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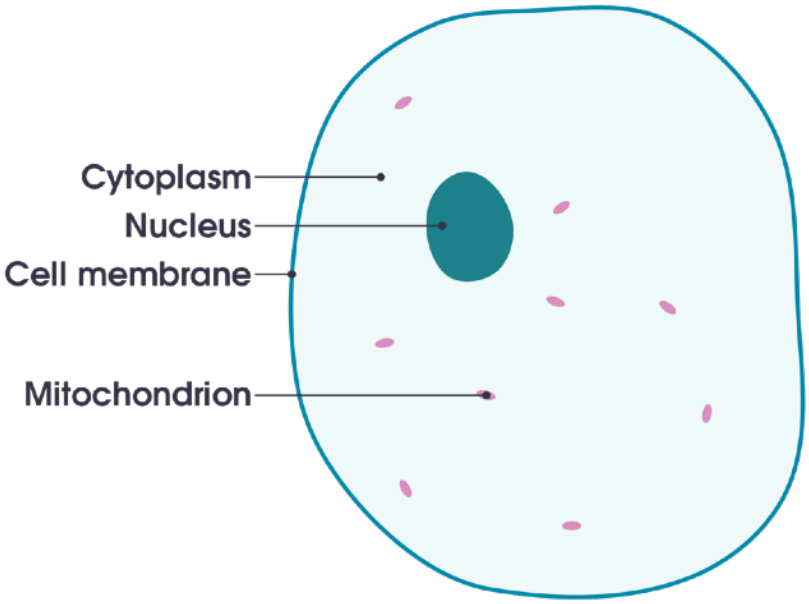

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Original Research

Comparing the effect of breastfeeding promotion interventions on exclusive breastfeeding: an experimental study

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Abstract

Introduction: Despite the proven risks associated with not breastfeeding, few mothers exclusively breastfeed their babies for six months as recommended by the World Health Organization. This study was conducted to compare the effect of breastfeeding promotion interventions on exclusive BMF among primiparous women. **Methods**: This quasi-experimental study was conducted on a sample of 93 primiparous women who were referred to health care centres, Mashhad, Iran, in 2010. Health care centres were selected by multistage sampling method, and then randomly allocated into two intervention groups (peer support group and health care provider's education group) and one control group. Primigravidae aged 18-35 years old, with singleton pregnancy, with gestational age of 35-36 weeks, and intending to breastfeed their children were randomly selected out of health care centres. The peer support group participants received supports from their peers four times and education group's participants received 4 training sessions by health care providers. The control group received only routine cares. Exclusive BMF duration and rate assessed at 4 and 8 weeks postpartum and collected data were analysed using SPSS (ver.11.5) software.

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Results: There were no significant differences in exclusive BMF duration at 4 and 8 weeks among the 3 groups (P=0.993, P=0.904). Exclusive BMF rate at 4 and 8 weeks after birth was significantly different among the 3 groups (P=0.043, P=0.023). No significant difference was found between peer support and healthcare provider's education groups with respect to BMF rate at 4 weeks (P=0.111), but the difference was significant at 8 weeks (P=0.027). **Conclusion:** All women should be offered education and peer support to breastfeed their babies to increase the exclusive breastfeeding rate. But to continue exclusive breastfeeding, and increase its duration, help of family is more important than education and peer support. Support that is only offered reactively, in which women are expected to initiate the contact, is unlikely to be effective; women should be offered ongoing support so they can predict that support will be available. Support should be tailored to the needs of the setting and the population group.

Keywords

Peer Support, Education, Health Care Providers, Exclusive Breast Feeding

Introduction

World Health Organization (WHO) recommended exclusive breast milk feeding (BMF) to the infant for the first six months of life to achieve optimal growth, development,911 and health. Nevertheless, exclusive BMF remains uncommon in most countries (both developed and developing), even in countries with high rates of breastfeeding initiation (Imdad et al., 2011). Recent data shows that the prevalence of exclusive BMF in developing countries has increased from 33% in 1995 to just 39% in 2010 (Haroon et al., 2013). Although using promoting programs, exclusive BMF has had a descending trend in Iran in 2000-2006, and an increasing trend in 2007-2011 (Motlaq Me, 2013). According to the results of the Demographic Health Survey (2000) (Olang et al., 2009), less than 45% of the infants who were younger than 6 months used exclusive BMF decreasing the rate to 27% in 2004 (Olang et al., 2009) and 23.1% in Integrated Monitoring Evaluation System Survey in 2006 (Esfahani Mm, 2009), but fortunately, prevalence of exclusive BMF increased to 53.1% in 2011 (Motlaq Me, 2013). Integrated Monitoring Evaluation System Survey in Razavi Khorasan Province, Iran showed that the prevalence of exclusive BMF was approximately 25% (Esfahani Mm, 2009).

BMF is natural, but it requires skill and education. Especially in primiparous women, limited information and inexperience about breastfeeding is the most common problem. This may lead to vicious ring of problems which may end in failure of establishment and continuation of breastfeeding (Najem, 2011; Translators-Group, 2008).

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WHO declared BMF promotion and support as a public health priority in 2003 following reduction in the rate and duration of exclusive BMF (Esfahani Mm, 2009). In order to achieve this, two vast groups of hospital and community-concentrated strategies, which will be done by professional and non-professional people, are recommended to promote exclusive BMF (2006, July 21).

One of the hospital-concentrated strategies is fulfilling educational programs by professionals (Translators-Group, 2008). BMF education is usually a formalised, defined, descriptive, and goal-oriented programme with a specific purpose and target audience, and given as part of routine antenatal care (Lumbiganon et al., 2012).

Breastfeeding education provided by the primary care physician during routine preventive visits is likely to have limited impact, compared with the effects of various barriers that negatively affect breastfeeding duration such as psychological factors, cultural factors, and returning to work. Studies have documented that infant feeding counselling is often associated with poor quality or unavailability for many women (Ansari et al., 2014; Owais Ahmad M, 2012). Although there is an education regarding exclusive breastfeeding during pregnancy by midwives in Iran, it did not result in great success in breastfeeding (Ansari et al., 2014).

Dyson's review study have shown that formal and informal education, based on the requirements, are effective in increasing BMF (Dyson et al., 2005). Belay showed that prenatal education could increase exclusive BMF rate (Belay and Haidar, 2013). Ansari reported that educational program could increase exclusive BMF duration (Ansari et al., 2014). Likewise, Artieta-Pinedo believes that antenatal education might increase breastfeeding for first month after birth (Artieta–Pinedo et al., 2013).

On the other hand, peer support is an important element in offering health care (Dennis, 2003), and community education and support for target population (Mickens, 2008). Peer support in BMF includes emotional support, encouragement, BMF education, and help in resolving nursing mothers' difficulties by mothers who have had a BMF history (Mead et al., 2001; Muller et al., 2009). Peer support can be done during pregnancy and postpartum by an individual, one-to-one counselling, a support group, a phone call or a home visit (Dennis, 2003; Mead et al., 2001; Mickens, 2008) or a peer who is similar to the nursing mother in some special characteristics like age, sex, occupation, socioeconomic status, health status, etc (Dennis, 2003; Muller et al., 2009). Several studies have shown the effect of peer support on increasing early BMF initiation, its continuation up to 6-8 weeks, increasing the rate (Andrade S, 2008; Chung et al., 2008; Dennis et al., 2002; Dennis, 1999; Dennis and Kingston, 2008; Mickens, 2008; Palda et al., 2004) and duration of exclusive BMF (Andrade S, 2008; Dennis, 1999; Dennis and Kingston, 2008; Meglio et al., 2010; Palda et

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al., 2004; Persad and Mensinger, 2008) and decreasing BMF problems (Mickens, 2008; Rossman, 2007).

Although several studies have shown the effectiveness of support and education for nursing mothers by professionals or non-professionals on all patterns of infant feeding (exclusive, predominant, complete, and partial) (Chung et al., 2008; Sikorski et al., 2003), especially exclusive BMF (Sikorski et al., 2003), but in relation to breastfeeding promotion, there is little information as to which type of strategies are most effective in promoting exclusive breastfeeding and achieving high and equitable coverage (Chung et al., 2008; Sikorski et al., 2003). Therefore, this study is aimed at comparing the effect of breastfeeding promotion interventions on exclusive BMF in primiparous women settled in Mashhad, a city in the North East of Iran, in 2010.

Methods

Design and participants

This quasi-experimental study was conducted on 108 primigravidae who were referred to health care centres in Mashhad in 2010. Research ethics approval has been obtained from Mashhad University of Medical Sciences (No: 12–082) on September 24, 2010. All pregnant women participated and peer supports were requested to give their written informed consent prior to any study procedure. To secure confidentiality, all identifying information of participants, including name, medical, and contact information as well as all collected data will be kept in patient's privacy.

Multistage sampling was done. First, the city of Mashhad was divided into three clusters. Each cluster contained a health centre, which consisted of 40 to 60 small health centres. In every cluster, a list of all health centres was prepared using random numbers table, and finally three health centres were randomly selected. The selected centres were randomly assigned into 3 groups of peer support, education by health care providers, and control. A total of 9 health centres were included in the study.

The research was conducted in three stages, including peer selection and training, selection and training of the health care providers, and sample selection and intervention.

Peer selection and training

In the first stage, among the people covered by health care centres dedicated to the peer support group, 23 Iranian female volunteers living in Mashhad, with at least a fifth grade education, a BMF history, and good attitude towards BMF, were selected through a public invitation. Then, their knowledge and attitude

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towards BMF and communication skills were tested using a pre-test by researcher.

To prepare volunteers for support, the researcher held training classes in five group sessions with 7 to 8 persons in each group. Daily sessions over 3 hours with a 45-minute break was held every 90 minutes separately by the investigator at each centre.

The education was on the definition of peer support; that is, the purpose, role, and responsibilities of peer volunteers; communication skills; and the basics of BMF (including the benefits of BMF; the anatomy and physiology of lactation; conditions of feeding and breast insertion; proper establishment of BMF; barriers to support and encourage; questions, common concerns, and stories; and false beliefs about BMF), in the mentioned health care with the lecture approach, using educational slides and images, questions and answers, and role playing. Sixteen out of the twenty three enrolled participants completed the course, and obtained at least 75% of the post-test scores of knowledge, attitude, and communication skills and 75 percent of the self-assessment score, and took responsibility as a peer to support introduced mothers.

Selection and training of the health care providers

In the second stage, 7 midwives were selected among official or contract healthcare providers who had passed BMF counselling classes, and employed in health care centres allocated to health care provider's education group. After obtaining written consent, in order to integrate the presented materials to the participants, they received necessary education for 2 hours according to BMF education guide for mothers.

Sample selection and intervention

After education of the peers and health care providers, the sample size was calculated with a pilot study. At first, 30 pregnant women with eligibility criteria were randomly divided into three groups of peer support (10), education by health care providers (n=10), and control (n = 10).

Sample size was calculated on the basis of the results of pilot study and using the formula for the difference between three means with the following assumption of 3.8±2.66 days duration of exclusive breastfeeding. We estimated that a sample of 27 mothers in each group would have 85% power with a 2-tailed α error of <0.05 to detect a 21.8 relative increase in the duration of exclusive breastfeeding at 8 weeks.

We assumed that nearly 30% of mothers would be lost to follow-up monitoring; therefore, we planned to include 36 eligible mothers in each group. At last 108 women were enrolled the study.

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Inclusion criteria were as follows: Iranian primigravidae who were 18-35 years old, living in Mashhad with at least a fifth-grade education, normal BMI, singleton pregnancy, and intended to breast-feed their children with gestational age between 35-36 weeks.

The researcher referred to the selected health care centres, and prepared a list of eligible pregnant women from the office of maternal care; then, 12 participants were randomly selected at each using a random number table centre. The eligible women were invited by phone to the health centres. After explaining the objectives and the application of research results, they were enrolled, and informed consent was obtained from them.

Selected mothers in the centre assigned to the peer support were introduced to a peer volunteer with regard to cultural, social, and economical similarities, by the researcher or similar coordinated peer. The first support was conducted at 36-38 weeks of pregnancy, and three latter supports in 1, 2 and 3 interval weeks after birth, by peer. The first and third supports were done by face to face, and second and fourth supports by phone.

Selected mothers in the centre assigned to the training health care providers received the first training at 36-38 weeks of pregnancy, and three latter training sessions in 1, 2 and 3 interval weeks after birth by health care provider. The first and third training sessions were done by face to face, and second and fourth by phone.

Selected mothers in the centre assigned to the control just received routine prenatal (including nursing training in 35-37 weeks of pregnancy) and postpartum care (including nursing training in 1-3, 10-15 and 42-60th day postpartum).

Instruments

Attitude of peer volunteers, health care providers, and pregnant women towards breastfeeding was evaluated by a 20-question questionnaire. The responses were based on 3-point Likert scale (agree, no idea, and disagree) with score of 1 to 3; the minimum score was 20 and the highest score 60. In other words, attitude was classified as poor (20-33.3), moderate (33.4-46.6), and good 46.7-60). Content validity and reliability were confirmed using the Cronbach's Alpha coefficient of 0.74. The feeling of mother and husband towards pregnancy was examined with a 5-point Likert-type scale as, "very happy", "happy", "indifferent", "sad", and "very sad".

Baseline characteristics regarding mother and her pregnancy were gathered using a questionnaire at the beginning of the study. Baseline characteristics regarding delivery and infant were gathered using a questionnaire during postpartum period. Breast milk feeding information was gathered by a daily reporting questionnaire form on infant nutrition during 8 weeks after postpartum. Exclusive BMF duration and rate were assessed at 4 and 8 weeks

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postpartum. The daily reporting questionnaire was approved by 13 faculty members in Mashhad University of Medical Sciences with content validity and reliability with test-retest reliability (r = 0.9) and Cronbach's alpha (r = 0.7).

Statistical analysis

SPSS (version 11.5, SPSS, Inc, Chicago, IL, USA) software package was used for all statistical analyses. Normality of quantitative variables was evaluated by Kolmogorov-Smirnov test. Quantitative variables have been presented as mean and standard deviation, and qualitative variables as number and percent. The main dependent variables were exclusive BMF duration and rate at 4 and 8 weeks postpartum. The analysis of variance (ANOVA) test was used to compare mother's age and birth weight of newborn among groups. The Kruskal Wallis test was used to compare participant's education and occupation, the first time of BMF, participant's feeling towards pregnancy, the first prenatal care visit, spouse's idea about BMF, others' idea about BMF, location of prenatal care among groups. The chi-square was used to compare delivery mode, spouse's feeling toward pregnancy, assistant person in caring infant after birth among groups. The Fisher exact test was used to compare family income, unplanned pregnancy among groups. The chi-square was used to compare exclusive BMF rates among groups at 4 and 8 weeks postpartum, and exclusive BMF rates between 4 and 8 weeks were compared in each group using McNemar test. The ANOVA was used to compare exclusive BMF duration among groups at 4 and 8 weeks postpartum. Exclusive BMF duration between 4 and 8 weeks were compared in each group using paired t-test. Confidence coefficient 95% and α level of 0.05 were used for all statistical tests.

Results

Sixteen out of the 23 enrolled peer supporters completed the educational course. Their mean age was 34.7±6.36, most of the women had 2 children (50%), the mean duration of BMF for children was 21.3±5.51 months, and majority of them had elementary (37.5%) and high school (37.5%) education.

The average age and work experience of health care providers participated were 33.6±4.40 and 7.9±3.98 years, respectively.

Fifteen women out of 108 participants were excluded because of the exclusion criteria during the research such as being hospitalised, neonatal death or still birth, lack of tendency to keep cooperation, or not receiving education or support **(Fig. 1)**. So the analyses were performed on 93 participants. There were no differences in baseline characteristics regarding maternal and infant data between the 3 groups such as participant's age, birth weight **(Table 1)**, education, occupation, family income, planned pregnancy, when to decide for BMF, delivery mode, and the first time of BMF **(Table 2)**. However, three groups

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had significant differences in some variables like participant's feeling toward pregnancy (P=0.039), spouse's feeling about pregnancy (P=0.009), prenatal care for the first time (P=0.002), the spouse's attitude towards breast milk feeding (P=0.038), the others' attitude towards breast milk feeding (P=0.022), place of prenatal care (P=0.029), and assistant help in child care at and after birth (P=0.023) **(Table 3)**. The effect of these variables was analysed on two dependent variables (duration and rate of exclusive BMF). None of the heterogeneous variables affect the duration and rate of exclusive BMF except assistant help in child care at and after birth; which just affected the duration of exclusive BMF and not its rate **(Table 3)**.

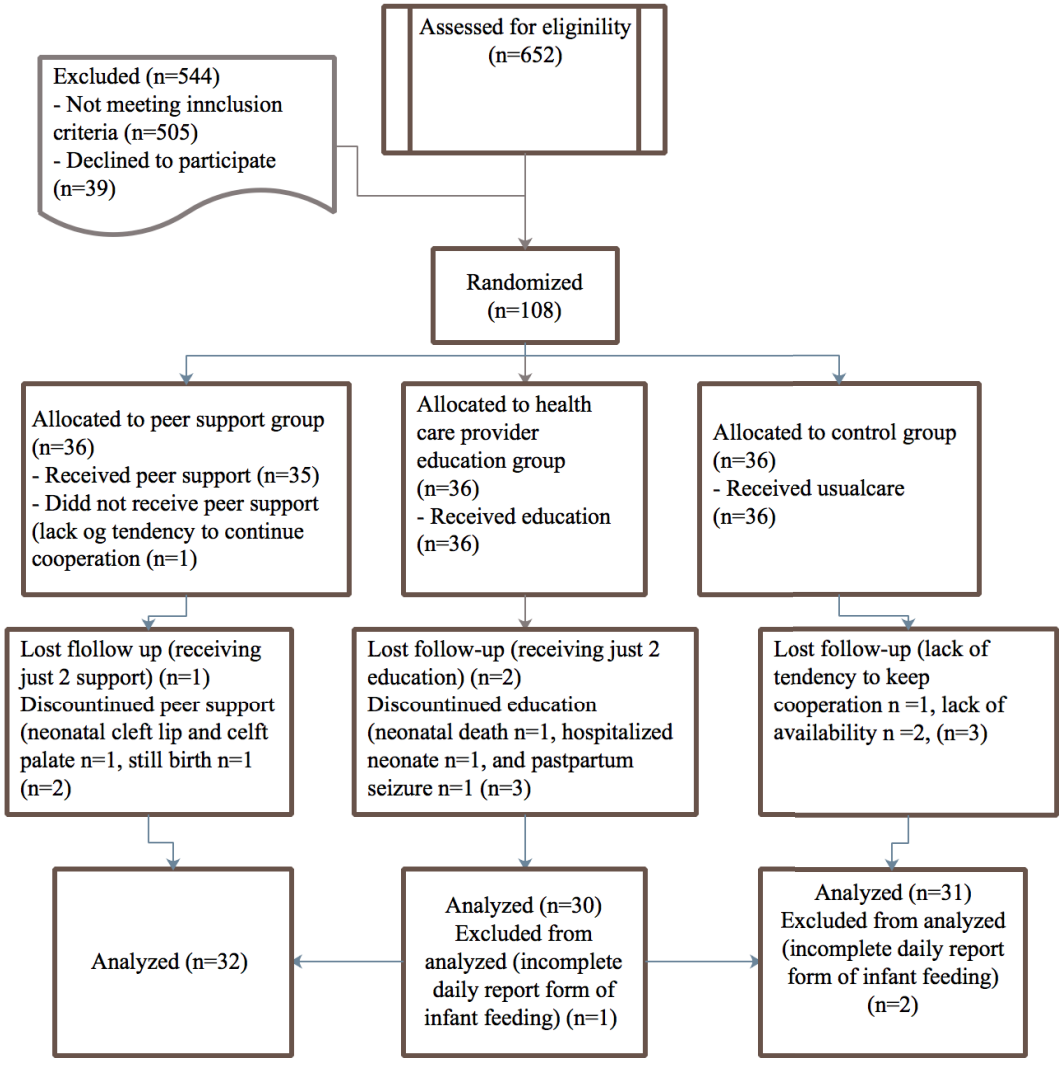

Figure 1. Consort flow diagram. Enrollment, Allocation, Follow-Up patients in the study.

