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Original Research Article

Laboratory Use of Lectin Mitogens for Mitotic Stimulation of Human Lymphocytes

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HIGHLIGHTS

- Lectins act as essential factor of the normal differentiation and growth of all humans and animals.
- Phytohemagglutinin (PHA) is a lectin (mucoprotein) from *Phaseolus vulgaris*.
- Crude extract of PHA could be used in human leucocyte cultures as mitotic stimulator.
- The indigenous PHA have used to identify chromosome preparation in normal conditions and malignancies.

ABSTRACT

Lectins are believed to act as modulations of cell substratum interactions and to be essential for the normal differentiation and growth of all multicellular humans and animals. Although several lectins have been reported from microfungi, many more genera remain unexplored and their physiological role is also uncertain. The aim of this laboratory work was to make a comparison between self-made lectins (Indigenous) and commercial ones, following High Resolution Cell Synchronization technique (HRCS). Cytogenetic studies were performed in 175 normal healthy blood donor individuals of both genders and statistical analysis was performed. Our results indicated that the preparation of fresh phytohemagglutinin at the time of cell division and cell culture procedure reveals a satisfactory score. The overall frequency of mitotic index in our study was higher when compared with commercial imported Lectins ($p < 0.05$). The significant differences in the results may be due to fresh preparation. However, the cost effective, easy and nearest approach of this indigenous product, as well as the high demand for this product, among health care services can be considered.

Introduction

Phytohemagglutinin (PHA) is a lectin (mucoprotein) from *Phaseolus vulgaris* and comprises two 30-kDa subunits along with an N-terminal sequence (Goossens et al., 1994). PHA-P is the protein form and PHA-M is the mucoprotein form of these isolectins (Pandit et al., 2016).

The commercially available PHA has been used for many years in human leucocyte cultures for cytogenetic studies, but it is very expensive; therefore, an attempt was made to utilize the crude seed extract of *P. vulgaris* in the leucocyte cultures, which is less expensive. Also, there has been very few and inconclusive studies using the crude extract of PHA in human leucocyte cultures (Sharon and Lis, 2004). Therefore, Pokeweed

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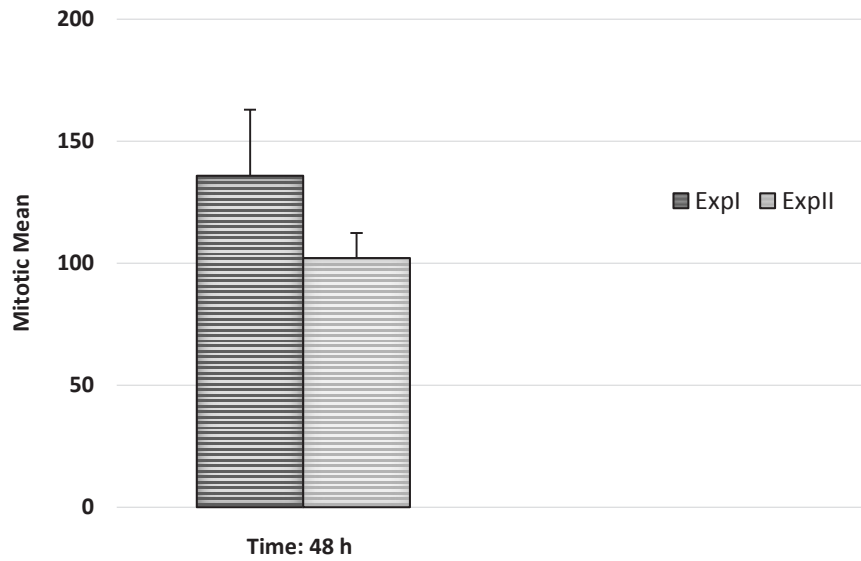

Figure 1. Comparison of mitotic index (MI) between Exp-I and Exp-II ($p < 0.001$).

mitogen (PWM), which was known to stimulate T and B-lymphocytes, and some other mitogens such as Concavalin A (Con-A), lipopolysaccharide (LPS), Wheat Germ Agglutinin (WGA) and Soybean Agglutinin (SBA) were used and their mitotic stimulating effects in single use and combined use of these mitogens were compared (Sofuni and Yoshida, 1992). One of the mitogens, PHA, has been widely used for mitotic stimulation of human lymphocytes, and several different types of PHA, such as PHA-P, M,W and others, comparing its ability to induce mitoses, were presented by other workers (Morgan and Watkins, 2000; Velloso et al., 2002; Onaga and Taira, 2008; Espinosa et al., 2009; Meesmann et al., 2010; Beinke et al., 2016). Dectin-1, a β-glucan receptor, is the mammalian lectin that was identified by Gordon and Brown (Caudill et al., 2015). A new type of plant root lectin found in different leguminous plants but not in plants of different family (Kalsi and Etzler, 2000).

PHA has the potential to induce closer contacts between adjacent cell membranes; it is an N-aceteylgalactosamine/galactose sugar-specific lectin with wide variety of biological activities (Zhang et al., 2008). PHA has been successfully used for membrane-induced fusion in human oocytes (Tesarik et al., 2000), bovine oocyt (Hong et al., 2005), and caprine oocytes (Begin et al., 2003). It was the first direct evidence of the involvement of bacterial lectins in the initiation of infection, as well as the basis for the present attempts in academia and industry to apply carbohydrates for antiadhesion therapy of such diseases reviewed by Mulvey and co-workers (Mulvey et al., 2001).

