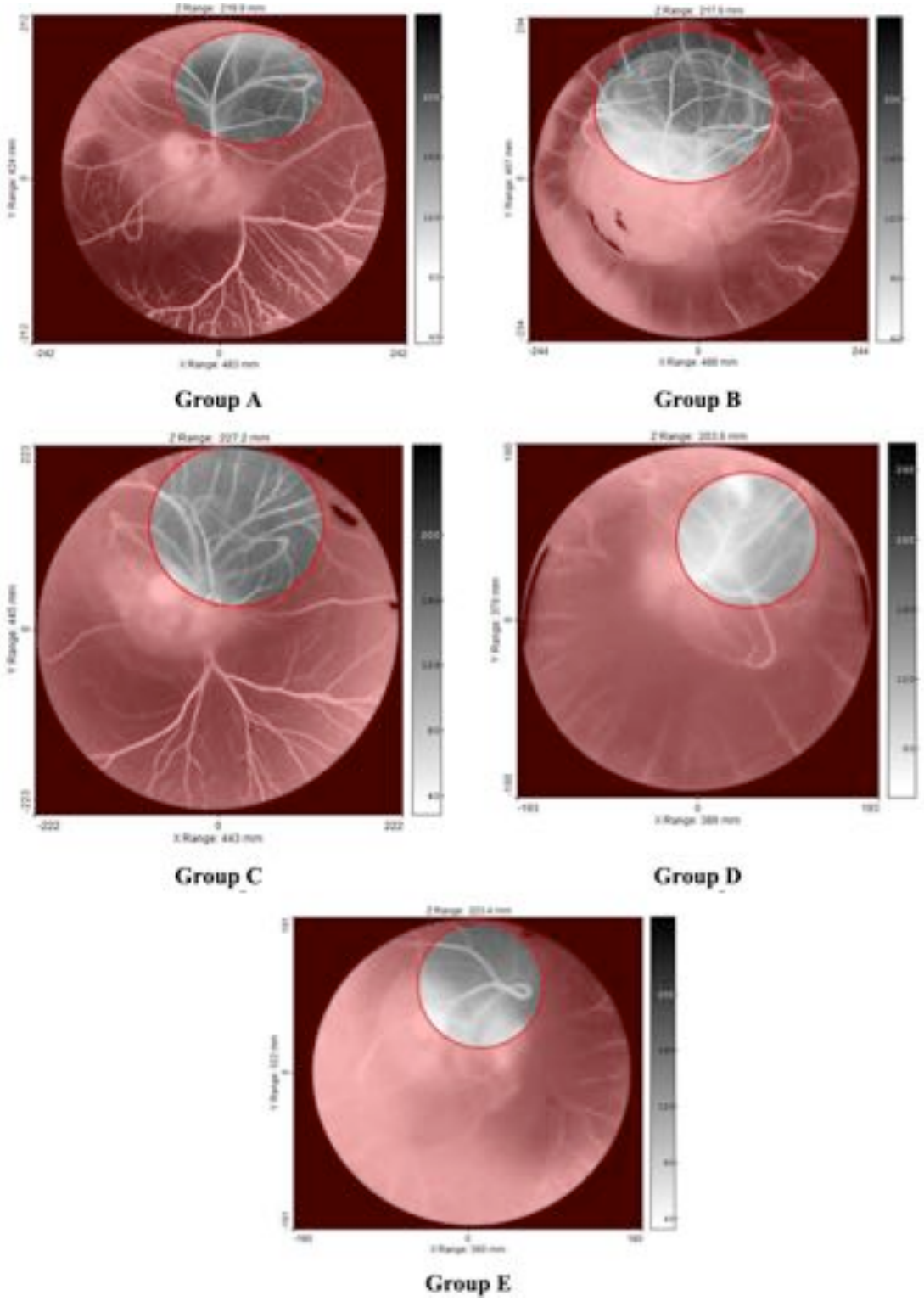

Figure 5. The figure shows the branching pattern in all groups.

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Discussion

The CAM model is widely used in angiogenic investigation and is principally appropriate for the study of diseases categorized by proliferative retinal vasculature, such as age-related macular degeneration (Tufan and Satiroglu-Tufan, 2005). In the current effort, this model was used in order to determine the angiogenic and/or anti-angiogenic effect of Naproxen sodium (NapS) and determine the effective dose of NapS for angiogenesis.