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Table 1. Mean of participants' age and birth weight of newborn in assessed groups

Variable	Group			P values
	Peer support	Health care provider's education	Control	
	Mean ± SD	Mean ± SD	Mean ± SD	
Mother's age (year)	24.0±3.78	24.3±3.85	23.0±4.06	0.389
Birth weight of newborn (kg)	3.19±0.394	3.24±0.452	3.38±0.387	0.189

Table 2. Education, occupation, family income, unplanned pregnancy, delivery mode, and the first time of BMF in assessed groups

Varibale		Groups						Test results
		Peer support		Health care provider's education		Control		
		N	%	N	%	N	%	
Education	Basic	1	3.1	1	3.3	3	9.7	x^2=3.787 df=2 P=0.151
	Junior high school	7	21.9	6	20.0	11	35.5	
	High school	17	53.3	18	60.0	13	41.9	
	Academic	7	21.9	5	16.7	4	12.9	
Ocupation	Homemaker	29	90.6	25	83.3	27	87.1	x^2=0.020 df=4 P=0.762
	Student	1	3.1	1	3.3	0	0	
	Employed	2	6.3	4	13.3	4	12.9	
Family income	Inadequate	5	15.6	3	10.0	2	6.5	X^2= 1.392 df=2 P=0.499
	Adequate	27	84.4	27	90.0	29	93.5	
Unplanned prenancy	Yes	3	9.4	5	16.7	3	9.7	x^2=0.996 df=2 P=0.665
	No	29	90.6	25	83.3	28	90.3	
Delivery mode	NVD	16	50.0	11	36.7	18	58.1	X^2=2.846 df=2 P=0.241
	CS	16	50.0	19	63.3	13	41.9	
The first time of BMF	First half an hour after birth	7	22.6	5	16.7	6	19.4	x^2=0.258 df=2 P=0.879
	30-60 min after birth	7	22.6	7	23.3	7	22.6	
	1-24hr after birth	17	54.8	18	60.0	18	58.1	

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Table 3. Heterogeneous variables and their effect on the duration and rate of exclusive BMF in assessed groups

Variable	Groups						Test results	Variable effects on exclusive BMF			
	Peer support		Health Care provider's education		Control			Rate of EBMF		Duration of EBMF	
	N	%	N	%	N	%		4 wks	8 wks	4 wks	8 wks
Participant 's feeling toward pregnancy	Very pleased	13	40.6	4	13.3	10	x^2=6.465 df=2 P=0.039	x^2=2.539 df=3 P=0.506	x^2=2.801 df=3 P=0.372	x^2=2.957 df=3 P=0.398	x^2=2.899 df=3 P=0.407
	Pleased	18	56.3	22	73.3	18					
	Neutral	0	0	3	10.0	2					
	Sad	1	3.1	1	3.3	1					
Spouse's feeling toward pregnancy	Very pleased	19	59.4	7	23.3	17	x^2=9.47 df=2 P=0.009	x^2=0.164 df=1 P=0.686	x^2=0.000 df=1 P=0.995	Z=-1.221 P=0.222	Z=-1.156 P=0.248
	Pleased	13	40.6	23	76.7	14					
The first prenatal care visit	First trimester	15	46.9	9	30.0	24	77.4	x^2=12.783	x^2=1.899	x^2=1.099	x^2=4.176
	Second trimester	16	50.0	20	66.7	6	19.4	df=2	df=2	df=2	df=2
	Third trimester	1	3.1	1	3.3	1	3.2	P=0.002	P=0.318	P=0.448	P=0.124
Spouse's idea about BMF	Strongly agree	10	31.3	4	13.3	7	22.6	x^2=6.547	x^2=1.713	x^2=1.430	x^2=4.242
	Agree	22	68.8	21	70.0	23	74.2	df=2	df=2	df=2	df=2
	No comment	0	0	5	16.7	1	3.2	P=0.038	P=0.566	P=0.589	P=0.120
Family's idea about BMF	Strongly agree	8	25.0	1	3.3	4	12.9	x^2=7.661	x^2=6.406	x^2=1.019	x^2=0.851
	Agree	24	75.0	27	90.0	26	83.9	df=2	df=2	df=2	df=2
	No comment	0	0	2	6.7	1	3.2	P=0.022	P=0.620	P=0.394	P=0.653
Location of prenatal care	Health care centre	4	12.5	10	33.3	14	45.2	x^2=7.048	x^2=1.408	x^2=1.038	x^2=5.928
	Health care centre+ physician	26	81.3	19	63.3	16	51.6	df=2	df=2	df=2	df=2
	Health care+ midwife	2	6.3	1	3.3	1	3.2	P=0.029	P=0.656	P=0.749	P=0.053
Assistant person in caring infant at birth	Yes	23	71.9	28	93.3	20	64.5	x^2=7.550	x^2=2.173	x^2=2.132	
	No	9	28.1	2	6.7	11	35.5	df=2	df=1	df=1	Z=-2.500
								P=0.023	P=0.237	P=0.288	P=0.012

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Table 4. Mean of the duration of EBMF at 4 and 8 weeks after birth in assessed groups

The duration of EBMF (day)	Total mothers in assessed groups				Mothers who had no assistant help in caring their children at after birth				Mothers who received an assistant help in caring their children at after birth			
	Peer Support	Health care provider's education	Control	P value	Peer Support	Health care provider's education	Control	P value	Peer Support	Health care provider's education	Control	P value
	Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	Mean ± SD	
4 wks after birth	13.0 ± 12.65	7.8 ± 10.66	6.5 ± 9.47	P=0.056	5.9 ± 10.04	5.0 ± 7.07	5.8 ± 9.53	P=0.993	15.7 ± 12.67	8.0 ± 10.93	7.0 ± 9.67	P=0.020
8 wks after birth	21.7 ± 24.44	10.10 ± 16.23	8.5 ± 14.93	P=0.014	5.9 ± 10.04	5.0 ± 7.07	8.4 ± 16.92	P=0.904	27.9 ± 25.75	10.5 ± 16.70	8.5 ± 14.19	P=0.002
P value	P=0.001	P=0.088	P=0.140		P=1.000*	P=1.000*	P=0.341		P=0.000	P=0.088	P=0.282	

The SE of difference is 0.

Table 5. Exclusive BMF rate at 4 and 8 weeks after birth in assessed groups

Exclusive BMF		Groups						
		Peer Support		Health Care Provider Education		Control		P values
		Percent	N	Percent	N	Percent	N	
4 wks after birth	3	9.7	5	16.7	11	34.4	Yes	X^2=6.294
	28	90.3	25	83.3	21	65.6	NO	df=2
	100	31	30	100	32	100	Total	P= 0.043
8 wks after birth	2	6.5	2	6.7	9	28.1	Yes	X^2=8.120 (Fisher exact)
	29	93.5	28	93.3	23	71.9	NO	df= 2
	31	100	30	100	32	100	Total	P=0.023
P values		P = 0.500		P = 0.250		P = 1.000		

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About 22.6% of the mothers of peer support group, 16.7% of the mothers of health care provider's education group, and 19.4% of the mothers of control group initiated breastfeeding within the first hour after birth. However, there were no significant differences between the groups (p=0.879). The mean duration of exclusive BMF of total participants at 4 weeks after birth was 9.2 ± 11.26 days, and the mean duration of exclusive BMF of total participants at 8 weeks was 13.5 ± 19.81 days. The three groups had no significant difference in terms of duration of exclusive BMF at 4 weeks (P=0.056), but they had significant difference at 8 weeks (P=0.014) **(Table 4)**. The variable "assistant help in child care at and after birth to ward their family members" was heterogeneous in assessed groups. Also, it was a confounding variable on the duration of exclusive BMF. Therefore, its effect was assessed on the duration of exclusive BMF according to **Table 4**. The duration of exclusive BMF at 4 and 8 weeks had no significant difference in groups who did not receive assistant help to care their child at and after birth to ward their family members (P=0.993 and P=0.904) **(Table 4)**. The mentioned variable in groups who received assistant help to take care of their child at and after birth was P=0.020 and P=0.002, respectively **(Table 4)**.

In this study, 20.4% of subjects had exclusive BMF at 4 weeks after birth. In peer support group - 34.4%, in health care provider's education group - 16.7%, and in control group - 9.7% of research subjects had exclusive BMF 4 weeks after birth. Fourteen percent of subjects had exclusive BMF at 8 weeks after birth. Chi square test showed that three groups had significant differences in the rate of exclusive BMF at 4 and 8 weeks after birth (P=0.043 and P=0.023) **(Table 5)**.

Both peer support and health care provider's education had no significant difference in the rate of exclusive BMF at 4 weeks after birth (P=0.111), but they significantly differ in terms of the rate of exclusive BMF at 8 weeks after birth (P=0.027).

Discussion

These findings demonstrated that peer support and health care provider's education were similar in exclusive BMF duration. However, peer support was more effective than health care provider's education in increasing exclusive BMF rate. Compared with routine care, both groups of peer support and health care provider's education show some effect in extending the rate of exclusive breastfeeding. Assessing the effect of confounding variable "assistant help in child care at and after birth" on the duration of exclusive BMF showed that receiving peer support or training by health care providers did not increase the duration of exclusive BMF; but increased remarkably the rate of exclusive BMF.

Partners are particularly important because their approval means so much to a mother, and her partner is often her primary source of support. The baby's

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grandmothers are also very influential because mothers who have recently given birth rely on them for support and advice. To make breastfeeding successful, mothers need the support and encouragement of all of these people (Health and Services, 2011).

Dennis (2002) and Anderson (2005) concluded that peer support increases the rate of exclusive BMF that are in agreement with our result (Anderson et al., 2005; Dennis et al., 2002).

The findings of Meglio (2010) and Vari (2000) showed significant improvements in exclusive BMF duration resulting from peer support, whereas Graffy (2004), Muirhead (2006) and our research did not (Graffy et al., 2004; Meglio et al., 2010; Muirhead et al., 2006; Vari et al., 2000). Given the diversity of cultures and philosophies underpinning health service systems in different countries, it is unlikely that one generalised intervention will provide a magic bullet to increase breastfeeding (Hoddinott et al., 2011).

In addition, Vari examined breastfeeding status with the question "To provide feeding your infant, how long (the number of weeks) you use your milk?" Megilo investigated at the end of each week lactation status by the phone (Haroon et al., 2013; Vari et al., 2000), but in our study, daily feeding reports were used because that is closer to evaluation of infants' feeding pattern (Sheehan, 1999).

Belay (2013) and Artieta-Pinedo (2012) showed that education could increase exclusive BMF rates (Artieta–Pinedo et al., 2013; Belay and Haidar, 2013) which were consistent with the present study.

But Ansari (2014) and Sheehan (1999) concluded that breastfeeding education could increase the duration of exclusive BMF (Ansari et al., 2014; Sheehan, 1999) that was in contrast with the present study. Ansari utilised an integration method of peer education and professional education for intervention (Ansari et al., 2014) and Sheehan used peer education group in the presence of their spouses and in the education group by midwives. It seemed interventions that combine health professional and peer may be effective in increasing breastfeeding (Jolly et al., 2012). Moreover, women should make their spouse and family involved in deciding on BMF because their support affects initiating and continuing of BMF and to increase the duration of exclusive BMF (Esfahani Mm, 2009; Olang et al., 2009; Raine, 2003; Uchendu et al., 2009).

Su's study (2007) showed that exclusive BMF rate did not differ significantly between education and professional support groups (Su et al., 2007). Also, in the present study, the rate of exclusive BMF at 4 weeks after birth did not significantly differ between health care provider's education and peer support groups, but peer support increased the rate of exclusive BMF at 8 weeks more than health care provider's education. In the present study, peer supporters emphasised to each mother that they are available and dedicated to help them with their breastfeeding needs and challenges. The supportive actions of the

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peer supporters vary according to the mother's needs at the moment and peer supporters' assessment of the situation.

Because of sampling selection in health care centres, the effect of some prenatal factors, such as drugs during delivery and neonatal Apgar score, were not assessed on exclusive BMF. However, some factors such as mode of delivery, interval between birth and infant's first contact with the mother, interval between birth and the first baby BMF, duration of the first BMF, mother's satisfaction with delivery, and postpartum complications were controlled as much as possible.

Conclusion

In this study, education and peer support increased the number of women who fed exclusively with breast milk, to continue exclusive breastfeeding, and to increase its duration. The help of family is more important than education and peer support. Therefore, it is suggested that families are educated about the importance of supporting and helping lactating mothers. All women should be offered education and peer support to breastfeed their babies to increase the exclusive breastfeeding rate. But to continue exclusive breastfeeding, and increase its duration, the help of family is more important than education and peer support. Support that is only offered reactively, in which women are expected to initiate the contact, is unlikely to be effective; women should be offered ongoing support so they can predict that support will be available. Support should be tailored to the needs of the setting and the population group. The strength of the study is that it was conducted in 3 groups by multistage sampling method, and then the groups were randomly allocated into peer support, health care provider's education, and control groups. The generalisation of our findings to other breastfeeding women is limited by the small sample size.

Abbreviations

WHO: World Health Organization; BMF: breast milk feeding, ANOVA: The analysis of variance

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Author Contributions

Asieh Moudi contributed to study design, data acquisition and analysis, and wrote the manuscript. Mahin Tafazoli contributed to write and edited the manuscript. Hasan Boskabadi to reviewed and edited the manuscript for intellectual content. Saeed Ebrahimzadeh contributed to data analysis and reviewed and edited the manuscript for intellectual content. Hamid Salehiniya designed the study, analyzed data, and wrote the manuscript. All authors gave final approval of the version to be published. Asieh Moudi is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors reed the final version of article.

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Original Research

A preliminary evaluation of the effects of *Camellia sinensis* on stroke induced rat model

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Abstract

Introduction: The objectives of current study are to test for Neuroprotective activity of *Camellia sinensis* in rat model of stroke and to evaluate the effect of *Camellia Sinensis* as anti-thrombolytic agent and in lowering the impact of disease with the behavioural changes before and after the induction of Stroke. **Methods**: Forty male albino rats were subjected to middle cerebral artery occlusion method for induction of stroke. *Camellia sinensis* extract was administered orally for 21 consecutive days prophylactically. Ischaemic rats administered the same volume of tap water were used as a control group. Functional outcome tests (Pasta, forelimb flexion, cylinder, staircase) were performed. Rats were subjected to surgical procedures after 21 days' treatment for analysis of stroke recovery. **Results**: Treatment with *Camellia sinensis* extract of 400 mg/kg PO significantly (*P=0.000*) enhanced neurological recovery in all tests performed. There was no significant difference of infarct volume among the experimental groups treated with *Camellia sinensis* extract 200 mg/kg PO. **Conclusion**: The outcomes of this study was vivid that *Camellia sinensis* extract is safe and effective mediator in clot dissolution and stroke reversal in rat model. It is the first agent found effective in no behavioural modification or adverse effects using its extract. Therefore, there is a need to evaluate, assess and appraise its desired characteristics and therapeutics in human subjects.

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Keywords

Camellia sinensis, Pasta, Forelimb flexion, Cylinder, Staircase

Introduction

Stroke is the third leading cause of death in the developed world. Stroke is characterised by the World Health Organisation (WHO) as "the fast interruption of brain capacity with indications of central (or global) development with side effects enduring 24 hours or more, or that prompts passing with no other obvious cause other than that of vascular starting point." In industrialised nations, this wellbeing issue is the essential driver of building up a long haul handicap and the third driving reason for death; the primary spot being taken by coronary illness and second place of all other diseases together (Warlow et al., 2011). Stroke comprises of two obsessive subtypes: Ischaemic and haemorrhagic (Gomes and Wachsman, 2013). Atherosclerosis is the major risk factor; ischaemic stroke and its risk elements are in this manner imparted to all other infectious states brought on by atherosclerosis including myocardial dead tissue (Gorelick, 1993).

David Suzuki said, "The medical literature tells us the most effective way to reduce the risk of heart disease, cancer, stroke, diabetes, Alzheimer's and many more problems is through diet and exercise." One of these is green tea (*Camellia sinensis*). The therapeutic impacts of tea have a history going back right around 5000 years. The concoction parts of green tea primarily incorporate polyphenols, caffeine and amino acids. *Radix Salviae Miltiorrhizae* (RSM) is a powder which is extracted and processed from dried root and rhizome of *Salvia miltiorrhiza Bunge*, family *Labiatae*. It has been used for increasing the cerebral blood flow and therefore has a potential against cerebral ischaemia (Tang et al., 2002). It has been tried against various models of cerebral ischaemia like ligation of the carotid artery in gerbils and 4-vessel occlusion model in rats and has been found to be effective. Kuang et al demonstrated that RSM reduced the lipid peroxidation and afforded cerebral protection against reperfusion injury (Kuang et al., 1996). Recently, it has been shown that RSM has the actions of improving blood circulation and resolving stasis to promote regeneration in traumatic intracranial haematoma (Sun et al., 2009) and also has been used for the management of cardiovascular disease (O'Brien et al., 2011). Tetramethylpyrazine (TMP) is widely used in the treatment of ischaemic stroke by Chinese herbalists and is one of the most important active ingredients of the traditional Chinese herbal medicine *Ligusticum wallichii* Franchat (Chung Xiong). However, the mechanism by which TMP protects the brain is still not clear,

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although neuroprotective effects of TMP against ischaemic brain injury might involve its anti-inflammatory potential (Liao et al., 2004). Experimentally, TMP has been shown to induce vasodilatation, to increase coronary blood flow and inhibit ADP induced platelet aggregation. These properties of TMP apparently account for its efficacy in the treatment of disorders associated with blood vessel occlusion like cerebral ischaemia (Luo et al., 1994). Ginseng, the root of *Panax ginseng*, is a well-known traditional Chinese herbal medicine. It is a slow-growing perennial plant with fleshy roots, in the *Panax* genus, in the family Araliaceae. Ginsenoside Rd (GSRd), one of the main active ingredients in *Panax ginseng*, exhibited remarkable neuroprotection when presented during oxygen glucose deprivation and reoxygenation, which may be ascribed to its anti-oxidative properties by reducing the intracellular reactive oxygen species and malondialdehyde production; increasing glutathione content; and enhancing the antioxidant enzymatic activities of catalase, superoxide dismutase and glutathione peroxidase. These findings suggest that it may be a potential neuroprotective agent for cerebral ischaemic injury and further studies are required to explore the potential neuroprotective efficacy of GSRd (Son et al., 2009).

Tea likewise contains flavonoids, mixes answered to have hostile to oxidant properties having numerous valuable impacts. Tea flavonoids decrease irritation, has antimicrobial impacts and counteracts tooth rot. A related compound found in tea is theophylline, an authorised prescription for the treatment of respiratory maladies, for example asthma. Today's PC driven world creates muddled way of life and utilisation of certain characteristic item like tea might just supplant the evil impacts of substance medications prompting a more secure world with more joyful life (Sharangi, 2009). The water concentrate of green tea and lemon grass was examined for their cancer prevention agent, impacts on pale skinned person, rats with 100 mg/kg body weight of green tea (Ojo et al., 2006).

The objectives of the present study are to assess the neuroprotective activity of *Camellia sinensis* in a rat model of Stroke.

Materials-Methods

Total forty male albino rats, aged 12 months, weighed ranging from 290 – 300 g were taken and kept in standard cages (L = 595 mm x W = 380 mm x H = 200 mm), ten rats per cage. Animal cages were maintained in a controlled environment at a temperature of 22° C, humidity 40 - 60% and light between the hours 07.00 - 19.00. This study was conducted in three phases.

During **Phase-I**, rats were acclimatised for seven days and behavioural modulation of the rats was done. Four tests were implemented to rats. These include cylinder test, staircase test, pasta test and forelimb flexion test. The Cylinder test is designed to evaluate locomotor asymmetry in rat models having

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CNS disorders. The staircase test is designed for the measurement of side-specific deficits in coordinated paw reaching in rats and has been shown to reveal impairments on the contralateral side following unilateral lesions in a wide range of motor structures of the brain. Pasta test develops a simple quantitative measure of forepaw dexterity that is sensitive to lateralised impairment changes. Similarly, a very basic assessment used to detect neurological deficits is a test of forelimb flexion.

In **Phase-II** these rats were trained and divided into four groups, each comprising of 10 rats. Group A received normal saline, while Group B was Sham group. Sham surgery (placebo surgery) group is a fake surgical intervention that omits the step that is believed to be therapeutically necessary. In clinical trials of surgical interventions, Sham surgery is an important scientific control. This is because it isolates the specific effects of the treatment as opposed to the incidental effects caused by anaesthesia, the incisional trauma, pre- and post-operative care and the patient's perception of having had a regular operation. Thus, Sham surgery serves an analogous purpose to placebo drugs, neutralising biases such as the placebo effect. Group C received *Camellia sinensis* 200 mg/kg, and group D received 400 mg/kg *Camellia sinensis* extract orally. Extract of *Camellia sinensis* was prepared as specified by Cheruiyot et al (2015) (Sigei et al., 2015). Prepared soluble granules of both black and green tea samples sealed in silver lined sachets stored at room temperature were obtained. Cold aqueous crude extracts were made by soaking weighed amount of dry soluble granules of tea (10 g) in 100 mL of sterile distilled water and shaken for half an hour in an electric shaker. The extracts were filtered using Whatman No. 1 filter paper to exclude any suspending particles. Crude extract, supernatant was then transferred to sterile screw cap bottles, labelled and stored under refrigerated condition (40°C) until use. Crude extract, filtrate was then transferred to sterile screw cap bottles, labelled and stored under refrigerated condition (40°C) until use. Only fresh extracts were used in the experiment, as marked chemical changes occurred when tea was allowed to stand (Sigei et al., 2015).

During **Phase-III**, ischaemic stroke was induced through Middle Cerebral Artery Occlusion (MCAO) method. The MCAO method involves the threading of carotid artery resulting in cessation of blood flow and subsequent brain infarction in the MCA territory. This technique was used for transient occlusion. The suture was removed after 60 minutes, reperfusion was achieved. The highlights of surgical procedure are shown in **Fig. 1**. To evaluate the extent of cerebral infarction, we stained brain slices with 2, 3, 5-triphenyltetrazolium chloride (TTC) to identify ischaemic brain area as shown in **Fig. 2**.

Data was analysed on SPSS statistical software version 22.0 using One-Way ANOVA. Tukey range Post Hoc test was applied considering $P < 0.05$ as significant.

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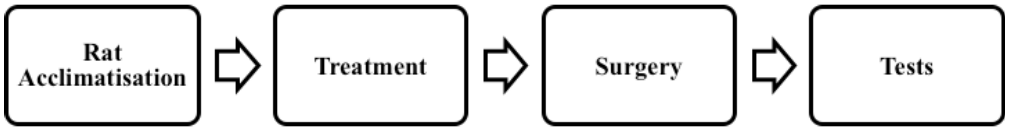
Ethical Committee Statement

Research protocol and use of animals (Rats) was approved by the Committee on Animal Ethics, Hajvery University, Lahore-Pakistan.



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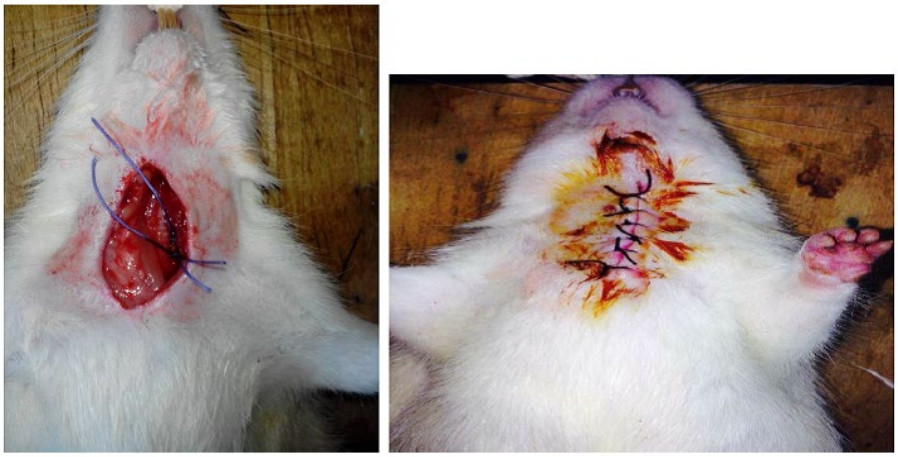

Figure 1. Surgical procedure for induction of ischemic stroke.

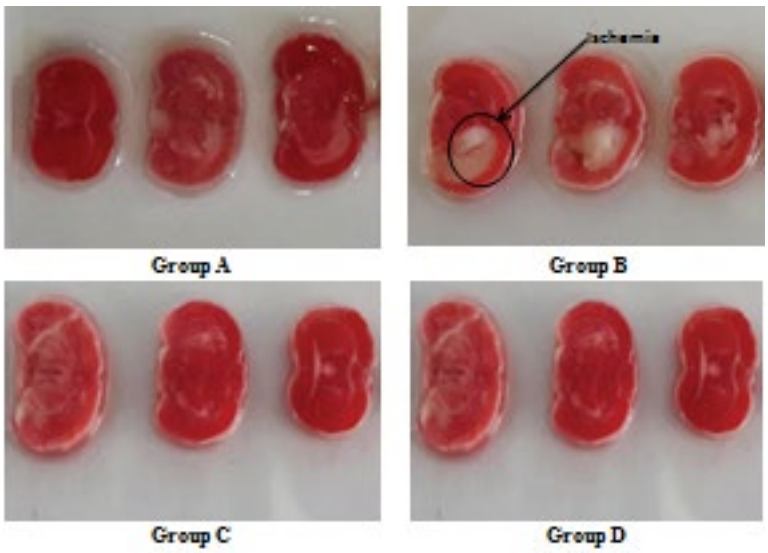

Figure 2. Brain slicing of rats. Area shown in white shows ischemic region as clearly seen in Group B.

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Results

This study was designed for the evaluation of neuroprotective activity of *Camellia sinensis* extract (CSE) in rat model of stroke. Behavioural modulation of rats was performed and following four tests were applied. These include cylinder test, forelimb flexion test, staircase test and pasta test.

Overall performance was measured as the total number of falls, movements and grips in relation to time was observed and recorded in a row of 1st, 4th, 7th, 10th and 13th day. All four tests were performed and their behaviour was observed as shown in **Fig. 03**.

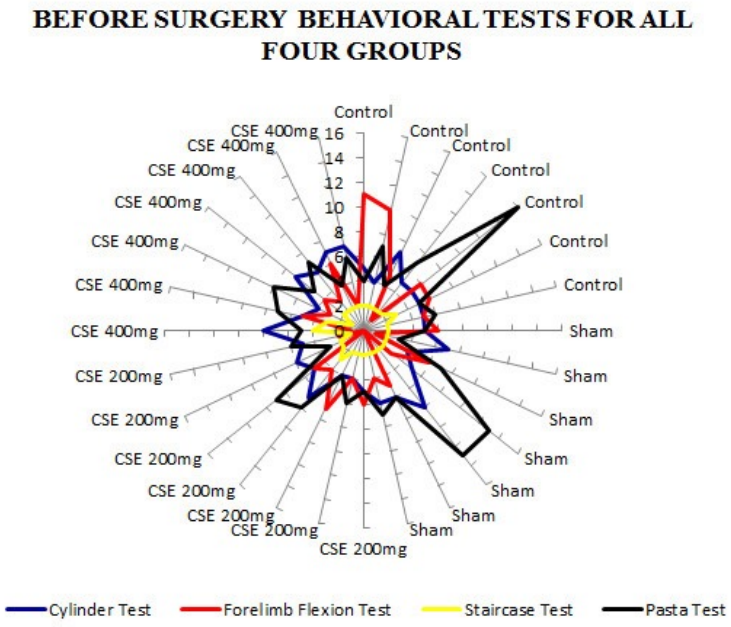

Figure 3. Shows the rat behavior and task performance before induction of stroke.

It is shown from **Fig. 3** that all rats acquired the basic task. However, few rats in control and Sham groups in Forelimb Flexion and Pasta tests showed quicker response and behaviour. It may be caused by their physiological function. It was also noted that those rats that were administered with CSE showed a little aggressive behaviour, which may be further evaluated in future research. In short, our finding has no significant difference in behaviours of animals of all the groups and they were in appropriate condition for surgical procedure.

Cylinder Test

Cylinder test is intended to evaluate locomotor asymmetry in rat models having CNS disorders. It was observed during experimentation that there was significant difference between CSE 200 mg (0.714 $\pm$ 0.420, 1.142 $\pm$ 0.260, 1.142

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± 0.142) and control (5.142 ± 0.340) as well as Sham groups (1.142 ± 0.260, 0.857 ± 0.260, 1.285 ± 0.285) in 24, 48 and 72 hours intervals *(p = 0.000, p = 0.000, p = 0.000)* as shown in **Fig. 4**. However, results of CSE 400 mg (5.142 ± 0.260, 3.857 ± 0.670, 5.571 ± 0.297) and control (5.142 ± 0.340) are not significant *(p = 1.000, p = 0.072, p = 0.953)*. This indicates that CSE 400 mg has high influence in the recovery of stroke at all three time intervals among all defined treatment groups. Moreover, CSE 200 mg (0.714 ± 0.420, 1.142 ± 0.260, 1.142 ± 0.142) did not produce an exemplary effect on stroke and remained almost parallel to Sham (1.142 ± 0.260, 0.857 ± 0.260, 1.285 ± 0.285) group. Data also show that homogeneity of variance in the cylinder test *(p = 0.459)* is far from the level of significance declared, i.e. 0.05.

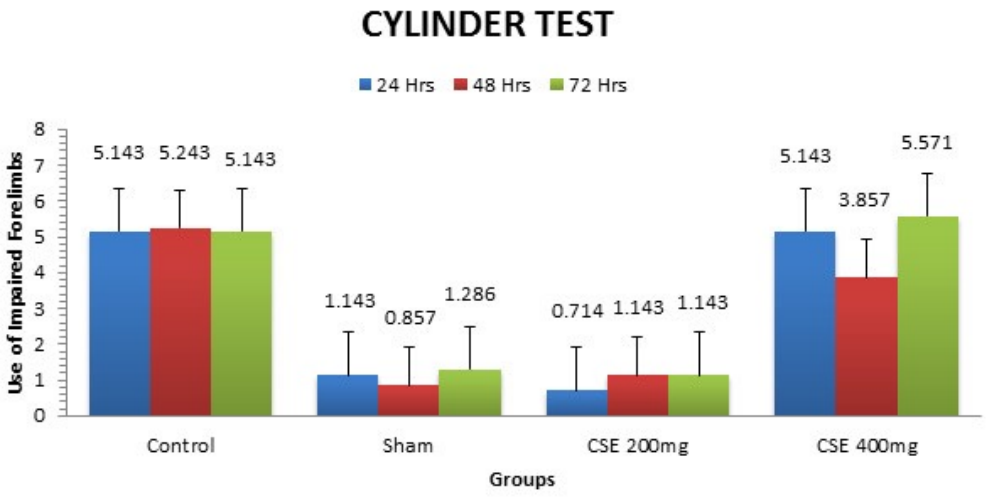

Figure 4. Cylinder test. Figure shows that the cyclinder test results at 24, 48 and 72 hrs intervals. This indicates that CSE 400 mg has high influence in recovery of stroke at all three time intervals among all defined treatment groups.

Forelimb Flexion Test

A very elementary evaluation used to distinguish neurological discrepancies is the test of Forelimb Flexion. Significant difference was found among CSE 200 mg (1.428 ± 0.202, 1.428 ± 0.202, 1.571 ± 0.202), control (6.285 ± 1.267) and Sham (0.571 ± 0.202, 0.428 ± 0.202, 0.714 ± 0.184) groups in 24, 48 and 72 hours intervals *(p = 0.000)* respectively as demonstrated in Fig. 05. However, CSE 400 mg (4.428 ± 0.202, 5.000 ± 0.308, 5.428 ± 0.368) and control (6.285 ± 1.267) groups are non-significant *(p = 0.040, p = 0.275, p = 0.710)*, which shows that CSE 400 mg is very effective in induced stroke at all levels of treatment intervals. On the other hand, CSE 200 mg (1.428 ± 0.202, 1.428 ± 0.202, 1.571 ± 0.202) showed little improvement in stroke recovery. Data of Forelimb Flexion show homogeneity of variance *(p = 0.000)*.

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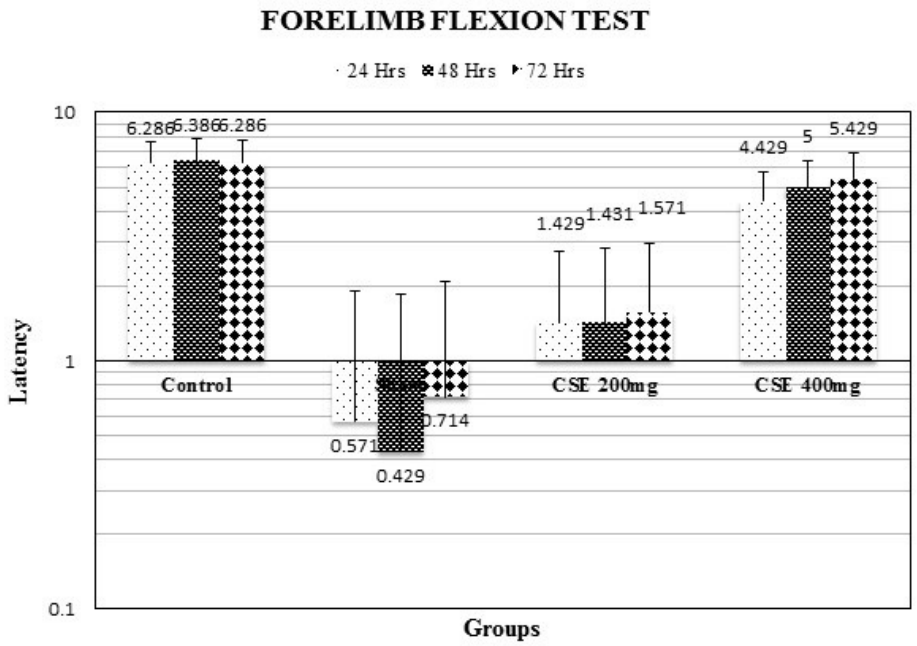

Figure 5. Forelimb flexion test. Figure demonstrates the Forelimb Flexion test results at 24, 48 and 72 hours intervals. CSE 400mg found effective in induced stroke at all levels of treatment intervals.

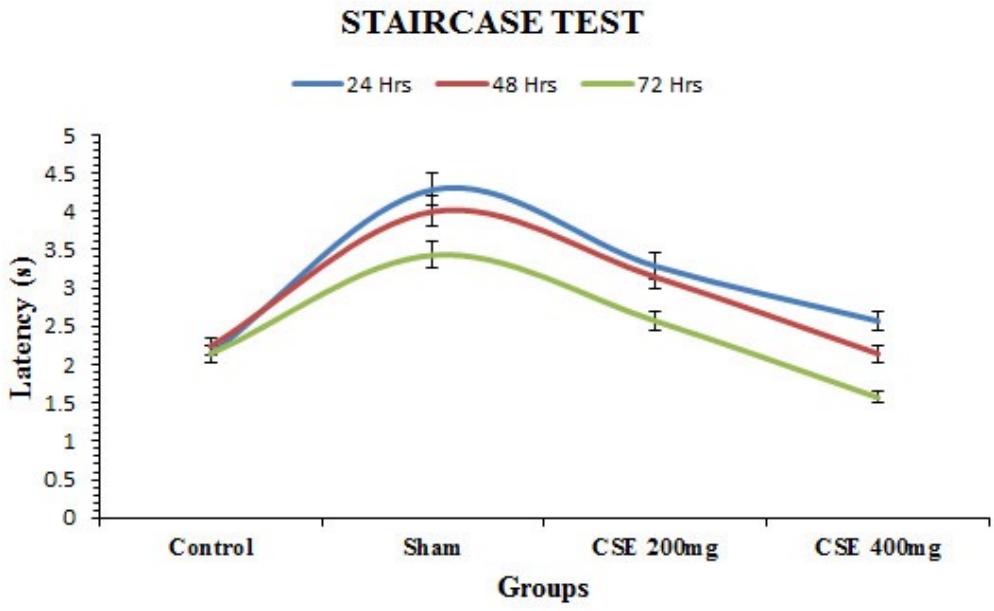

Figure 6. Staircase test. Figure illustrates the Staircase test results at 24, 48 and 72 hours intervals. CSE 400mg had a positive response in nervous coordination.

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Staircase Test

Staircase test is a well-known test for the measurement of deficits in coordinated paw reaching in rats and shown to expose damages on the contralateral side following unilateral lesions in a wide range of motor structures of the brain. When we statistically analysed the control group with all other three groups, we found no really significant results with CSE 200 mg *(p = 0.163, p = 0.134, p = 0.904)* and CSE 400 mg *(p = 0.904, p = 1.000, p = 0.695)* irrespective of Sham that was highly significant *(p = 0.000, p = 0.000, p = 0.027)*. Staircase test showed that although the results are not significant in all experimental groups, but CSE 400 mg had a positive response in nervous coordination as illustrated in **Fig. 6**.

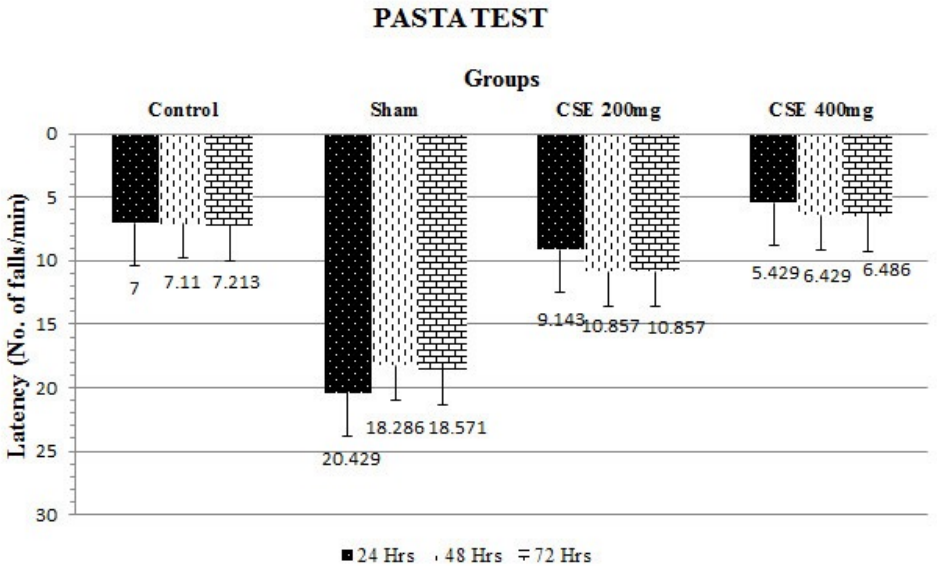

Figure 7. Pasta test. Figure shows the Pasta test results at 24, 48 and 72 hours intervals. CSE 400mg showed the best strength in comparison to all groups.

Pasta Test

Pasta test is used for simple quantitative extent of forepaw dexterity that is sensitive to lateral impaired changes. Statistical analysis showed significant results between Control (7.000 ± 1.573) and Sham (20.428 ± 2.158, 18.285 ± 1.569, 18.571 ± 1.411) group *(p = 0.000, p = 0.000, p = 0.000)*, while other groups showed insignificant results in comparison to control. As the **Fig. 7** demonstrated, CSE 400 mg (5.428 ± 0.368, 6.428 ± 0.368, 5.285 ± 0.285) showed the best strength in comparison to all groups. In addition, level of homogeneity of variance is highly significant in Pasta test *(p = 0.000)*.

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Discussion

The objective of the present research study was to evaluate the neuroprotective activity of *Camellia sinensis* extract (CSE) in stroke induced rat model. The data was subjected to analysis at different levels of complexity according to the requirements of the specific experimental investigations. After administration of CSE for the specific period of time not more than 21 days, rats were subjected to surgery according to the procedure as specified in previous sections. CSE administered was 200 mg and 400 mg/kg. Two doses were selected on the basis of dose ranging study. A dose-ranging study is a clinical trial where different doses of an agent (e.g. a drug) are tested against each other to establish which dose works best and/or is least harmful. Typically, a dose ranging study will include a placebo group of subjects and a few groups that receive different doses of the test drug. For instance, a typical dose-ranging study may include four groups: a placebo group, low-dose group, medium-dose group and a high-dose group. The maximum tolerable dose information is necessary to be able to design such groups and therefore dose-ranging studies are usually designed (Ting, 2006).

The subjects were evaluated with different tests and their behaviour was observed post-operatively. Cylinder test is aimed to assess locomotor asymmetry in rat models having CNS disorders. This test has additionally been found to be able to recognise even shallow neurological impedance. It was observed during experimentation that there was much significant difference between CSE 200 mg and control as well as Sham groups at 24, 48 and 72 hours intervals ($p = 0.000$). However, results of CSE 400 mg and control are not significant ($p = 1.000$, $p = 0.072$, $p = 0.953$). This indicates that CSE 400 mg has high influence in the recovery of stroke at all three time intervals among all defined treatment groups. Moreover, CSE 200 mg did not produce an exemplary effect on stroke and remained almost parallel to the Sham group. CSE composition has flavonoids that are involved in improvement of brain function and reduction in stroke damage. The neuroprotective actions of dietary flavonoids involve a number of effects within the brain including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuro-inflammation and the potential to promote memory, learning and cognitive function. This multiplicity of effects appears to be underpinned by two processes. Firstly, they interact with important neuronal signalling cascades leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and differentiation. These interactions include selective actions on a number of protein kinase and lipid kinase signalling cascades, most notably the PI3K/Akt and MAP kinase pathways, which regulate pro-survival transcription factors and gene expression. Secondly, they induce peripheral and cerebral vascular blood flow in a manner which may lead to the induction of angiogenesis and new nerve cell growth in the hippocampus. Therefore, the consumption of flavonoid-rich foods such as berries and cocoa throughout life holds a potential to limit the neuro-degeneration associated with a variety of neurological

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disorders and to prevent or reverse normal or abnormal deteriorations in cognitive performance (Spencer, 2009). In relation to this study, our vivisection found effective as CSE 400 mg installed a stroke reversal program in rat brain.

Forelimb Flexion test is an elementary evaluation for detection of neurological dysfunction. It is one of the sensorimotor tests used to assess brain function with limb coordination. Significant difference was found among CSE 200 mg, control and Sham groups at 24, 48 and 72 hours intervals *(p = 0.000)* respectively. However, CSE 400 mg and control groups are non-significant *(p = 0.040, p = 0.275, p = 0.710)*, which shows that CSE 400 mg is very effective as surgical assault in induced stroke at all levels of treatment intervals. On the other hand, CSE 200 mg showed little improvement in stroke recovery. Parallel research is piloted by Zhang et al (2004) for the investigation of neuroprotective effect. The effect of Crataegus flavonoids on brain ischaemic insults were investigated in Mongolian gerbil stroke model and results suggest that oral administration of this antioxidant increases the antioxidant level in the brain and protects the brain against delayed cell death caused by ischaemia/reperfusion injury (Zhang et al., 2004; Zhao, 2005). Our exploration established the better bridge among paws' grasping and brain function in rat in association with previous studies.

Staircase test is recognised for the evaluation of side-specific discrepancies in synchronised paw reaching in rats, which show the impairments on the contralateral side following individual lesions in an extensive range of motor structures of the brain. No really significant results were found with CSE 200 mg *(p = 0.163, p = 0.134, p = 0.904)* and CSE 400 mg *(p = 0.904, p = 1.000, p = 0.695)* irrespective of Sham that was highly noteworthy *(p = 0.000, p = 0.000, p = 0.027)*. Staircase test showed that although the results are not significant in all experimental groups, but CSE 400 mg had positive response in nervous coordination. CSE 400 mg positively declared the sensory and forelimb reaching capacities along with motor coordination. Similar results was also observed by Freret et al (2006). They used staircase test for the evaluation of above defined boundaries (Freret et al., 2006). Another vital indicator observed in our study was the ability to assess practiced use of the limbs autonomously following impairment. CSE 400 mg made more sensitive restoration efforts for unilateral brain damage when evaluating independent limb use of the contralateral limb. This feature was also observed by Grabowski (Grabowski et al., 1993) with staircase test that optimises our study.