The effects of drug visualized in Figure 1 show that NapS is angiogenic at higher concentration and anti-angiogenic at low concentrations. Similar studies were conducted by Blacher et al. (2005) (Blacher et al., 2011). They used new advances in imaging investigation to quantify CAM angiogenic response, covering all vascular components, from the large supplying and feeding vessels down to the capillary plexus. Their morphometric investigation highlighted that a precise quantification of the CAM vasculature desires to be executed at several scales (Blacher et al., 2011).

Quantitative analysis was performed including imaging studies, roughness analysis and diameter of blood vessels. The NapS was applied on CAM and images were captured at uniform time intervals. The images captured were then analyzed with the software. These were transformed into three dimensional views as shown in **Fig. 2**. These were prepared to know the better understanding of blood vessels networks.

There are no worldwide gauges for roughness investigation in light of surface pictures yet, however the actualized calculations are in assertion suggestions from driving specialists and a large portion of the definitions are regular augmentations of the ISO norms for surface profiles. With respect to all other expository outcomes the roughness parameters are composed to records, which can be transported in by worksheet suites and utilized for factual purposes. We also performed this parameter to check the roughness of images. These factors illuminate the variances in surface roughness between control and treated CAMs. The Abbott curve, a graphical picture of roughness, was also dignified to appraise even slight changes in the stature of blood vessels on the surface of CAMs. Abbott curve heights for control and treated CAMs were observed as shown in figure 3. This shows that roughness was found high in group A and B while in group C, D and E, roughness was comparatively low. Similarly, same roughness parameter was observed by Hussain *et al*. (2011) (Hussain, 2011). In their study, the average values of roughness in the control group were high in relation to treated CAMs. It portrayed that surface roughness analysis, demonstrating neo-vascularization of treated CAMs was expressively ($P<0.05$) less than that of control CAMs (Hussain, 2011).

The captured images were assessed with the software to determine the vessel diameters. Different segments in a selected area for measurement were identified. The respective diameters of each vessel portion located in distinct

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segments were identified using the software. Using this procedure, the diameter of a specific vessel was determined automatically at each time point for the respective drug concentration. The dispersal of the vessel diameter of the CAM was observed to be usual. From the investigations, it was obvious that the vessel diameters of the CAM did not modify meaningfully over time. It was also observed that in Group A and B diameter increased which shows that NapS increases diameter in higher concentrations. However, there is no significant statistical difference between both groups. On the other hand, in low concentrations, NapS worked as an anti-angiogenic agent and diameter of blood vessels decreased with a significant difference among control and treated groups (*P=0.000*) as shown in figure 4. Another study was conducted by Salas (2015) (Salas, 2015) to assess the phytochemical components and properties of herbal plant extracts, such as *Gynura nepalensis*, *Pandanus odoratissimus L.* and *Carmona retusa masam*, as potential angiogenesis inhibitors using the CAM assay. They found similar reduction in diameter with *Carmona retusa masam*.

The current research was also executed to investigate that blood vessels are fashioned by the capillaries selection in the plexus. To recognize the developmental phenomena of blood vessel branching pattern, a series of radiographs was successfully recorded, and a computer simulation was carried out by the process of *in vivo* vascularization. The simulation established that a positive feedback system contributed in the development of a branching pattern. As the embryo grew, it was witnessed that in Groups A and B, there was a fork branching pattern while in group C, D and E, there was polygonal and/or tree capillary networks as shown in **Fig. 5**. An area where progress was rapidly received much blood flow and produced finer networks of capillaries.

Conclusion

It is concluded that the presentation of Naproxen sodium on CAM on 6th day of incubation showed angiogenic effects in high concentration and anti-angiogenic effect in low concentration. Results indicated noticeable modifications in CAM design, weakening of blood vessels, reduction in surface roughness parameters, and decrease in Abbott curve. The significant amounts of Naproxen sodium may reveal anti-angiogenic activity in the similar fashion as those seen *in vitro* and describe its clinical efficacy.

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Abbreviations

AG: Angiogenesis
CAM: Chorioallantoic membrane
NapS: Naproxen sodium
SPSS: Statistical package for social sciences

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Author contribution

Hafiz Muhammad Shoaib Zahid conducted the research, Tanveer Ahmed Khan performed data analysis, Muhammad Ijaz-ul-Haq drafted the manuscript, Humayun Riaz reviewed the manuscript, Syed Atif Raza supervised the study, and Zia Mohy-ud-din Khan performed graphical interpretation.

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