Pasta test is used to measure a modest quantitative measure of forepaw dexterity that is delicate to lateral impaired changes. Statistical analysis showed significant results between Control and Sham group *(p = 0.000)*, while other groups showed insignificant results in comparison to control. CSE 400 mg showed the best strength in comparison to all groups. However, caution should be used in attempting to identify neuroprotective drugs. Neural tissue is complex and a large number of animals/samples are needed before meaningful results can be obtained.

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Conclusion

This research was conducted with the aim to evaluate the neuroprotective activity of *Camellia sinensis* extract prophylactically in surgically induced stroke in rat model. The outcomes of this study point out to the fact that *Camellia sinensis* extract is safe and effective mediator in clot dissolution and stroke reversal in rat model. It is the first agent found effective with out behavioural modification or adverse effects using its extract. Therefore, there is a need to evaluate, assess and appraise its desired characteristics and therapeutics in human subjects.

List of Abbreviations

RSM: Radix Salviae Miltiorrhizae; TMP : Tetramethylpyrazine; GSRd : Ginsenoside Rd; CSE: Camellia sinensis extract; MCAO : Middle Cerebral Artery Occlusion; TTC : 2, 3, 5-triphenyltetrazolium chloride

Author's Contribution

Arsalan Ali designed the study and performed surgery. Tanveer Ahmed Khan wrote the draft of manuscript and statistically analysed the data. Lubna Shakir and Mehtab Ahmad Khan supervised the study. Komal Najam and Fouzia Karim performed the tests. Muhammad Yousaf and Atif Saeed extracted the *Camellia sinensis*. Saad Nabeel collected the data. Awais Ali Zaidi reviewed the manuscript.

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Case report

Pitfalls in fine needle aspiration cytology diagnosis of hyalinizing trabecular tumour - A report of two cases with review of literature

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Abstract

Hyalinizing Trabecular Tumour (HTT) is rare among follicular cell neoplasm and contributes less than 1% of all primary thyroid gland tumours. It is commonly seen in middle age with female prepondenrance. HTT is a benign to malignant potential tumour. The tumour has definite histomorphological features. However cytological diagnosis is challenging. The definite cytological diagnosis of HTT is important to plan the surgical treatment. Here we present two case reports; one in 72 years male and other in 22 years female highlighting the pitfalls in the cytological diagnosis of HTT.

Keywords

Aspiration cytology, hyalinizing trabecular tumour, thyroid

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Introduction

Hyalinizing Trabecular Tumour (HTT) is rare among follicular cell neoplasm and contributes less than 1% of all primary thyroid gland tumours (Gupta et al., 2010; Li et al., 2012; Thompson, 2011). It is considered as a unique entity. However some consider it as a variant of papillary thyroid carcinoma (PTC). It coexist with PTC and also seen with a variety of thyroid lesion (Li et al., 2012; Ninan et al., 2011). Majority are benign. A few cases with metastatic deposits in lymph node / lung are reported.

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Hence it is considered as tumour of uncertain malignant potential and termed as HTT which is adopted by many Pathologists and WHO (Howard et al., 2013; Li et al., 2012). The tumour has definite histomorphological features. However cytological diagnosis of this unusual tumour is difficult (Santosh et al., 2011). Here we present two case reports; one in 72 years male and other in 22 years female highlighting the pitfalls in the cytological diagnosis HTT.

Case report 1

72 year male presented with swelling in the midline of neck, gradually increasing in size since 5 years. No history of pain in swelling or difficulty in swallowing. Patient gives history of Diabetes mellitus and is on treatment since 2 years. Local examination showed a solitary nodule on left lobe of thyroid measuring 8x8 cms. A provisional clinical diagnosis of solitary thyroid nodule was given. Laboratory haematological investigations were within normal limits. Thyroid profile showed; T_3 – 1.2 ng/ml, T_4 – 7.8 mcg/dl, TSH – 1.76 mcIU/ml and anti- TPO- 10.2 IU/ml. Ultrasound showed a well-defined hypoechoic lesion on left lobe of thyroid measuring 2.5x3.6x4.5 cms with increased vascularity. Right lobe showed 3 hypoechoic well-defined nodules, largest measuring 8x8 cms. A radiological diagnosis of multinodular goitre was offered. Fine needle aspiration cytology (FNAC) of the left lobe of thyroid showed highly cellular smear with follicular cells in sheets and clusters having marked Hurthle cell change. Focal area showed follicular cells in radiating/pallisading pattern around thick hyalinized fibrocollagenous tissue (**Fig. 1**). The background showed erythrocytes and scanty colloid. A cytological diagnosis of Hurthle cell neoplasm was considered.

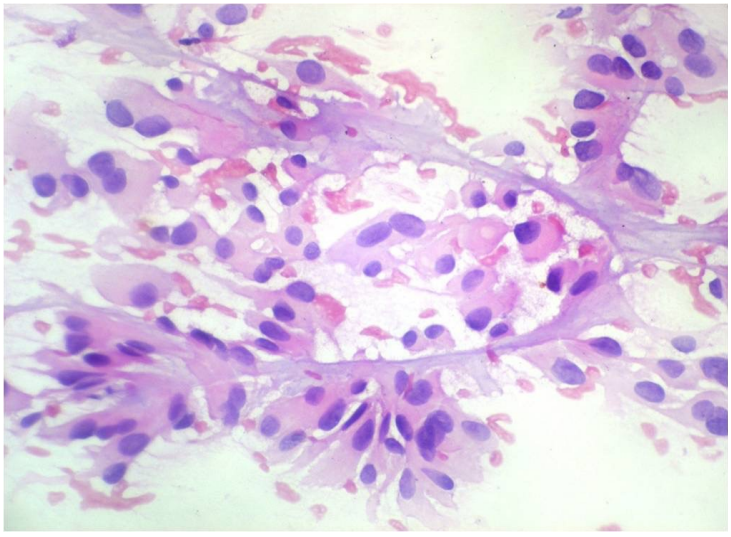

Figure 1. Microphotograph showing follicular cells with abundant amount of cytoplasm arranged in pallisading pattern. H&E X400

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Patient underwent left hemithyroidectomy and specimen was subjected to histopathological examination. Grossly specimen measured 6x5.5x3cm. Cut section showed encapsulated gray white nodular lesion measuring 5x3.5cm with normal thyroid tissue only at one pole (**Fig. 2A & 2B**). Microscopy showed an encapsulated neoplasm composed of tumour cells arranged in cords/ trabecular pattern and in follicular pattern separated by fibrocollagenous tissue. The nuclei showed longitudinally placed nuclear grooves and occasionally intranuclear inclusions. Cytoplasm was moderate and eosinophilic. The tumour cells showed minimal capsular invasion. However tumour cells had not breached the capsule. No vascular invasion was noted. The diagnosis of HTT, with uncertain malignant potential was given (**Fig. 3**). Patient was followed for one year and was uneventful.

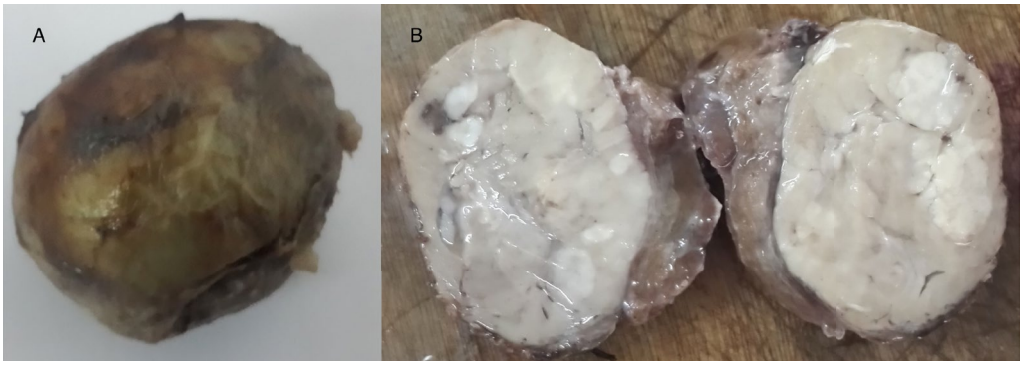

Figure 2. A. Gross photograph showing well encapsulated lobectomy specimen. B. Cut surface shows well defined gray white tumour with normal thyroid tissue at one margin.

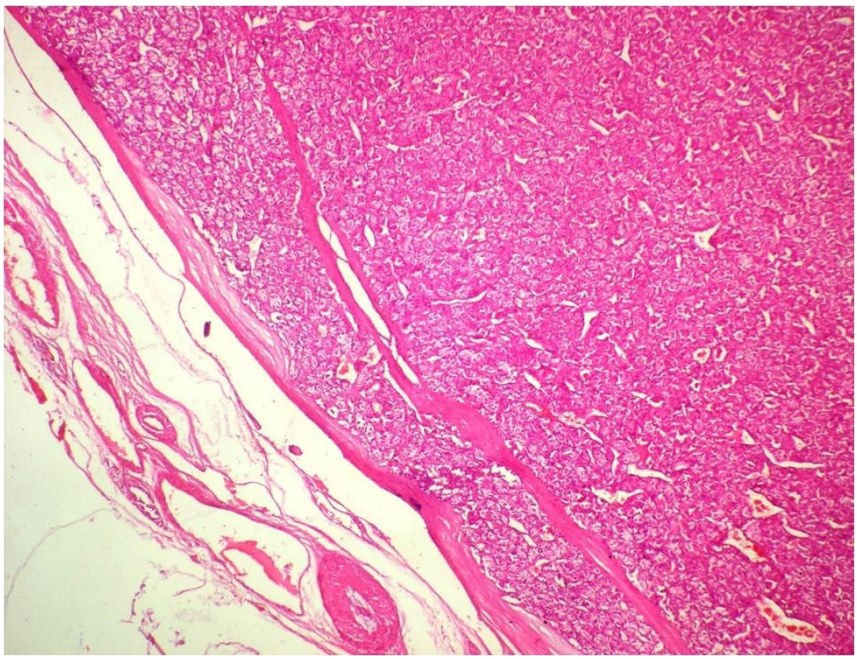

Figure 3. Microphotograph showing tumour cells arranged predominanatly in trabecular pattern with minimal capsular invasion. H&E X100

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Case report 2

22 years female presented with swelling in neck since 4 years with difficulty in breathing. Local examination showed single well defined swelling predominantly in left lobe of thyroid measuring 6x6cm, freely mobile and firm in consistency. A provisional clinical diagnosis of nodular goitre was given.

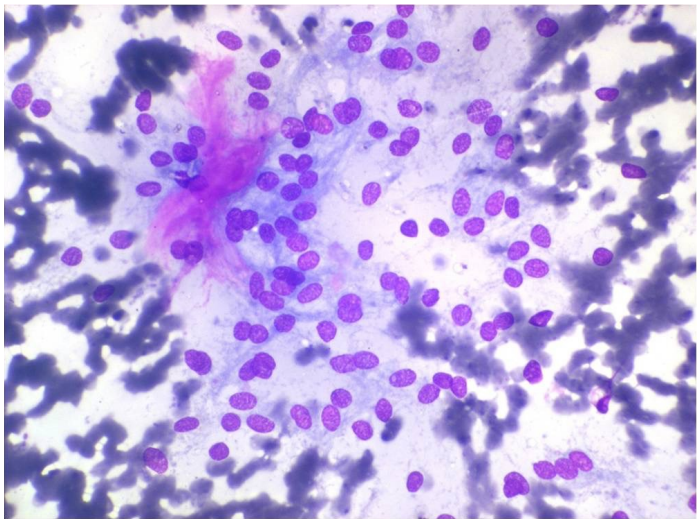

Figure 4. Microphotograph showing tumour cells with abundant amount of cytoplasm, ill-defined cell margin with hyaline material. H&E X400

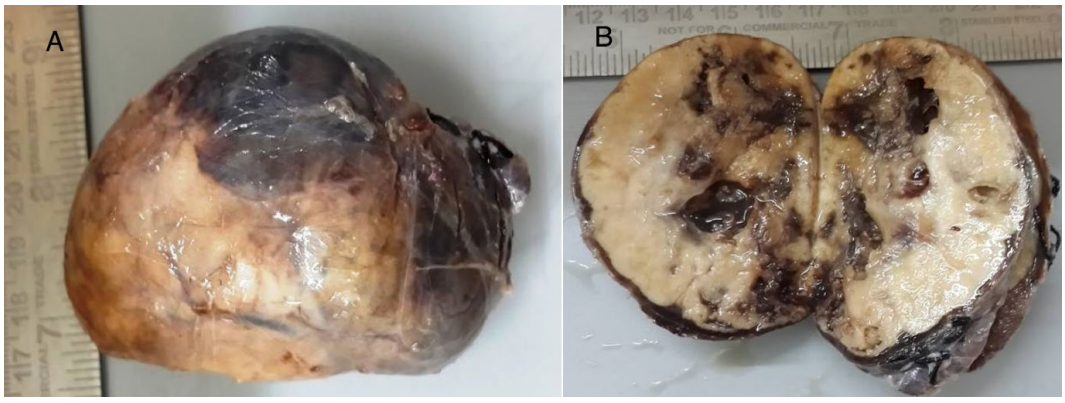

Figure 5. A. Gross photograph showing well encapsulated lobectomy specimen. B. Cut surface shows well defined gray white tumour with focal cystic change.

Laboratory investigations were within normal limits. Ultrasound showed unilateral left thyroidomegaly with features of multinodular goitre. FNAC showed satisfactory cell yield consisting of follicular cells having isonucleosis and scanty cytoplasm. Some of the follicular cells showed Hurthle cell change. Background showed colloid and erythrocytes (**Fig. 4**).

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FNAC diagnosis of nodular goitre with Hurthle cell change was given. Patient underwent left hemithyroidectomy and specimen grossly showed single lobe of thyroid measuring 8.5x 5.3x4 cms. External surface showed intact capsule. Cut section was gray white with one area showed cystic degeneration (**Fig. 5A & 5B**). Microscopy showed an encapsulated tumour consisting of round to oval to spindly cells having fine, granular chromatin, longitudinal nuclear grooves, intranuclear inclusions with moderate to abundant eosinophilic cytoplasm. The cells were predominantly arranged in trabecular pattern with intra and intertrabecular hyaline material. The cells in trabeculae were arranged parallel to each other (**Fig. 6**). A focal area of cystic degeneration was seen. The adjacent thyroid tissue was within normal limits. The tumour did not show capsular/ vascular invasion. A diagnosis of HTT was given. Patient was followed up for 8 months which was uneventful.

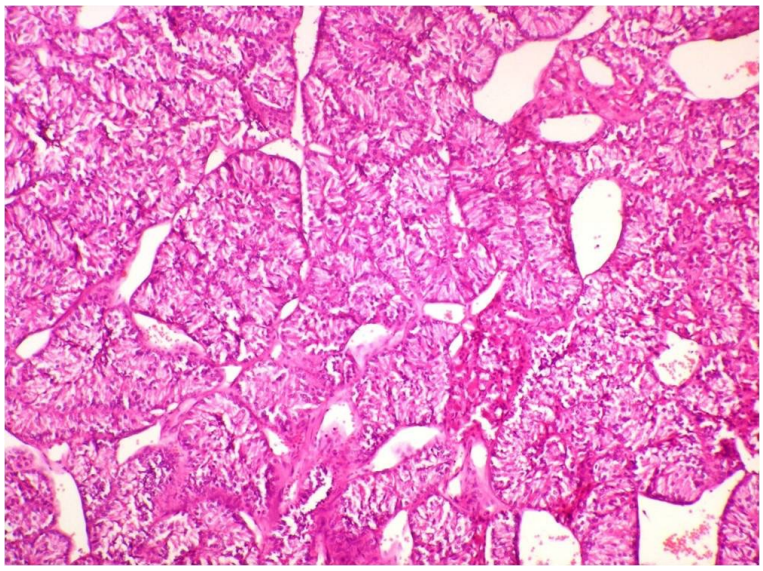

Figure 6. Microphotograph showing tumour cells arranged in pallisading pattern within the trabeculae. H&E X400

Discussion

HTT was originally reported in 1905 (Howard et al., 2013). Later it was described in detail about the trabecular growth pattern and marked intratrabecular hyalinizing stroma in 1987 by Carney (Casey et al., 2004; Gupta et al., 2010; Howard et al., 2013; Jang et al., 2016; Li et al., 2012; McCarthy et al.; Ninan et al., 2011; Santosh et al., 2011). Various etiological factors are put forth as; radiation exposure, arising in a background of chronic lymphocytic thyroiditis and multinodular goitre (MNG). HTT and lymphocytic thyroiditis are proved to have similar molecular genetics, age and gender distribution.

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It can present as solitary/prominent nodule in MNG. There are evidence of possible association with PTC and sometimes considered as variant of PTC due to similar nuclear cytology, immunoprofile and RET oncogene (Gupta et al., 2010; Li et al., 2012; Ninan et al., 2011; Thompson, 2011).

HTT are commonly seen in middle age i.e. in 4th and 5th decade of age and are common in females with female:male ratio of 6:1. The tumour presents as asymptomatic/incidental onset or as an incidental finding in a thyroidectomy specimen (Gupta et al., 2010; Li et al., 2012; Ninan et al., 2011; Thompson, 2011). In the present case reports, one case is 72 years male and other is 22 years female both presenting as incidental finding.

Cytological diagnosis is challenging. The smears are usually hypercellular with polygonal or elongated spindle cells. Nucleus is hyperchromatic with longitudinally placed grooves, pseudoinclusions (cytoplasmic inclusions), perinuclear halo, perinuclear yellow bodies (represent lysososmes) and fine chromatin without optically clear chromatin.

Cells have low nuclear to cytoplasmic ratio. Cytoplasm is abundant. Cell border is ill defined. Sometimes psammoma bodies are seen. These cells will be arranged in a radial pattern around the hyaline material with vague curved nuclear pallisading. The cells will also arranged in sheets along with hyaline material with lack of papillary pattern and in bloody background (Casey et al., 2004; Gupta et al., 2010; Howard et al., 2013; Li et al., 2012; McCarthy et al.; Ninan et al., 2011; Santosh et al., 2011). The cytological differential diagnoses are PTC and medullary thyroid carcinoma (MTC). HTT is mistaken for PTC because of nuclear features and presence of psammoma bodies. It is mistaken for MTC for the elongated/ spindle cells and the hyaline material. However the hyaline material in HTT is solid/homogenous and well defined than amyloid and not contain the streaked nuclear material as in amyloid. Cells in HTT do not have cytoplasmic granules (Gupta et al., 2010; Howard et al., 2013; Jang et al., 2016; McCarthy et al.). In the present case reports, in the first case the cells showed cytoplasmic features as mentioned above, however the nuclear features were absent and hence the cells were considered as Hurthle cells. The cells were arranged in pallisading pattern around thick hyaline fibrocollagenous tissue which was not accounted. The diagnosis of Hurthle cell neoplasm was given. In the second case a few follicular cells showed Hurthle cell change along with erythrocytes and colloid in background. The arrangement of cells did not show any definite pattern. The diagnosis of nodular goitre with Hurthle cell change was given. In both cases the cells did not show cytoplasmic granules.

The tumor grossly presents as solitary/multiple nodules with average size of 2.5cm. Cut section is well circumscribed/encapsulated usually solitary, solid, yellow tan, lobulated and well defined with foci of calcification. Capsule is thin irregular and uneven (Gupta et al., 2010; Howard et al., 2013; Thompson, 2011).

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In the present two cases, both presented as solitary nodule in left lobe of thyroid of more than 2.5 cms size, well defined, encapsulated and gray white on cut surface. Histomorphologically the cells will be polygonal with oval to elongated nuclei (as in cytological feature) with intracellular and extracellular hyaline material. These cells arranged in trabecular, alveolar and in insular pattern. In trabecular pattern, the cells will be seen as straight/pallisading pattern with nuclear grooves perpendicular to the long axis of the trabeculae. Fibrovascular stroma will be seen in intra and intertrabecular areas with marked hyalinization. The hyalinization shows greater deposition at the periphery of the trabeculae. Mitoses are rare. Psammoma bodies may be seen. Usually no vascular/capsular invasion seen (Gupta et al., 2010; Howard et al., 2013; Ninan et al., 2011; Thompson, 2011). In the present two cases all the mentioned histomorphological classical features were present. However this shows the poor cyto-histo correlation in the present cases considering cytological diagnosis of HTT. One study has shown the poor performance of even ultrasound guided FNAC in cytological diagnosis of HTT (Jang et al., 2016).

The definite diagnosis of HTT is important as it determines the treatment. The treatment of choice for HTT is lobectomy. Misdiagnosis gives rise to mistreatment and total thyroidectomy which is reported in 44-71% of cases.

Cytopathologists should know the cytomorphological features of HTT for precise cytological diagnosis which helps to plan the surgery and avoid surgical overtreatment, postoperative levothyroxine supplementation / radiodine ablation therapy or follow-up with serial thyroglobulin antibody levels. Hence expertise of cytopathologist is very important. Clinicians should be aware that HTT is a benign entity, however it can behave as a potentially malignant tumour (Howard et al., 2013; Li et al., 2012; McCarthy et al.; Thompson, 2011). The prognosis depends on careful screening of tissue sections for vascular, capsular and parenchymal invasion to eliminate the possibility of malignancy (Gupta et al., 2010; Howard et al., 2013; Li et al., 2012).

Conclusion

Even though HTT is a rare entity, one should be aware of this tumour, its presentation and cytomorphological features by which definite cytological diagnosis can be offered and surgery can be planned appropriately.

List of Abbreviations

FNAC: Fine Needle Aspiration Cytology; MNG: Multinodular Goitre; MTC Medullary Thyroid Carcinoma; HTT: Hyalinizing Trabecular Tumour; PTC: Papillary Thyroid

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Carcinoma; TPO: Thryoid Peroxidase; TSH: Thyroid Stimulating Hormone; WHO: World Health Organization

Author Contribution

Kalyani. R.: Data collection, literature search, concept, manuscript writing, editing and review; Sweta Sinha: Data collection, typing of manuscript.

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Review

The effects of transplanted cells in stem cell therapy for myocardial ischemia

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Abstract

It is known that myocardial infarction (MI) causes damages to the heart tissue and that present medical therapies, such as medication, stenting and coronary artery bypass surgery, cannot recover the injured heart. Fortunately, advances in stem cell research have brought hope of full heart recovery for myocardial ischemia patients. There have been many studies using cell therapies for myocardial ischemia, from preclinical trials to clinical trials. However, the biggest concern is the effect of transplanted cells in myocardial recovery. This review will focus on analyzing both the positive and negative effects of transplanted cells in myocardial recovery to better understand the underlying biological mechanisms and ways to evaluate safety and efficacy of cell transplantation in myocardial ischemia treatment.

Keywords

Biological mechanism, effect, myocardial ischemia, stem cell therapy, transplanted cells

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Introduction

Myocardial infarction (MI) kills cardiac cells and forms scars in the heart tissue. Although the formation of scars helps the injured heart cope with damages quickly, protects healthy tissue from further damage and prevents a cascade of adverse uncontrollable events (Azouz et al., 2004), the biochemical reactions during scar formation remain unclear. In fact, the physical and functional properties during heart tissue scarring is similar to those of normal tissues. Scarring has negative effects on the structure and activity of the infarcted heart (Cregg et al., 2014; Silver and Miller, 2004; Xu et al., 2004).

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Current treatments such as lifestyle changes, medication, stent intervention or artery bypass surgery can only support the heart to slow the failure process, but cannot recover damaged heart tissues. The successful rate of all heart transplantation was very low. The number of novel postnatal heart tisue was low too and it was around 1% of total myocardium and decreased with age (Garbern and Lee, 2013).

Nowadays with the development of regenerative medicine, cell therapy can be expected to completely restore the structure and function of the damaged heart. Cell transplantations into ischemic areas have been investigated in small animal models (Avolio et al., 2015; Kim et al., 2015; Tang et al., 2015), large animal models (Kanazawa et al., 2015; Yee et al., 2014) and in clinical trials (Karantalis et al., 2012; Perin et al., 2015). Various cell types have been used in implantation, including mesenchymal stem cells (MSCs) (Kocher et al., 2001), induced pluripotent stem cells (iPSCs) (Cantz and Martin, 2010), cardiac progenitor cells (CPCs) (Garbern and Lee, 2013), cells derived from fetal tissue and adult cardiomyocytes (Soonpaa et al., 1994; Zhang et al., 2001), skeletal myoblasts (Menasche et al., 2001), muscle cells (Condorelli et al., 2001), embryonic-derived endothelial cells (ECs) (Condorelli et al., 2001), bone marrow-derived immature cells (Hattan et al., 2005), fibroblasts (Galli et al., 2005), smooth muscle cells (SMCs) (Harada et al., 2016) and bone marrow-c-kit positive and negative progenitor cells (Fazel et al., 2008; Fernandez-Aviles et al., 2004). In spite of the fact that results with the above have varied, they were largely similar in that there is a positive impact of cell transplantation on the recipients. It is important to understand how the transplanted cells act in heart wound healing and their efficacy. This review will focus on those issues.

Effects of transplanted cells

Most likely transplanted cells reduce negative remodeling by reducing the stiffness of the ventricular wall scar and restoring the lost heart muscle. Proposed mechanisms for this process include: **(1)** the transplanted cells secrete paracrine factors to protect the cells from apoptosis, mobilize the available cardiac stem cells, activate their proliferation and differentiation into heart cells, partake in neovascularization, reduce scar formation and limit inflammation; **(2)** the transplanted cells can fuse with host graft; **(3)** the transplanted cells can differentiate into cardiac muscle cells (**Fig. 1**).

Secretion of growth factors

Secretion of implanted cells plays an important role in repairing heart tissue damage. Adult stem cells, particularly MSCs, after transplantation can release a variety of cytokines, chemokines and growth factors involved in heart repair process (Deb et al., 2008; Li et al., 2012; Loffredo et al., 2011). These factors, in turn, induce neighboring stem cells to secrete cytokines and induce changes in the microenvironment which promote proliferation and differentiation of stem

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cells (Behfar et al., 2002; Doyle et al., 2016; Gude et al., 2015; Kinnaird et al., 2004). In particular, properties such as myocardial protection and neovascularization of paracrine factors currently have been most widely studied. Moreover, the secreted factors also impact positively on the inflammation process, fibrogenic process, heart metabolism, heart contractions and/or endogenous cardiac regeneration. These effects may occur in different ways and are dependent on the microenvironment after infarction. These factors may also act in an autocrine fashion, impacting the cells which secrete them (Deb et al., 2008).

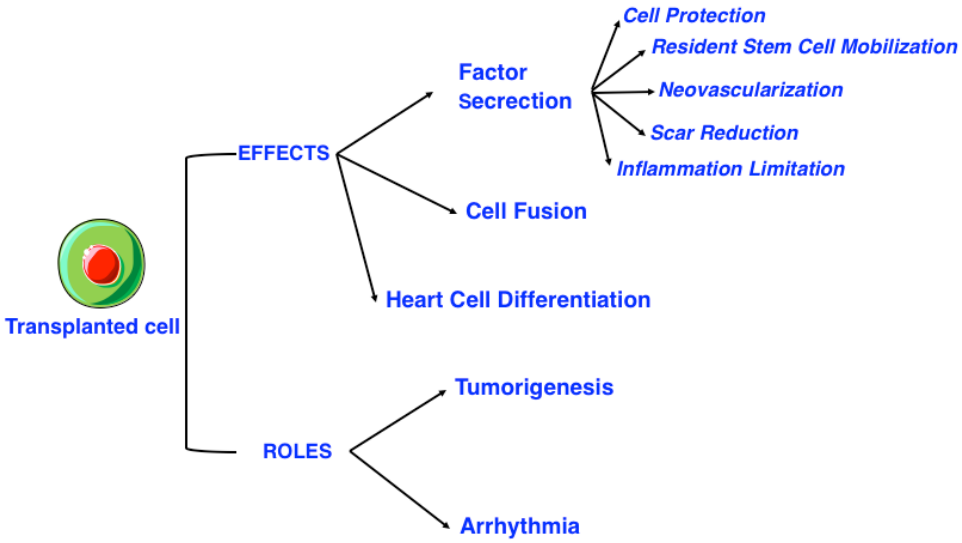

Figure 1. Effects and roles of transplanted cells in myocardial ischemia treatment. Transplanted cells can secrete useful factors to protect cells from appoptosis, mobilize resident stem cells, participate in the process of forming new vessels, reduce the size of the scar in the infarction area or limit the inflammation ; these cells can also fuse with the host cells; or differentiate into heart cells. However, there are some things to consider when choosing the type of cells for transplantation such as their capable of tumorigenesis or arrhythmia causing.

Protection of heart tissue

The immediate impact of stem cells after grafting into the heart muscle is to release cytoprotective molecules to increase myocardial viability. These molecules inhibit apoptosis by activating AKT/PKB signaling pathway (Rosenberg et al., 2012; Yang et al., 2012). Some studies have shown that Akt-overexpressing MSCs and exosomes secreted from CXCR4-overexpressing or GATA4-overexpressing MSCs are able to significantly prevent apoptosis, thereby reducing infarction size (Kang et al., 2015; Noiseux et al., 2006; Yu et al., 2015). Other studies have shown that elements secreted from grafted bone marrow

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stem cells induce cardiomyocyte protection in the ischemic region (Broughton and Sussman, 2016; Dai et al., 2008; Xu et al., 2007). Cardiomyocytes differentiated from bone marrow monocytes (BM-MNCs) cultured under hypoxic conditions show they have inhibitory effects on apoptosis and can reduce infarction size when transplanted into the body (Kubal et al., 2006). When heart muscle cells and BM-MNCs obtained from the same patients were co-cultured, cell necrosis and apoptosis were significantly reduced; cell protection, however, did not occur when heart muscle cells with co-cultured with ECs or keratinocytes (Yoon et al., 2005).

Moreover, transplanted Akt-expressing MSCs also expressed secreted frizzled related protein 2 (Sfrp2), which increases cellular total -catenin of cardiomyocytes. The -catenin protected cardiomyocytes of newborn rats were stable against hypoxia and reoxygenation-induced apoptosis by blocking the pre-apoptotic effects of Wnt3a (Mirotsou et al., 2007; Zhang et al., 2009). Akt-MSCs regulated the increase of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and thymosin 4 (TB4). In turn, these factors promoted neovascularization, protection of myocardium and cardiac contractility (Gnecchi et al., 2006) (**Fig. 2**).

The overexpression of bFGF can enhance the recovery of contractile function and reduce infarction area after reperfusion. In fact, bFGF reduces the protein kinase C delta (PKC-d) displacements but does not affect PKC-alpha (PKC-a), PKC-epsilon (PKC-e), or PKC-zeta (PKC-z). PKC-d reduction protects heart cells and decreases the number of dead cells. In addition, bFGF has been shown to be related to the MAPK/ERK signaling pathway in heart cell protection, although the mechanism remains unclear (Baines et al., 2002; House et al., 2007; Padua et al., 1998; Rose et al., 2010; Srisakuldee et al., 2014). Akt activates a number of substrates, including members of the B-cell lymphoma 2 (Bcl-2) protein family, glycogen synthase kinase 3 beta (GSK-3) and endothelial nitric oxide synthase (eNOS). Nitric oxide (NO) synthesized from eNOS activates PKG through intracellular cGMP increasing. The substrates for protein kinase G (PKG) are thought to include SR regulation proteins and phospholamban, which promotes SR Ca^{2+} absorption thereby reducing the overload of cytosolic Ca^{2+}. PKG is the final component of the signal transduction leading to activation of PKC-epsilon (PKC-e) mitochondrial pool. Activated PKC-e in turn activates mitochondrial ATP-dependent potassium channels (mK_{ATP}), promoting the reactive oxygen species (ROS) formation (**Fig. 2**).

The inhibition of mitochondrial permeability transition pore (MPTP) may occur as a result of PKC-e activation. Sarcolemmal K_{ATP} (sK_{ATP}) and mitochondrial connexin-43 (Cx43) are also considered components of the pre-regulation mechanism. The formation of ROS and reactive nitrogen species (RNS) are the results of mK_{ATP} opening and are mandatory components of the signaling cascade. It seems that ROS /RNS signaling is related to activation of kinases, such as p38, MAPK, PKC and JAK/STAT (Ferdinandy et al., 2007).

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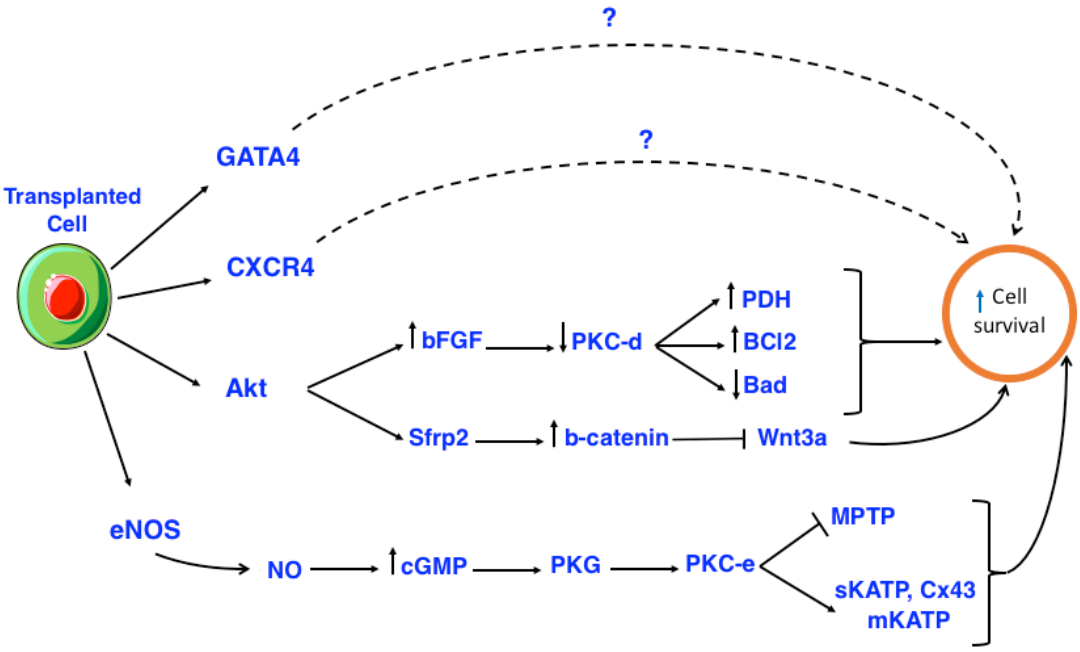

Figure 2. Transplanted cells protect heart tissue by increasing cell survival. CXCR4 or GATA4 overexpressing Mesenchymal Stem Cells released exosomes that prevented cells from appoptosis; the mechenism is unknown. Akt overexpressing MSCs increased bFGF leading to PKC-d reducing that increased PDH, Bcl2 and decreased Bad to protect cell survival. Akt overexpressing MSCs also secreted Sfrp2 which increased cellular total -catenin of cardiomyocytes that blocked pre-apoptotic effects of Wnt3a and thereby protected cardiomyocytes. Akt activated eNOS and NO synthesized from eNOS activated PKG through intracellular cGMP increasing leading to activate PKC-e. PKC-e, in its turn, activate sKATP, mKATP, Cx43 and inhibit MPTP leading to cell protection.

The heart protects extracellular ligands, including adenosine, bradykinin and opioids, while activating multiple kinases, including p42/p44, MAPK/ERK, PI3K/Akt and PKC. The substrates of PKC related to the regulating protective mechanisms are still unknown. There is also evidence that the activation of PKG might occur as part of the post-regulating mechanism downstream of Akt/NO/cGMP, and may play a role in promoting the absorption of sarcoplasmic reticulum Ca^{2+}. It is known that activation of Akt inhibits GSK-3 and members of the chemokine (C-C motif) ligand 2 (Ccl-2) protein family, leading to inhibition of the formation of mK_{ATP}-mediated MPTP (Ferdinandy et al., 2007).

Normally, PKC exists in a non-activated state until it is stimulated by phospholipid diacyl glycerol (DAG)-derived second messengers. Intracellular ROS translocates to the subcellular target locations, including mitochondria, sarcolemmal membrane and gap junctions. Indeed, sK_{ATP}-mediated phosphorylation leads to shortening of the action potential, reducing the Ca^{2+}

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overload during ischemia. Moreover, PKC-mediated phosphorylation of Cx43 reduces connexon permeability and prevents the expansion of damages between co-joined cells. PKC-mediated phosphorylation opens the mK_{ATP}, conserves mitochondria function and forms local ROS. In turn, ROS may initiate PKC signaling via positive feedback. MPTP inhibition can occur by both direct mechanisms (e.g. via PKC phosphorylation) and indirect mechanisms (e.g. via mK_{ATP} opening which reduces cell death after infarction). Receptor for activated c-kinase (RACK) treatment and erythropoietin also promote PKC activation, resulting in heart cell protection (Bearzi et al., 2007; Budas et al., 2007).

In summary, during ischemia reperfusion activated PKC-e in turn activates sK_{ATP} and mK_{ATP}, phosphorylates Cx43 and inhibits MPTP, leading to cell protection. However, activated PKC-d inhibits pyruvate dehydrogenase (PDH), decreases Bcl-2, increases Bcl-2-associated death promoter (Bad) protein, causing apoptosis and necrosis. Thus, transplanted cells protect myocardium by secreting various factors that activate PKC-e and inhibit PKC-d.

Mobilization of resident stem cells

The discovery and recognition of the existence of cardiac stem cells led to a shift by researchers and physicians to explore the use of stem cells for new cardiovascular disease therapies (Bearzi et al., 2009; Hosoda et al., 2009; Urbanek et al., 2006). In the heart, cardiac stem cells (CSCs) have existed in their niches. Normally, these cells have kept "silent" in their niches, and have been nourished and controlled by feeding cells within the niches. Upon receiving trigger signals, CSCs undergo a symmetrical or asymmetrical proliferation; they separate from their niches and migrate to areas where they were needed to replace damaged or dying heart cells. However, the number of CSCs are very rare, at approximately 1 stem cell per 30,000 heart cells (Beltrami et al., 2003; Urbanek et al., 2006). Therefore, based on the normal growth rate of resident CSCs, when infarction occurs the number of cells needed to be replaced is much greater than the number of available cells, leading to a lack of intrinsic cardiac stem cells for replacement. This means that while the injured heart may be delayed in damages in the short term, heart failure cannot be reversed in the long term (Urbanek et al., 2003).

Studies have shown that when MSCs are grafted into the body in the absence of oxygen, they release HGF and IGF-1 to mobilize and activate resident CSCs (Gómez-Mauricio et al., 2016; Linke et al., 2005). Besides MSCs, endothelial progenitor cells (EPCs) play an important role. EPCs activate cardiac regenerative pools and promote the migration, proliferation and differentiation of CSCs (via secretion of cytokines such as VEGF, IGF-1 and SDF-1) (Urbich et al., 2005). These factors induce interstitial CSCs to move through the myocardium to necrotic myocardium and scar areas. There, they divide and differentiate into heart cells and become involved in the process of new blood vessel formation (Bian et al., 2014; Hosoda et al., 2009; Tillmanns et al., 2008). CSC invasion to

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scar tissue is believed to be related to matrix metalloproteinase (MMP)-9 and -14 mediated regulation (Bax et al., 2012; Huang et al., 2011; Rota et al., 2008).

Neovascularization

MSC transplantation improves reperfusion efficiency by increasing the formation of new blood vessels. However, MSCs are rarely present in the new vessels; their main activity is to secrete angiogenic factors, such as VEGF, bFGF, angiopoietin-1, NO and HGF (Kinnaird et al., 2004; Zhao et al., 2010). These factors increase the permeability of the capillary wall, activate MMP, promote the proliferation and migration of ECs and vascular smooth muscle cells (VSMCs), and form new vessels in the lesions (Jiang et al., 2006; Louis and Zahradka, 2010; Nagaya et al., 2004). Besides MSCs, transplanted EPCs also enhance new vessel formation by releasing VEGF and stromal cell-derived factor 1 (SDF-1) into the cellular matrix, thereby promoting the migration and maturation of EPCs into ECs (Cochain et al., 2013; Urbich et al., 2005; Wu et al., 2006). Angiogenic cells have been implanted as hydrogel supplying scaffolds to increase microvasculature along infarction areas, thereby significantly improving coronary blood flow and ejection fraction after MI (Kim et al., 2012; Leblanc et al., 2013; Levit et al., 2013). In addition to angiogenesis factors, adult stem cells secrete TB4 and erythropoietin (EPO) (Lv et al., 2015; Smart et al., 2007). TB4 induces the proliferation and circuit network formation of epicardium-derived cells (EPDCs) and is involved in the intermediate PKC signaling pathway (Smart et al., 2013; Smart et al., 2007). Meanwhile, granulocyte colony stimulating factor (G-CSF) and EPO mobilize hematopoietic stem cells (HSCs) and EPCs from bone marrow for angiogenesis by activating Janus-activated tyrosine kinase 2 (JAK2) through STAT, PI3K/Akt and MAPK signaling pathways (Nagai and Komuro, 2012).

Impact on extracellular matrix (ECM) and reduction of scar formation

After infarction, scars are formed to replace injury tissues damaged by myocardial ischemia. During ischemia, the ECM secretion process is disordered due to heart cells dying and the body's self-regulation. These impact the thickness of the developing scar, leading to an effect on the contraction of the surrounding heart tissue. A decrease in the ECM makes the ventricular wall thinner, causing left ventricular (LV) rupture while an increase in the ECM enhances fibrosis, leading to heart failure over time (Zamilpa and Lindsey, 2010).

From studies, it has been observed that grafted cells are capable of regulating scar formation through inhibition of fibroblast proliferation; furthermore, it has been shown that paracrine factor secretion alters ECM to improve cardiac functions (Berry et al., 2006). Transplanted MSCs reduce myofibroblasts through releasing MMPs (Almalki and Agrawal, 2016; Mias et al., 2009). In MI rat models, implanted MSCs reduce the expression of collagen types I and III, tissue inhibitor of metalloproteinase-1 (TIMP-1), MMP2, MMP9, and transforming growth factor-beta (TGF-beta) (Nagaya et al., 2005; Xu et al., 2005). It is

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interesting that transplanted MSC cardiomyocyte (MSC-CM) also express the ability to reduce the scarring process by downregulating fibroblast proliferation and inhibiting the expression of collagen type I and type III in myofibroblasts (Ohnishi et al., 2007a; Ohnishi et al., 2007b). In addition, embryonic stem cell cardiomyocyte (ESC-CM) also show the ability to reduce scarring after MI (Leor et al., 2007). In sheep, MSCs injected one hour after MI also show changes in MMP-1, -2, -3, -7, -9, -13, membrane type 1-MMP (MT1-MMP), and TIMPs-1, -2, -4, at the border zone and infarct zone (Dixon et al., 2009).

Limiting inflammation

After MI, the inflammatory process is needed to mobilize immune cells to clear out dead heart cells and debris, and to stimulate ventricular remodeling (Frangogiannis, 2012; Frangogiannis et al., 2002). However, prolonged inflammatory responses would be detrimental to the remodeling process and ventricular function due to heart cell loss leading to negative impacts on ECM as well as formation of new vessels (Frangogiannis et al., 2002). Transplanted cells, such as MSCs or MSC-CMs, are able to limit the inflammatory process in the injured tissue. They weaken the proliferation of inflammatory CD^{68+} cells, decrease the expression of monocyte chemoattractant protein (MCP-1), increase the expression of genes involved in DNA repair, increase antioxidant enzymes and stimulate detoxifier systems, thereby improving cardiac function (Fuse et al., 2001; Ohnishi et al., 2007b; Ramalho-Santos et al., 2002).

Transplanted MSCs increase the number of M2 macrophages which were at the anti-inflammatory stage. The mechanism of this process is related to a variety of paracrine factors derived from transplanted MSCs, such as CCL2, galectin-1, interferon-γ, interleukin (IL)-1β, indoleamine-2,3-dioxygenase, IL-4, IL-6, IL-10, IL-13, prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), NO, heme oxygenase-1, HGF, and TGF-beta (Ben-Mordechai et al., 2013; Bernardo and Fibbe, 2013; Du et al., 2008). Normally, the pro-inflammatory cytokines (e.g. NF-κB, IL-6 and TNF-) poison the heart muscle cells, causing detrimental effects on cardiac contractile function. Transplanted MSCs have been shown to inhibit activation of NF-κB, reduce production of TNF- α and IL-6, and increase the expression of anti-inflammatory cytokine IL-10, thereby limiting inflammation after infarction (Du et al., 2008; Onai et al., 2007). Moreover, the increase of indoleamine-pyrrole 2,3-dioxygenase (IDO) and PGE2 secretion also reduces T-cell activity and NK cell proliferation (Glennie et al., 2005; Nauta et al., 2006; Pradier et al., 2011; van den Akker et al., 2013).

Cell fusion

After infarction, grafted stem cells reduce cardiomyocyte apoptosis and stimulate cell proliferation via cell fusion (Alvarez-Dolado et al., 2003; Yang et al.,

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2012). Studies of bone marrow transplantation have proven that there is cell fusion between donor cells and recipient cells to form multi-nuclear cells (Alvarez-Dolado et al., 2003; Mayourian et al., 2016). Some studies have shown that stem cell transplantation can reprogram recipient cells, causing cardiomyocytes to re-enter the cell cycle; this contributes to regeneration of cardiac muscle and improvement of cardiac function (Yeh and Zhang, 2006).

Manuel Alvarez-Dolado et al. demonstrated that bone marrow stromal cells (BMSCs) derived from R26R mice (i.e. a Cre reporter mouse line) could fuse with Cre+ neurosphere cells after 4 days of co-cuture. Moreover, BMSCs could fuse with Cre+ fibroblasts in primary cuture. In vivo studies have revealed that bone marrow of actin-Cre-GFP mice could be grafted to irradiated R26R mice. The transplanted cells were shown to fuse with and exhibit similar morphology as local mature cardiomyocytes. The fused cells expressed GFP at 2 months after implantation; however, GFP expression was absent in most of the fused cells at 4 months after implantation, suggesting that interesting changes may occur inside the cells (Alvarez-Dolado et al., 2003). In accordance with Manuel et al., Yang et al. reported that transplanted bone marrow cells tend to integrate with local heart cells in infarcted regions rather then in healthy tissues. The infusion contributed to prevention of apoptosis of intergrated cardiomyocytes (Yang et al., 2012). In another interesting study, from Nygren et al., it was shown that transplanted HSC survive but do not transdifferentiate into cardiomyocytes within the infarcted myocardium, whereas X-gal and GFP positive cardiomyocytes or fused cardiomyocytes are seen outside the infarcted zone (Nygren et al., 2004).

Differentiation of transplanted cells into heart cells

Transplantation of stem cells or progenitor cells restore structure and function of heart tissue after infarction; this has been demonstrated both by preclinical studies and clinical trials. One of the proposed mechanisms is that transplanted cells and mobilized cells have the ability to differentiate into heart cells to replace damaged or necrotic cells (Kajstura et al., 2005; Nagata et al., 2016; Suzuki et al., 2015), possibly forming links with neighboring heart cells (Dimmeler et al., 2005).

So far, stem cell differentiation into heart muscle cells is proposed to be related to four major signaling pathways: canonical/non-canonical Wnt signaling pathway, bone morphogenetic protein (BMP) signaling pathway, fibroblast growth factor (FGF) signaling pathway, and Notch signaling pathway. Firstly, when the canonical Wnt pathway is inhibited through Wnt3A and b-catenin inhibition, this stimulates the differentiation of stem cells/precursor cells into cardiomyocytes (Pagliari et al., 2014). In addition, when non-canonical Wnt signaling pathway is activated through Wnt11 and Wnt5A activation, this also increases cell differentiation into cardiomyocytes (Pagliari et al., 2014). Secondly,

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BMP-Smad1 inhibits Wnt/b-catenin and activates non-canonical Smad binding factors, leading to the transcription of activating transcription factor-2 (ATF2), while promoting major histocompatibility complex b (b-MHC) expression, contributing to heart cell differentiation (Parikh et al., 2015). Thirdly, FGF activates the PI3K/Akt signaling pathway to preserve stem cell properties (Parikh et al., 2015); moreover, via the MAPK/ERK signaling pathway they cause proliferation of cardiac progenitor cells but inhibition of their final differentiation into mature cardiomyocytes (Tirosh-Finkel et al., 2010). Repression of FGF signaling, therefore, accelerates the differentiation process of cardiac precursor cells (Tirosh-Finkel et al., 2010). Finally, Notch signaling plays a very important role in the regulation of stem cell differentiation into cardiomyocytes. It affects many kinds of cells- from transplanted cells, such as EPCs, MSCs and CPCs, to resident CSCs, immature cardiomyocytes and quiescent cardiomyocytes. Notch signaling is mediated by Jagged 1, NICD, Wnts, cyclin D1, RBP-Jk and Nkx2.5 (Gude et al., 2015; Luxan et al., 2016; Zhou and Liu, 2014).

Tumorigenesis of transplanted cells

Besides the positive effects of transplanted cells in experimental treatments for myocardial ischemia, there are negative impact, such as fertility tumorigenesis and cardiac arrhythmias.

Almost all adult stem cells and cardiac progenitor cells differentiated from stem cells have been shown to be safe; they did not produce tumors when transplanted into recipients (Ghodsizad et al., 2013; Huber et al., 2007). However, ESCs and iPSCs, have exhibited a high fertility tumorigenesis after transplantation (Ben-David and Benvenisty, 2011; Lee et al., 2013). Nussbaum et al. showed that after injection of undifferentiated-ESCs into rat models, tumor formation developed after transplantation (Nussbaum et al., 2007). In another study, Blin et al. grafted incompletely purified human ESC-derived cardiomyocytes into immune suppressed monkey models and found tumor formation in the transplanted monkeys (Blin et al., 2010). This suggests that unless the ESC-derived cardiomyocyte population is comprised of completely purified embryonic stem cells, it is very easy to form tumors from residual, unpurified ESCs (Tohyama et al., 2013). To date, there has been studies conducted to generate iPSCs without using viral vectors to circumvent the tumorigenesis of iPSCs (Okita et al., 2008).

Arrhythmia

Besides tumorigenesis, the potential of arrhythmia induction from transplanted cells has also received wide attention. In clinical trials, it was shown that transplant of myoblasts could led to arrhythmia occurrence (Leobon et al., 2003).

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Additionally, a number of other studies also confirmed that transplanted beating heart cells could give rise to arrhythmia (Gillum and Sarvazyan, 2008; Menasche et al., 2008), while transplantation of human ESC (hESC)-derived cardiovascular progenitors into human fetal hearts showed harmony of the structure and function between transplanted cells and cardiac host cells (Ardehali et al., 2013). Moreover, the electromechanical coupling of hESC-derived cardiomyocytes and the suppression of cardiac arrhythmias in transplanted pig models seems to be related to the formation of electrical conduction bridges through scar tissues (Shiba et al., 2012). The problem of arrhythmia after transplantation has now become a major problem, attracting scientists and clinicians to investigate mechanisms to overcome it.

Conclusion

In conclusion, transplanted cells delivered by several different pathways have shown that they could recover the structure and function of the damaged heart. These mechanisms include secretion of factors that protect heart cells, neovascularization, prevention of the fibrosis process, limiting the inflammatory processes, and mobilizing resident stem cells to repair the heart (by fusing with host cells or differentiating into heart cells for cell loss replacement). However, in addition to positive effects, transplanted cells can also have undesirable roles, such as induction of tumorigenesis or arrhythmia. The chosen cell type for transplantation needs to be considered carefully before clinical application.

Abbreviations

AKT/PKB: serine/threonine-specific protein kinase/ protein kinase B; ATF2: activating transcription factor-2; b-MHC: major histocompatibility complex b; Bad: Bcl-2-associated death promoter; Bcl-2: B-cell lymphoma 2 ; bFGF: basic fibroblast growth factor; BM-MNCs: bone marrow monocytes; BMP: bone morphogenetic protein; BMSCs: bone marrow stromal cells; Ccl-2: chemokine (C-C motif) ligand 2; cGMP: cyclic guanosine monophosphate; CPCs: cardiac progenitor cells; CPCs: cardiac progenitor cells; CSCs: cardiac stem cells ; Cx43: mitochondrial connexin 43; CXCR4: C-X-C chemokine receptor type 4; DAG: phospholipid diacyl glycerol ; ECM: extracellular matrix; Ecs: endothelial cells; eNOS: endothelial nitric oxide synthase; EPCs: endothelial progenitor cells; EPDCs: epicardium-derived cells; EPO: erythropoietin; ESC-CM: embryonic stem cell cardiomyocyte; FGF: fibroblast growth factor; G-CSF: granulocyte colony stimulating factor; GATA4: GATA Binding Protein 4; GFP: green fluorescent protein; GSK-3: Glycogen synthase kinase 3; GSK-3: glycogen synthase kinase 3 beta; hESC: human embryonic stem cell; HGF: hepatocyte growth factor; HSCs: hematopoietic stem cells; IDO: indoleamine pyrrole 2,3-dioxygenase; IGF-1: insulin-like growth factor-1; IL: interleukin; iPSCs: induced pluripotent stem cells; JAK/STAT: Janus kinase/ signal transducers and activators of transcription; JAK2: Janus-activated tyrosine kinase 2; LV:

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left ventricular; MAPK/ERK: Mitogen-Activated Protein Kinase/ extracellular signal–regulated kinases; MCP-1: monocyte chemoattractant protein 1; MI: myocardial infarction; mK_{ATP}: mitochondrial ATP-dependent potassium channel; MMP: matrix metalloproteinase; MPTP: mitochondrial permeability transition pore; MSC-CM: MSC cardiomyocyte; MSCs: mesenchymal stem cells; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; NICD: Notch intracellular domain; NO: Nitric oxide; PDH: pyruvate dehydrogenase; PGE2: prostaglandin E2; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; PKC-a: protein kinase C alpha; PKC-d: protein kinase C delta; PKC-e: protein kinase C epsilon; PKC-z: protein kinase C zeta; PKG: protein kinase G; RACK: Receptor for activated c-kinase; RNS: reactive nitrogen species; ROS: reactive oxygen species; SDF-1: stromal cell-derived factor 1; Sfrp2: frizzled related protein 2; sK_{ATP}: sarcolemmal $K_{ATP;}$ SMCs: smooth muscle cells; TB4: thymosin 4; TGF-beta: transforming growth factor-beta; TIMP-1: tissue inhibitor of metalloproteinase-1; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor; VSMCs: vascular smooth muscle cells; Wnt3a: Wnt Family Member 3A

Author Contributions

Truc Le-Buu Pham wrote and participated in editing the review. Phuc Van Pham oriented, gave important idea and revised the manuscript of this review.

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Original Research

Association of a single nucleotide polymorphism at 6q25.1, rs2046210 with breast cancer risk among Vietnamese population

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Abstract

Introduction: This study aims to confirm the association between SNP rs2046210 and breast cancer in the Vietnamese population. **Methods**: A case/control study has been performed with the sample size of 300 cases and 325 controls. High Resolution Melting method is optimised for SNP genotyping. Statistic logistic regression analysis was used along with dominant and recessive analysis to analyse the association between alleles and genotypes of the selected SNP and breast cancer risk. **Results:** With the selection population and the optimal HRM method, genotyping resulted in precise data for association analysis as the HWE test showed the distribution of genotypes in the population is in equilibrium ($p > 0.05$). The logistic regression association analysis showed SNP rs2046210 strongly associated with breast cancer risk in both allelic ($p = 0.0015$) and genotypic ($p = 0.0064$) analysis. The risk allele A, like other previous studies, strongly associated with increasing the risk of breast cancer up to 1.425 folds (OR [95%CI] = 1.425 [1.143- 1.777], $p = 0.0015$). However, it has shown the recessive effect in the additive analysis. Genotype AA showed stronger effect on the risk of breast cancer while the heterozygous AG genotypes showed weaker effect. Due to the small sample size, statistical power of this study is not high as expected (58.67%). But this case/control study confirms the association between SNP rs2046210 and the risk of breast cancer in the Vietnamese population. **Conclusion**: SNP rs2046210 is associated with breast cancer risk in the Vietnamese population. The risk allele A plays a role in increasing the risk of breast cancer in Vietnamese women.

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Keywords

Breast cancer, rs2046210, Estrogen receptor 1 (ESR1), HRM, Melting curves analysis, Vietnamese population

Introduction

Cancer is the disease that has received the most attention recently because of its complications. The mechanism of cancer is not clear. Treatment of cancer fails if the disease is found in later stages. Within cancers, breast cancer (BC) is the second most common cancer as well as the most common cancer in women worldwide. According to Globocan 2012 (WHO), BC was the third prominent cancer in Vietnam. It is expected to increase in the future and become the most prominent cancer in women (Torre et al., 2015). Fighting breast cancer requires early diagnosis. According to WHO, the best protection from cancer is early detection which greatly increases the chances for successful treatment (WHO, 2016). When treated at an early stage, many patients survive at least 5 years after diagnosis (the 5-year survival rate). This rate is relatively high with patients who are diagnosed in stage 0-I: 100%; in stage II: 93%; and is very low at the late stage III: 72%; and stage IV with 22% (DeSantis et al., 2013).

Breast tumorogenesis involved both extrinsic factors (e.g. environmental factors) and intrinsic factors (e.g. genetic factors). Among which about 5% to 10% of breast cancer cases are thought to be hereditary (Pei et al., 2013). Besides, DNA variations appearing may be caused by intracellular exposure to endogenous and exogenous mutagens, which can cause genetic instability and carcinogenesis. Understanding about the DNA damage or variations in the cancer development is an important study which provides information for early diagnosis and genetic therapy in the future.

Single Nucleotide Polymorphism (or SNP) is a common variation with small change in the DNA sequence; however, recently its role in development of several diseases has been demonstrated. It may play a crucial role in the regulation of the gene expression, which may alter the protein level or structure leading to development of certain diseases. One SNP may contribute a bit to the development of the disease, but the haplotype of several SNPs have been known to play the main role in disease development or resistance to a certain disease (Dunstan et al., 2014). GWA studies have been performed and identified several SNPs associated with cancer and particularly breast cancer (Easton et al., 2007; Gold et al., 2008; Hunter et al., 2007; Long et al., 2012; Stacey et al., 2007; Zheng et al., 2009). In a smaller scale, many other studies have demonstrated that some individual SNPs are very strongly associated with breast cancer. SNP rs2046210 is known as one of the very strong candidates for breast

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cancer association study. In 2009, Zheng et al has identified SNP rs2046210 to be highly associated with breast cancer in Asian population (Zheng et al., 2009), especially in Chinese [ORs (95%CI) = 1.35(1.17-1.57), p = 4.1.10-5] (Lin et al., 2014) or [ORs (95%CI) = 1.30(1.22–1.38) and 1.64(1.50–1.80) for the AG and AA genotypes, respectively, p = 1.54×10−30]; Japanese women [ORs (95%CI) = 1.37(1.11-1.70), p = 0.05] (Mizoo et al., 2013) or [ORs (95%CI) = 1.31(1.13–1.52) and 1.37(1.06–1.76), p = 2.51×10−4] (Cai et al., 2011); Korean women [ORs (95%CI) = 1.31 (1.19–1.45), p = 7.91 × 10−8 for dominant model] (Han et al., 2011). This SNP also has a positive association with breast cancer risk in European-ancestry American women with [ORs (95% CI) = 1.07 (0.99 – 1.16) and 1.18 (1.04 – 1.34), P for trend = 0.0069]. However, there was no association observed in African American women [ORs (95% CI) = 0.81 (0.63 – 1.06) and 0.85 (0.65 – 1.11) for the AG and AA genotypes, respectively, P for trend = 0.4027] (Cai et al., 2011).

SNP rs2046210 is located in 6q25.1 locus of chromosome 6, approximately 29 kb upstream of the estrogen receptor 1 (ESR1) gene, which is responsible for expressing ERα - the cofactor of estrogen hormone. Due to its close proximity to ESR1, rs2046210 is suspected to alter ESR1 expression thereby causing uncontrolled proliferation of breast epithelial cells, which leads to breast tumor formation (Dunbier et al., 2011; Zhou et al., 2013). Its A-allele was associated with a population attributable risk of 18.9% and an estimated 2.1% excess familial risk of breast cancer, especially for the AG and AA genotypes (Zheng et al., 2009). Nearly 60% elevated risk of breast cancer was found among women homozygous for the variant A allele in rs2046210 (Cai et al., 2011). This study aimed to demonstrate the association between the SNP rs2046210 and the breast cancer in the Vietnamese population.

Methods

Subjects

300 women patients who confirmed having breast cancer and preparing for surgery were informed to participate in this study. The other group of 325 volunteers indicated as healthy persons and without breast cancer also joined this study. The blood samples were collected from all participants satisfying the sample selection criteria including matching in sex, Kinh - Vietnamese were willing to sign the consent form, which was approved by the Ethical Committee of Oncology Hospital – HCMC Vietnam under the decision number 177/HĐĐĐ-CĐT, 18th November 2014.

2 mL of whole blood samples were collected by vein puncture and put in EDTA container till DNA extraction was performed. The DNA extraction was then performed using the salting out method (Hue et al., 2012) with some modifications for whole blood samples. 500 µL whole blood was used for each

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extraction. The DNA extracted samples were then stored at -20°C until used for PCR assay.

SNP selection

This SNP was chosen for its ubiquity in East Pacific Asian breast cancer incidence. Being one of the oldest countries in East, it is likely that Vietnam also shares the same association with rs2046210 as other Asian populations. There were many studies which demonstrated that SNP rs2046210 associated with breast cancer, thus it is possible this happens in Vietnamese. The SNP rs2046210 is selected for this study aim to confirm the association of this SNP with breast cancer, particularly in the Vietnamese population.

Genotyping method

The genotyping method selected for this study is High Resolution Melting (HRM). The DNA sequence around SNP rs2046210 is obtained from the NCBI SNP database and then used for designing PCR primers using Primer3Plus. The web tool uMelt HETS (https://www.dna.utah.edu/hets/umh.php) was used for predicting the PCR products' melting curve (Wittwer et al., 2003). The best primer pair (rs2046210-F-5'-AAAGGCATGCTGGAAGAGTGTTTT-3' and rs2046210-R-5'- GGGTGCCTCAACTGTCTTGTGA-3') for HRM has been selected to amplify the fragment of 131 bp and give 3 distinct curves for the 3 genotypes GG, AA and GA.

The PCR was carried out in a LightCycler 96 Instrument (Roche Diagnostics, Penzberg, Germany) using Roche HRM master mix (Roche Diagnostics, Germany). Thermal cycling consisted of an initial pre-incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 10 s at 64°C (Ta) and elongation at 72°C for 10 s. Finally, heteroduplexes were generated by adding a step at 95°C for 1 minute and cooling the reaction to 40°C (Ramp rate of 2.2°C/s). The best conditions for HRM analysis have been optimised with the control samples (*data not shown*).

Association analysis

Statistical analysis was conducted STATA ver12. The Hardy–Weinberg equilibrium, the logistic regression analysis was performed. Associations among genotypes and breast cancer risk were then estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from the logistic regression analysis. The dominant, recessive and additive analysis was performed using website https://ihg.gsf.de/cgi-bin/hw/hwa1.pl to double check and clarify the role of allele in dominant or recessive affect. All statistical tests had a P-value of < 0.05 was considered statistically significant. Sample size and statistical power of the case-control study was computed by a genetic power calculator (http://sampsize.sourceforge.net/iface/s3.html#ccp). Genetic Power Calculator developed by Purcell et al. (http://pngu.mgh.harvard.edu/~purcell/gpc/).

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Results

Genotyping

Using the optimal HRM condition as mentioned in the method above, totally 625 samples were successfully genotyped, including 325 controls and 300 cases. Three different genotypes were identified due to different curve shapes in three analysis channels: melting curve, melting peak and different plot as described in **figure 1**. The genotyping result of 625 samples is very clear, and there is no confusion (compared to other SNPs which may appear in some confused samples – data not shown). Three different genotypes distinguished clearly along with three genotype controls (**Fig. 1**).

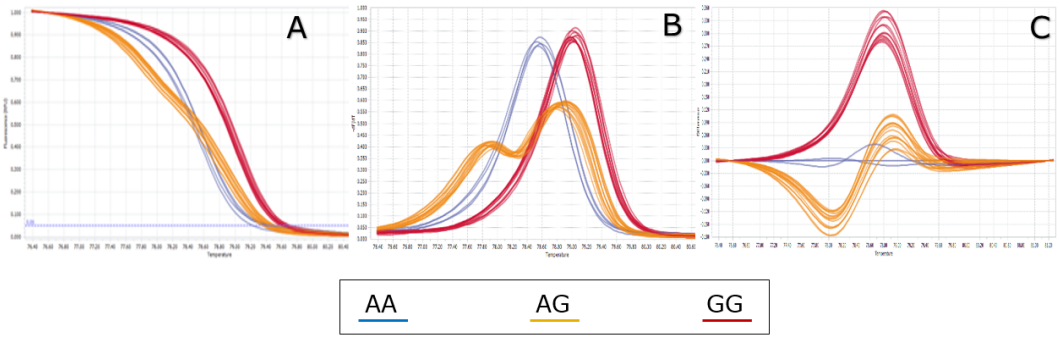

Figure 1. The melting curve (A) and melting peak (B) and different plot (C) analysis of rs2046210 by HRMA showed 3 different genotypes.

Alleles' frequency and HWE

As a result of the good genotyping, the minor allele (allele A) of the SNP rs2046210 occupied 37.69% in control group and 62.31% in case group (**Table 1**). Frequencies of three genotypes GG, AG and AA of this SNP in this population were 30.00%, 46.33% and 23.67% in case group and 40.00%, 44.62% and 15.38% in control group respectively (**Table 1**). The Hardy-Weinberg equilibrium was calculated for total sample set and for each group of the set and the result showed that the frequencies of 3 genotypes are in equilibrium. Particularly, in total sample set, p-value is 0.09 (pTotal = 0.09), in control-group pControl = 0.336, and in case-group pCase = 0.228. This result indicated that the genotyping data is matched with the requirement for further association analysis.

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Table 1. Logistic regression genotypic and allelic analyses

		Genotypes N (%)			Alleles N (%)		HWE p-value
		AA	AG	GG	A	G	
Cases	**300**	71(23.67)	139 (46.33)	90 (30.00)	281 (46.83)	319 (53.17)	0.228
Controls	**325**	50 (15.38)	145 (44.62)	130 (40.00)	245 (37.69)	405 (62.31)	0.366
Total	**625**	121 (19.36)	145 (45.44)	220 (35.20)	526 (42.08)	724 (57.92)	0.090
OR (95% CI)		2.05 (1.30-3.20)	1.38 (0.97-1.97)		1.425 (1.143-1.777)		
P-value		0.002	0.073				
		0.0064			0.0015		

Association analysis

The logistic regression analysis using STATA was applied for estimating the correlation between appearing of minor allele and the risk of getting the disease in the population. This statistic analysis showed that SNP rs2046210 is strongly associated with the disease, with very significant difference in allelic analysis [p = 0.0015, ORs (95% CI) = 1.43 (1.143 - 1.777)]. Genotypic analysis had also shown strong association between minor allele carriers and breast cancer with p = 0.0064. Homozygous genotypes of the minor allele (AA) have shown an increased risk of getting disease 2.05 folds compared to homozygous major allele (GG) with p = 0.002, ORs (95% CI) = 2.05[1.3 - 3.2] (**Table 1**). Minor allele A is the risk allele, but in the heterozygous genotype, the person carried only 1 minor allele, seems not to be associated with breast cancer risk or weak association (OR [95% CI] = 1.38 [0.97 - 1.97], p = 0.073) (**Table 1**). This indicated that the allele A has recessive effect in contribution to the breast cancer risk. The homozygous AA genotypes have shown strong effect (OR = 2.05) while the heterozygous AG genotypes have shown weaker effect (OR = 1.38).

The recessive effect of the allele A also is confirmed by the dominant analysis when all genotypes containing risk allele (AA and AG) is compared to the normal genotype (GG). Dominant analysis indicated that the risk allele carriers (women with the AG or AA genotypes), in general, increased 1.556 folds the risk of breast cancer (OR [95% CI] = 1.556 [1.116 - 2.168], p = 0.00892) (**Table 2**). However, the effect of both genotypes together was reduced with OR = 1.556 while the effect of AA genotype only has OR= 2.0556 in the additive (**Table 2**). Notably, recessive analysis does not show the complete recessive effect of the

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AG genotype. This recessive analysis demonstrated the weak effect of heterozygous AG genotypes to the risk of the diseases but has no effect on OR [95%CI] = 0.586 [0.392 - 0.877] (**Table 2**).

Table 2. Dominant and recessive association analysis

Analysis model		OR	95%CI	p value
Additive	**AA vs. GG**	2.051	1.307-3.219	0.00166
Additive	**AG vs. GG**	1.385	0.970-1.976	0.07239
Dominant	**[AA+AG] vs. [GG]**	1.556	1.116-2.168	0.00892
Recessive	**[AA] vs. [AG+GG]**	0.586	0.392-0.877	0.00884

Sample size and power estimation

In genetic association study to detect the causal genes of complex human disease, the sample size with sufficient statistical power is critical to the success of the study. Based on the current sample size used in the study, strength of the study is calculated, and the result shows that with recent sample size (300 cases/325 controls) and the OR = 1.425 in the allelic analysis, the power of this study is 58.67%. It seems below the expectation. To get higher power up to 70% a sample size of 406 cases/406 controls should be investigated (**Table 3**). With a bit bigger sample size, 456 cases/controls, the power of this study may go to 75%. And 80% or 90% power can be reached if the sample size is 516 cases/controls or 690 cases/controls (**Table 3**).

Table 3. Sample size and power calculation

Power	58.67%	70%	75%	80%	90%
Cases	300	406	456	516	690
Controls	325	406	456	516	690
Total	625	812	912	1032	1380

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Discussion

From this study, the SNP rs2046210 has been confirmed as the risk allele A in Vietnamese population along with other populations such as Caucasian, Asian, European, Chinese and Japanese. Presence of allele A points to susceptibility to and increased risk of breast cancer, which is also consistent with those populations where the association is identified. The frequency of allele A in this study is 42.8% in the total sample set and 37.69% in control group, supported the data of previous studies in the Asian population, which was about 34.8 - 39.3% (Chan et al., 2012; Han et al., 2011). Specifically, in this study, 37.69% allele A in the control group was in the same range as the reported 34% in Caucasian descendants, 37.8% in Chinese (Hap Map-HCB) and 30.0% in Japanese (Hap Map - JTP) (Mizoo et al., 2013). Distribution of this allele in Vietnamese which is the same as its distribution in other populations in Asia can be understood as they are in the same geography distribution and may share the same genetic distribution. Thus, the same genetic contribution to phenotype may also be the same. In fact, not only in Asian but this SNP was also found in European with nearly the same frequency (34%). However, in Africa, this ratio is relatively higher, around 69%, and this may lead to no association with breast cancer, while most of the previous studies in Chinese, Japanese, East-Asian, European, European-ancestry American, German women showed the association with the same frequency of risk allele (Cai et al., 2011; Stevens et al., 2011; Yang et al., 2013).

The SNP rs2046210 was first identified as a risk variant for breast cancer among Chinese women by Zheng et al. (Zheng et al., 2009), after that several studies have replicated, and similar results were achieved. This polymorphism was significantly associated with the risk of breast cancer in different Asian populations, including Chinese with strong association [ORs (95% CI) = 1.35 (1.17 - 1.57), p = 4.1×10-5 for allelic analysis and [P for trend = 1.54×10−30] (Lin et al., 2014). In Japanese women, the allele A has been strongly associated with breast cancer with [ORs (95% CI) = 1.37 (1.11 - 1.70), p = 0.05] (Mizoo et al., 2013) or [ORs (95% CI) = 1.31 (1.13 - 1.52) and 1.37 (1.06 - 1.76) for the AG and AA genotypes, p for trend = 2.51×10−4] (Cai et al., 2011). SNP rs2046210 had a population attributable risk of 18.9% plus an estimated 2.1% excess familial risk of breast cancer (Zheng et al., 2009). As a result, the association between this SNP, in particular the allele A with breast cancer in Vietnamese in this study was also demonstrated with higher OR, 1.425 in this study comparing to 1.35 to 1.37 in Chinese and Japanese.

The power of this case/control study was estimated at 58.67%. It seems quite low comparing to the previous study in Chinese population (Stevens et al., 2011). The power in Chinese study is around 77% with the sample size of 953 cases and 947 controls (Stevens et al., 2011) while in this study, we only used 300 cases and 325 controls. With the power calculation, in case Vietnamese population, if the sample size is increased to about 500 cases/controls the strong

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association between SNP rs2046210 with the breast cancer will be confirmed with about 80%.

It is clear that with the sample size of 300 cases/325 controls, this association together with other studies on other populations, SNP rs2046210 is confirmed to be associated with breast cancer even with a bit low power. The association can be analysed in a bigger sample size to increase the power and increase the reliability. With this result and previous studies' results, we believe that the allele A is the risk allele of breast cancer, not only for Vietnamese but also for other populations. Further functional studies are needed to confirm its function within the gene whether it alters the regulation of genes and leads to the breast cancer development. SNP rs2046210 is located 29 kb upstream of the first untranslated exon and 180 kb upstream of the transcription start site in the first exon of upstream of the estrogen receptor α (ESR1) gene on chromosome 6q25.1. This SNP is suspected to involve in regulating the expression of ESR1 gene suggesting its position within the binding site of a transcription factor (Zheng et al., 2009). The allele A, a result of changing from allele G, may relate to some changes in the binding site of the transcription factor, thereby not allowing the transcription factor bind to the target DNA, then inhibit or induce the appropriate expression level of the gene ESR1. If that happens, an over-expression of ERα, which is the product of ESR1 gene might result and it relates to increase in the risk of breast cancer among people with A allele compared to people without A allele. As suggested by other studies, a higher level of ERα is related to the uncontrolled proliferation of breast cells (Ellison-Zelski et al., 2009).

It is known that estrogen receptor positive (ER+ve) is one type of breast cancer in which the ESR 1 gene is expressed aberrantly high. This type of breast cancer can be considered as the most common one as it accounts for roughly 80% of human breast carcinomas (Dunbier et al., 2011; Russo and Russo, 2006). In this study, a population of breast cancer patients is selected as a representation for breast cancer disease in generally not separated in subtypes. Further analysis related to subtypes would be investigated to clarify this association. However, the association between SNP rs2046210 with breast cancer in this study indicated that the allele A of this SNP contributes to increase risk of the breast cancer development in Vietnamese in general. As it has been demonstrated to be associated with breast cancer in other populations (Antoniou et al., 2011; Long et al., 2010; Zheng et al., 2009) or in a special subtype ER negative (Yang et al., 2013) in the previous studies. Suggested that SNP rs2046210, particular allele A is a target marker for diagnosis breast cancer in the future.

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Conclusion

In conclusion, with the selected sample set and the optimal HRM method, the SNP genotyping produced a highly reliable data. This case/control study confirmed that SNP rs2046210 is associated with the risk of breast cancer in Vietnamese population and allele A of this SNP contributed to increased risk of breast cancer susceptibility in a recessive effect.

Abbreviations

BC: Breast cancer; 95% CI: 95% confidence interval; Erα: estrogen receptor α; ESR1 gene: Estrogen receptor 1 gene; GWAS: Genome Wide Association Study; HRM: High Resolution Melting; HWE: Hardy-Weinberg equilibrium; NCBI : National Center for Biotechnology Information; OR: Odd ratio; PCR: Polymerase Chain Reaction; SNP: Single nucleotide polymorphism; WHO: World Health Organization

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Ethical approval and informed consent

All patients gave informed consent and the studies were approved by the Local Ethics Committee of the Oncology Hospital of Ho Chi Minh City under the decision number 177/HĐĐĐ-CĐT, 18th November 2014.

Author Contributions

NTTL contributed to study design, statistically analysed the data, and wrote the manuscript. BTN contributed to the study. NTNT, NDTG, PNH reviewed and edited the manuscript for intellectual content. Tran Van Thiep involved in samples collection. Nguyen Thi Hue oriented, gave important idea and revised the manuscript of this review. All authors gave final approval of the version to be published.

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Original Research

Autologous osteochondral transplantation for treatment of cartilage defects in osteoarthritic knee: preliminary results

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Abstract

Introduction: Osteoarthritis is a contributing factor for pain and loss of function of the knee. Osteoarthritis results in many damages to the knee; one of the most common damages that is difficult to recover is cartilage injury. This study aims to apply autologous osteochondral transplantation (OAT) under knee arthroscopy for the treatment of knee cartilage defects. **Methods**: This was a prospective, descriptive and non-controlled study. Patients were diagnosed as having osteoarthritis, as confirmed by 1cm^2 – 3cm^2 cartilage defects. Arthroscopic OAT was performed on each patient. Treatment efficacy and safety were evaluated based on Lysholm, Oxford Knee Scores (OKS) and pain scales (VAS) after 3, 6, 12 and 18 months. **Results**: From 3/2014 - 8/2016, 61 cases (54 women and 7 men) participated in the study. The average age was 55 ± 8 years old. Most cases had cartilage defects in the medial condyle. Results showed that Lysholm, OKS scores and VAS scales improved after 12 months of treatment. Of the cases, 33 of 61 were followed out to 18 months; these patients showed improvement in knee function and pain scores. There was 1 case with incomplete matching between the plug and receiving site and 1 case with a broken plug. At the final stage of monitoring, there were no patients who experienced complications, such as broken instruments or fracture of condyle, nor who experienced early postoperative complications, such as infection and bleeding. **Conclusion**: Autologous osteochondral transplantation via arthroscopy is a safe and promising method for the treatment of knee cartilage defects in patients with average osteoarthritis.

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Keywords

Autologous osteochondral transplantation, cartilage defect , osteoarthritis, cartilage injury, mosaic plasty, OATS, OAT, osteochondral autograft transfer system

Introduction

Articular cartilage lesions cause pain and decreased mobility, affecting the working capacity and quality of life. To date, cartilage defects have been treated by different strategies, including debridement and lavage, microfracture, osteochondral autograft transplantation, osteochondral allograft transplantation, autologous chondrocyte implantation, and stem cell transplantation. Debridement and lavage are procedures of the oldest technique and typically reserved for low- demand older patients with small lesions (<2 to 3 cm^2) (Bert and Maschka, 1989; Federico and Reider, 1997; Freedman et al., 2004; Owens et al., 2002). Current research has suggested that the best candidates for debridement and lavage are those who suffer from mechanical symptoms (Moseley et al., 2002). Meanwhile, for patients with small to moderate sized lesions (1 to 5 cm^2), microfracture is a suitable treatment. The microfracture process helps stimulate fibrocartilage in-growth into the chondral defect to cover the underlying bone (Freedman et al., 2004; Gill and Macgillivray, 2001; Steadman et al., 2003). The procedure is performed by creating tiny fractures in the subchondral bone plate.

Moreover, osteochondral autograft plugs have been investigated as a means to restore cartilage defects. Osteochondral autograft transplantation has been most commonly applied to treat symptomatic lesions (Freedman et al., 2004; Hangody et al., 2001). The greatest advantage of osteochondral autografts is the use of live hyaline cartilage. This technique results in cartilage that is most similar to the injured cartilage. However, this technique also has disadvantages, namely donor site morbidity (pain and new cartilage defect), technical difficulty and risk of cartilage or bone collapse.

Fresh osteochondral allograft transplantation entails the implantation of a cadaveric osteochondral graft into the cartilage defect (Aubin et al., 2001; Bugbee, 2000; Garrett, 1994). This technique can be used for large articular cartilage defects (from 3 cm^2 up to an entire hemicondyle). The major advantage of osteochondral allografts is the ability to replace large osteochondral defects in a single-stage procedure.

Currently, autologous cultured chondrocyte implantation has also been explored for the treatment of cartilage defects. In this technique, a small piece of cartilage

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is harvested arthroscopically. Chondrocytes from the sample are isolated and grown expanded in culture over several weeks. In the next step, millions of autologous cultured cartilage cells are suspended in a solution of fibrin glue and later implanted into cartilage defects (Peterson et al., 2000). This technique is usually considered for intermediate to high-demand patients who have failed arthroscopic debridement or microfracture (Brittberg et al., 1994; Chu et al., 1999; Gillogly et al., 1998).

Stem cell transplantation is currently another promising therapy for osteoarthritis and cartilage defects. Some recent studies have shown that autologous adipose stem cell transplantation can improve osteoarthritis (Bui et al., 2014). Combination of stem cell transplantation and microfracture have also proven to be better than microfracture alone (Nguyen et al., 2016). However, similar to osteochondral allograft transplantation and autologous chondrocyte implantation, stem cell transplantation is expensive but yields promising results in clinical trials.

In this study, we aim to investigate the application of osteochondral transplantation to treat cartilage defects of osteoarthritic knee.

Methods

Inclusion criteria

From March 2014 to August 2016, 61 patients (54 women and 7 men) were enrolled in our study; all had degenerative knee of grades III or IV (classified by Outerbridge), with cartilage lesions with an area of 1- 3 cm^2 on the weight–bearing surface of the femoral condylar. All patients who participated in our study underwent arthroscopic osteochondral autologous transplantation. The mean age of the patients was 55 ± 8 years old.

Exclusion criteria

All patients with any of the following characteristics were excluded from our study: joint space ≤ 2mm, varus/valgus alignment > 5^0, knee stiffness, other joint diseases (e.g. rheumatoid arthritis, inflammation and neoplasm), and joint damage (e.g. caused by systemic diseases). Most patients had 1 or 2 plugs of osteochondral graft and 1 patient had 3 plugs. Moreover, 60 patients had lesions on the medial femoral condyle and 1 had lesions on the lateral femoral condyle (LFC). The mean size of cartilage defects was 1.54 cm^2. Patients were given clinical and functional evaluations pre-operatively and at 3, 6, and 12 months post-operation using the Lysholm, OKS and VAS scales.

Surgical procedure

Surgery was performed under arthroscopy. The location of the defect was determined. Remnants of residual cartilage were removed from the defect. The

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size of the defect was measured. The osteochondral grafts were later removed from the donor site on the superior-lateral aspect of the LFC or trochlea and transferred into the cartilage defect. The length of plug was at least 15 mm and similar to the recipient site depth (**Fig. 1, Fig. 2**).

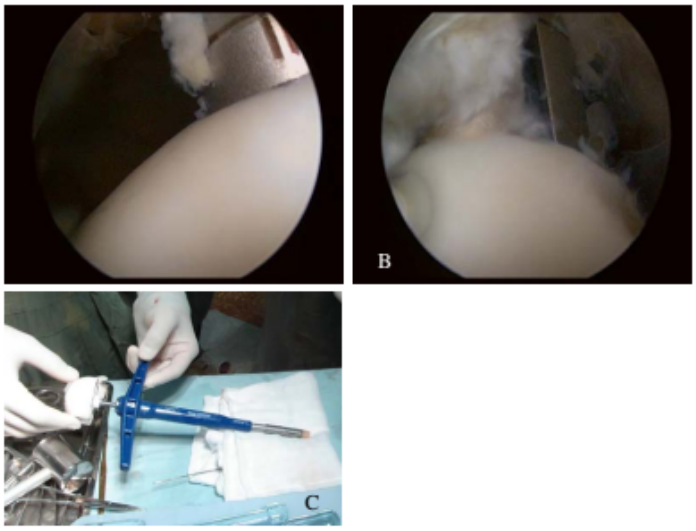

Figure 1. Havesting donor plug from lateral condyle. A, B : Graft is removed from superior-lateral aspect of the lateral femoral condyle; C : Graft is taken out.

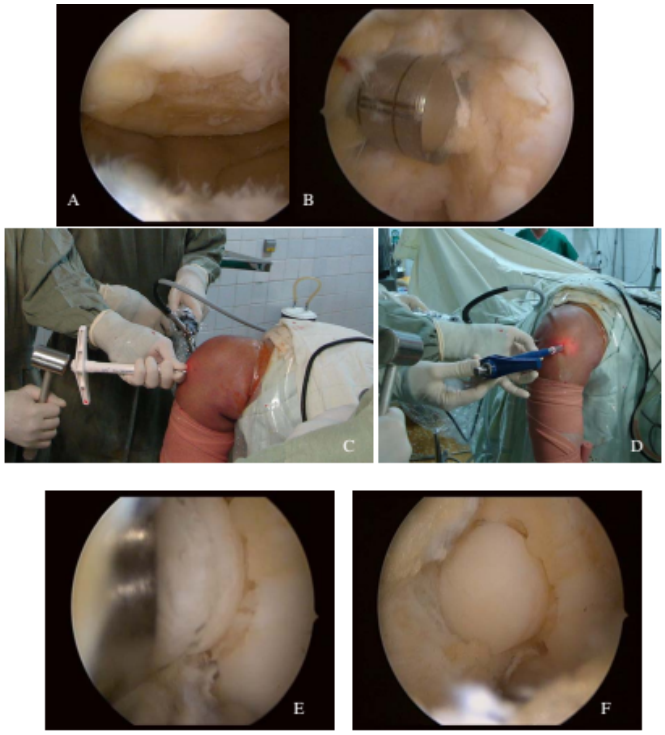

Figure 2. Donor plug is placed into the cartilage defect in the knee. A: Cartilage defect of femoral condyle; B : Measuring the size of defect; C: Havesting graft; D,E : Graft was transferred to recipient site; F : Recipient site after transfer

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Post-operative rehabilitation

The knee was passively mobilized on the second post-operative day. Touch-down weight bearing with crutches was allowed after 6 weeks, and the patient could then move gradually toward full weight bearing (at about 8 weeks).

Statistical Analysis

All continuous data were calculated as mean values and standard deviation of the mean. The Kolmogorov Smirnov test was performed to assess the normal distribution of the continuous variables. The normal distribution values were compared using t-tests. Non-normal distribution values or small numbers were compared using the Wilcoxon signed-rank-test and Mann-Whitney U test. Pearson correlation coefficients were used to determine the correlation between MOCART score and cartilage defect size, and between MOCART score and clinical outcomes. Confidence level for all analyses was set at $p < 0.05$. The statistical data was processed using the SPSS 16.0 software (IBM Corp., Armonk, NY, USA).

Results

Changes in Lysholm, OKS and VAS scores

The results showed an improvement in Lysholm, OKS and VAS scores at 3, 6, and 12 months after surgery. Specifically, the OKS score increased significantly from 24.9 ± 8.9 to 40.5 ± 5.5 after 12 months (paired t-test, $p<0.001$). Moreover, the VAS score decreased significantly from 6.2 ± 1.3 to 1.5 ± 1.3 after 12 months (paired t-test, $p<0.001$) (**Table 1**). In 33 patients who were followed out to 18 months, the same trend was observed. In fact, there was no significant difference when comparing the 12-month-follow up results with the 18-month results (paired t-test, $p>0.05$), thus demonstrating that improved outcomes were maintained out to 18 months post-operation (**Table 2**). The window in which patients felt improvement of symptoms was at about 3.7 months (1 to 7 months) after surgery.

The percentage of patients with normal or mild knee arthritis, based on the OKS scores, increased from 31.2% (pre-operation) to 95.1% (at 12 months post-operation) (**Table 4**). We also found that the percentage patients with no or mild pain, based on the VAS scores, went from 0% (pre-operation) to 93.4% (at 12 months post-operation) (**Table 5**). The percentage of patients with pre-operative good knee function, based on the Lysholm scores, was 3.3% (pre-operation) and 32.8% (at 12 months post-operation) and there was no patient with poor

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function (63.9% preoperative) (**Table 3**). Similar results was found in the 18-month follow-up group (**Table 2**).

There are 35 cases with one-plug OAT and 25 cases with double-plug OAT. Both groups showed improvement in function and VAS scores at 12 months post operation (paired t-test, $p<0.001$; Wilcoxon signed-rank test, $p>0.05$). Moreover there was no significant difference between those two groups at any follow-up time (Mann-Whitney U, $p>0.05$) (**Table 7**).

Table 1. Pre-operative and post-operative functional and pain outcomes

Unit (Point)	Preoperative	3 months	6 months	12 months
Lysholm	60.3 ± 12	72.8 ± 11.8	80.8 ± 9.6	83.3 ± 7.4
	(95% CI : 57.2–63.4)	(95%CI : 69.8-75.9)	(95%CI : 78.3-83.2)	(95%CI : 81.4-85.2)
OKS	24.9 ± 8.9	31.9 ± 7.5	38 ± 7	40.5 ± 5.5
	(95% CI: 22.7-27.2)	(95% CI: 30.0-33.8)	(95% CI: 36.2-9.8)	(95% CI: 39.1-41.9)
VAS	6.2 ± 1.3	3.1 ± 1.8	2.0 ± 1.6	1.5 ± 1.3
	(95% CI: 5.9-6.6)	(95% CI: 2.7-3.6)	(95% CI : 1.6-2.5)	(95% CI : 1.2-1.9)

Table 2. Pre-operative and post-operative outcomes out to 18-months of follow-up

Unit (Point)	Pre-operative	6 months	12 months	18 months
Lysholm	58.9 ± 12.3	79.3 ± 11.4	83.3 ± 8.5	83.9 ± 8.2
OKS	25.5 ± 9.1	37.3 ± 7.2	39.8 ± 6	40 ± 6.2
VAS	6.3 ± 1.4	2.2 ± 1.7	1.8 ± 1.5	1.5 ±1.7

When divided into two groups according to the size of lesion, (2 – 3 cm^2 group and <2 cm^2 group), we found that there was no significant difference in any of the scales at all follow-up times (Mann-Whitney U, $p>0.05$) (**Table 6**). Moreover, the size of defect had no correlation with clinical outcomes ($p > 0.05$). In the 18-month follow-up group, there were also no significant difference when comparing outcomes of single-plug group and double-plug group (Mann-

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Whitney U, $p > 0.05$) between the 2-3 cm^2 and < 2 cm^2 groups (Mann-Whitney U, $p > 0.05$) (**Tables 6 and 7**). There was no plug migration into the joint space, as assessed by clinical evaluation and knee X-ray after surgery.

MRI was performed for 39 patients at 6 months post-operation and for 25 patients at 12 months post-operation. For all cases, the MOCART scores were calculated, as well as assessment of integration of grafts into the receiving site, and intact cartilage surface. The mean MOCART was 61.8 ± 18 (95% CI: 55.9 – 67.7) at 6 months post-operation, and 62.4 ± 16 (95% CI: 55.5 – 69.3) at 12 months post-operation (**Table 8**).

Table 3. Results/grading of Lysholm scores (%)

	Poor (<65)	Fare (65-83)	Good (84-90)	Excellent (>90)
Pre-operative	63.9	32.8	3.3	0
3 months	24.5	52.5	16.4	6.6
6 months	6.6	47.5	31.1	14.8
12 months	0	49.2	32.8	18
18 months (33 cases)	0	42.4	30.3	27.3

Table 4. Results/grading of OKS scores (%)

	Severe (0-19)	Moderate (20-29)	Mild (30-39)	Normal (40-48)
Pre-operative	26.2	42.6	24.6	6.6
3 months	3.3	37.7	41	18
6 months	0	16.4	34.4	49.2
12 months	0	4.9	31.2	63.9
18 months (33 cases)	0	9.1	33.3	57.6

The rate of complete defect fill (100 – 125%) was 13% after 6 months and 8% after 12 months; the rate of partial cartilage defect fill (50 – 100%) was 69% after 6 months and 80% after 12 months (**Table 8**). At 6 months after surgery, the rate of complete integration of plug into subchondral bone was 56%, and 72% after 12 months. Eighteen patients had MRI performed at both 6 and 12 months post-

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operation; there was an observed increase of 3D MOCART, from 61.7 ± 18 to 64.4 ± 14.7, though the difference was not significant (Wilcoxon, p = 0.341).

Table 5. Results grading of VAS scores (%)

	None	Mild	Moderate	Severe
	0	(1-3)	(4-6)	(7-10)
Preoperative	0	0	57.4	42.6
3 months	8.2	52.5	37.7	1.6
6 months	18	60.7	21.3	0
12 months	19.7	73.7	6.6	0
18 months (33 cases)	30.3	60.6	6.1	3

Table 6. Outcomes of groups with defect size of < 2 cm^2 and 2-3 cm^2

Size	Time	Lysholm	OKS	VAS
< 2cm^2	**Pre-operative**	61.2	25.3	6.2
	3 months	73.8	32.5	3.2
	6 months	81.1	38.4	2.1
	12 months	83.2	40	1.6
	18 months (33 cases)	84.3	40.2	1.7
2-3cm^2	**Pre-operative**	57	23.1	6.3
	3 months	71.5	31.3	3.0
	6 months	80.4	37.4	2
	12 months	82.2	40.5	1.5
	18 months (33 cases)	85.5	41.3	0.8

Imaging results

There was no correlation between the size of lesion and MOCART score at 6 and 12 months post-operation (p=0.15 and p=0.263, respectively) (**Fig. 3**). We also found no correlation between MOCART score (or its variables) and clinical outcome scores (Lysholm, OKS and VAS) (p> 0.05 for all).

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Table 7. Outcomes of single-plug group and double-plug group

	Time	Lysholm	OKS	VAS
1 plug	Pre-operative	61.8	24.8	5.9
	3 months	73.2	32.5	3.1
	6 months	82	38.3	2
	12 months	84.7	41	1.3
	18 months (17 cases)	84.2	41.3	1.7
2 plugs	Pre-operative	58.8	25	6.6
	3 months	72.7	30.9	3.2
	6 months	79.2	37.5	2.1
	12 months	81.3	39.7	1.8
	18 months (15 cases)	83.5	39.5	1.4

Twenty single-plug cases and eighteen double-plug cases received an MRI evaluation at 6 months post-operation. The mean MOCART score for the single-plug group was 66.7 ± 17.9 and for the double-plug group was 56.4 ± 17.7; however, the difference was not significant (Mann-Whitney U, p=0.112). Similarly, the MRI results after 12 months for the 12 single-plug cases and 12 double-plug cases revealed no significant difference in MOCART score between the groups (Mann-Whitney U, p = 0.630).

Complications

We did not observe any complications during the operation, such as breakage of instrument, fracture of femoral condyle, and anterior cruciate ligament (ACL) or posterior cruciate ligament (PCL) attachment injury. There was one case of incomplete matching between plug and receiving site and one case of broken plug. Both instances entailed replacement of a new plug. None of the 61 experienced any early post-operative complications, such as infection, hemorrhaging or migration of plug.

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Table 8. 3D MOCART scores

	Point	6 months		12 months	
		Number	%	Number	%
1. Defect fill (degree of defect repair and filling if the defect in relation to the adjacent cartilage)					
0-25%	0	2	5	1	4
25-<50%	5	5	13	2	8
50-<100%	10	27	69	20	80
100- <125%	15	5	13	2	8
125- <150%	5	0	0	0	0
>150	0	0	0	0	0
2. Cartilage interface (integration with adjacent cartilage to border zone in two planes)					
Complete	15	4	10	3	12
Demarcating borders	10	24	61	11	44
Defect visible < 50%	5	10	26	9	36
Defect visible > 50%	0	1	3	2	8
3. Bone interface (Integration of the transplant to the subchondral bone, integration of a possible periosteal flap)					
Complete	5	22	56	18	72
Incomplete	0	17	44	7	28
4. Surface (constitution of the surface of the repair tissue)					
Surface intact	10	10	26	7	28
Surface damaged < 50% of depth	5	27	69	15	60
Surface damaged > 50% of depth or adhesions	0	2	5	3	12
5. Structure (constitution of the repair tissue)					
Homogeneous	5	19	49	16	64
Inhomogeneous or Cleft formation	0	20	51	9	36
6. Signal intensity (Intensity of MR signal of the repair tissue in comparison to the adjacent cartilage)					
Normal (identical to adjacent cartilage)	15	4	10	2	8
Nearly normal (slight areas of signal alteration)	10	32	82	21	84

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Abnormal (large areas of signal alteration)	0	3	8	2	8
7. Subchondral lamina (constitution of the subchondral lamina)					
Intact	5	24	62	16	64
Non-intact	0	15	38	9	36
8. Chondral osteophytes (Osteophytes within the cartilage repair area)					
Absent or Osteophyte with < 50% of the thickness of the cartilage transplant	5	39	100	25	100
Osteophyte with > 50% of the thickness of the cartilage transplant	0	0	0	0	0
9. Bone marrow edema (maximun size and location in relation to the cartilage repair tissue and other alternations)					
Absent	5	13	33	8	32
Edema	0	26	67	17	68
10. Subchrondral bone (constitution of the subchondral bone)					
Intact	5	15	38	8	32
Non-intact	0	24	62	17	68
11. Effusion (approx. size of joint effusion visualized in all planes)					
Absent	15	5	13	6	24
Small or medium	10	34	87	19	76
Large	0	0	0	0	0

Discussion

Hangody et al. recommended the use of OAT only for patients <40 years of age; there have been relative contraindications in patients ranging from 40-50 years of age, and contraindications in those >50 years of age (Hangody et al., 2001). Kish et al. (Kish et al., 1999) as well as Marcacci et al. (Marcacci et al., 2005) have also reported better results in younger patients. However, Chow et al. have found that age is not a factor which limits the procedure; old people with chondral defects and a stable knee joint can achieve good results (Chow et al., 2004). In our study, we also found in patients with a mean age of 55 ± 8 years old, clinical results as well as pain scores improved at 12 and 18 months post-operation. In a recent study, mosaicplasty for treatment of cartilage defects demonstrated promising results. In a multi-center study, Hangody et al. showed that mosaicplasty was better than other cartilage repair methods, including debridement, subchondral penetration and abrasion arthroplasty (Hangody et al., 2001). Similarly, Krych et al. saw better activity levels after osteochondral autograft transfer mosaicplasty than after microfracture (Krych et al., 2012).

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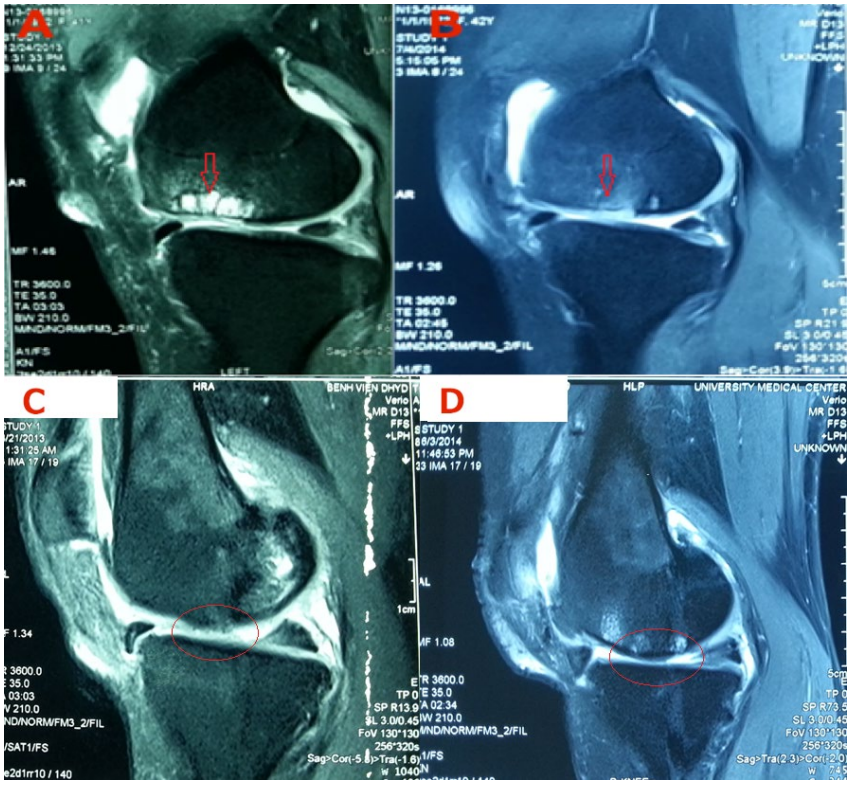

Figure 3. Pre-operative and 6-month post-operative MRI. Restoration of articular cartilage and subchondral bone could be observed (**C, D**) compared to pre-operation (**A, B**). A and B: Case 1, C and D: case 2

Several other studies have also demonstrated improved results after mid-term and long-term follow-up. Randomized studies with a control group have been performed. Horas et al. (Horas et al., 2003) and Dozin et al. (Dozin et al., 2005) concluded that the clinical outcome of mosaicplasty was equivalent to autologous chondrocyte implantation, with a high rate of hyaline cartilage. However, after early- stage promising results, Solheim et al. (Solheim et al., 2010) showed that there is a gradual reduction of efficacy after 10 to 14 years of follow-up; 40% of the 73 cases had poor outcome, and good outcome was often seen in younger patients with defect size <3 cm^2. In a study of 52 patients at 37 months follow-up, Jakob et al. found that the method was limited by the defect size and the number of plugs taken at the donor site (Jakob et al., 2002). Marcacci et al. studied 30 patients and confirmed better outcome was associated with small defect size and with only 1-3 plugs (Marcacci et al., 2005).

In our study, after the 12 month follow-up period, the percentage with good and excellent Lysholm score was 50.8%. The percentage having knee with normal or mild inflammation on the OKS scale was 95.1%, and the rate of mild pain or no pain on the VAS scale was 93%. Although the Lysholm scale results was lower than previous studies, the results of the VAS scores and OKS scores were

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equivalent to what other authors, such as Marcacci et al. (Marcacci et al., 2005) and Jakob et al. (Jakob et al., 2002) have published. The reason for the lower Lysholm scale scores in this study may be due to the fact that the patients in our study included those with knee osteoarthritis.

Osteochondral autograft transplantation for isolated cartilage defects with < 2-3 cm^2 lesion area in young people requiring high activity is nothing controversial. In our study, osteochondral autograft transplantation for grade III/IV cartilage defects with 1-3 cm^2 lesion area on the weight-bearing surfaces of femoral condylar in older adults with osteoarthritis is an expanded indication to delay knee replacement surgery. Initial results showed good results with lesion area of ≤ 3 cm^2 at 12 months post-operation and similar results in the 18 months post-operative group. In studies by Hangody et al., the authors only performed OAT for cartilage defect sizes from 1-4 cm^2, although it can be applied as a temporary method for 8 cm^2 cartilage defects (Hangody et al., 2001).

In this study, we compared the results in two groups of lesion defects: <2 cm^2 and 2-3 cm^2. We saw good results in both groups; there was no correlation between lesion size and clinical results. Marcacci et al. observed better results with smaller-sized lesions (Marcacci et al., 2005), but other authors have found (Jakob et al., 2002), as we did too, that there are no statistically significant correlation between clinical outcome and lesion size.

With the development of diagnostic imaging devices, MRI provides not only a non-invasive means to diagnose cartilage lesions but also a reliable tool for monitoring and evaluating results of articular cartilage lesion treatment. In particular, 3D MOCART is a good scale and most often used for evaluating results of OAT by MRI (Marlovits et al., 2006; Marlovits et al., 2004). 3D MOCART scale assesses many variables, including: degree of repair filling, integration of the cartilage repair tissue to the border zone, structure of the surface, structure of the whole repair tissue, and signal intensity. Thus, the scale can be used to evaluate the effectiveness, success or failure of treatment.

Rate of complete defect fill after 1 year according to research by Zak et al. is 50% (Zak et al., 2014). In our study, this rate was only 8%; most cases (80%) had defect fill from 50% to <100%. We found intact surface rate of cartilage after 1 year to be 28%; Zak group's found it to be 70% (Zak et al., 2014). This difference may be due to parameters in other studies which are not seen in osteoarthritis patients. The majority of patients in our study are older and osteoarthritic thus MRI results after 1 year were worse. However, the rate of complete bone interface was 72% and we did not have any complete delamination case, meaning that all plugs were stable and in place.

The average MOCART score after 12 months in our study was 62.4 points, not too much lower than 75 points in Zak et al.'s study (Zak et al., 2014) and Krusche-Mandl et al.'s study (Krusche-Mandl et al., 2012). We also did not find a correlation between MOCART score and function scores or VAS scores.

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Concerning the correlation between MOCART score and clinical outcome, Krusche-Mandl et al. did not find any correlation between MOCART score and Lysholm, IKDC or VAS scores (Krusche-Mandl et al., 2012). Tetta et al. also found that there is only a correlation with the IKDC scale but not with the Tegner scale (Tetta et al., 2010). A recent meta-analysis study also found that there is not enough evidence to confirm a correlation between morphological results of MRI and clinical outcome (Wakitani et al., 2002).

Ensuring the matching between plugs and the receiving site to create a smooth cartilage surface is a challenge in the OAT technique. Chow et al. showed that harvesting and transplanting of osteochondral plugs should be perpendicular to cartilage surface; in fact, wrong angle placement will reduce efficacy (Chow et al., 2004). Marcacci et al. also agreed with this assessment after 3 of their cases failed and were related to the surface matching problem (Marcacci et al., 2005). Hangody and Fules also emphasized the importance of matching between plugs and the receiving location (Hangody et al., 2001). Therefore, in our study, we carefully performed the OAT procedure, and only 1 case without matching after transplantation had to be replaced with another plug.

Conclusion

OAT procedure under arthroscopy was investigated as a treatment for osteoarthritis patients with grade III/IV cartilage defects (1-3 cm^2 lesion area) and showed promising initial results. OAT is an accepted and trusted method in the treatment of 1-3 cm^2 cartilage defects, helping to delay knee replacement surgery. There is no correlation between 3D MOCART and functional outcome, or to post-operative pain score. OAT under arthroscopy may be a promising procedure for the treatment of knee articular cartilage defects since it is a minimally invasive, low-priced, and one-stage procedure which yields some efficacy and few complications.

List of Abbreviations

OAT : autologous osteochondral transplantation; OKS : Oxford Knee Score; LFC : Lateral femoral condyle; MOCART :Magnetic resonance observation of cartilage repair tissue

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Ethical approval and informed consent

All patients gave informed consent and the studies were approved by the Committee of Department of Science and Technology, Ho Chi Minh city, Viet Nam.

Author Contribution

BHTK: collected, analyzed, data and wrote the manuscript; MTV, NDT, LTV, NPT: collected data, write the draft of manuscript; HNT: diagnosis, evaluated clinical scores; performed MRI evaluation. All authors approved this manuscript

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