Since the development in the early 1960s of convenient methods of culturing peripheral blood leukocytes (Nowell and Hungerford, 1960), the study of human chromosomes has been a rapidly advancing, exciting field. Earlier attempts were made to analyze human and other mammalian chromosomes by utilizing tissues, such as bone marrow and testicular tissue, in which cells in division were present. These investigators tried to adapt a plant chromosome methodology to the study of human tissues, but were largely unsuccessful. Frustration occurred because of the large number of chromosomes in the human complement, which meant that they could not be spread out and separated enough to visualize the entire complement of one cell. For the automation of human chromosome analyses, the accumulation of an enormous number of mitoses is thought to be a great advantage (Misawa et al., 1988; Kushida et al., 2000; Horwitz, 2007; Ikehara, 2009; Tsao et al., 2009; Kim and Yu, 2014; Schreiber et al., 2014; Nath et al., 2015).

In addition to the well-established effect of PHA on mitotic stimulation, and based on the current literature, the advantages of the PHA agent would indicate the potential sources for developing a novel pharmaceutical preparation. However, reports in which the culture condition of human lymphocytes, including the stimulating condition by mitogens, was examined in detail were quite limited, and the purpose of the present study is to establish the most appropriate condition in mitotic stimulation to human lymphocytes.

Materials and Methods

Materials

PHA, Colcemid, RPMI 1640 and fetal bovine serum were

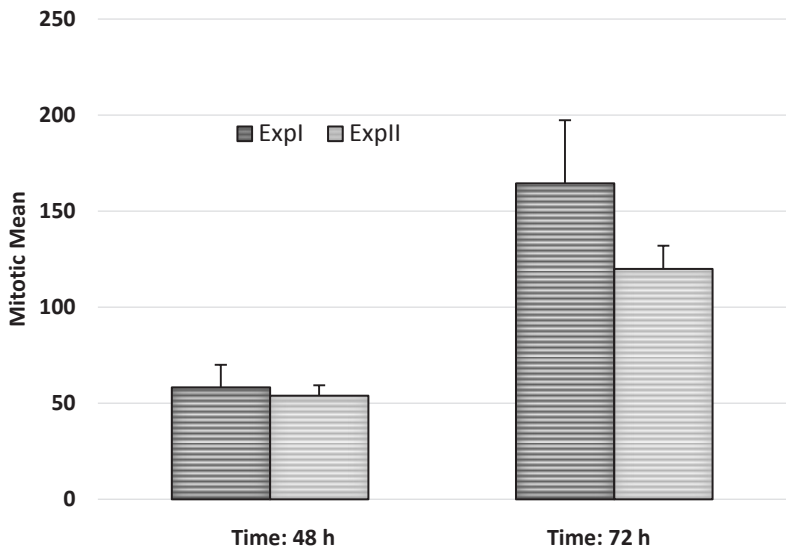

Figure 2. Comparison of mitotic index (MI) in Exp-I and Exp-II with two different culture periods ($p < 0.001$).

supplied from Gibco-BRL (Grand Island, NY, USA). All other chemicals were supplied form Merck Chemicals (Germany) and Sigma (USA).

Sample information

We studied 175 normal adults in order to determine the simultaneous effect of two different mitogens for cell division and karyotype procedure. Of these, 85 were males and 90 were females. The experiment was evaluated at Tehran during the year 2004-2015. The samples were taken from the individual with their consent. Personal information of the individual was kept confidential. No medicines or drugs were taken by this group for at least one month prior to sampling. Also, those suffering from genetic disorders, cancers, chronic and acute leukemia, syndromes, current viral infections, or who had a general or dental X-ray in less than a month were excluded from the study; also, they were to have had no recorded over-exposure in their personnel medical documents.

Study design

In each sample, 0.5-1.0 ml peripheral blood was obtained from each individual. For the culture, 3-5 × 10^6 cells were cultured in 4 ml medium (RPMI 1640) supplemented with 15% heat inactivated fetal bovine serum and the total preparation of PHA (5 µl/ml according to the manufacture's description). Also, 5µl/ml PHA with similar concentration was prepared by ourselves in a research laboratory at 37 ºC in an atmosphere containing 5% CO2. Briefly, the cultured cells were treated with Colcemid at the final concentration of 10 µg/ml and incubated at 37 ºC for an additional 20 min. The contents of the tube were then centrifuged for 10 min at 1000 rpm and re-suspended in 10 ml of 75 mM KCl (0.56%) pre-warmed to 37 ºC for 20 min. At this stage, 1 ml of Carnoys Fixative (3:1 methanol: acetic acid) was added into the tube, and this fixation step was repeated four times. Ten slides were prepared for each culture and stained for 3 min with Giemsa. Slides were examined with an Olympus model BH-2 light microscope. Experimental examination of mitotic division was described according to ISCN (Brothman, Persons et al., 2010).

Statistical analysis

The data of mitotic index (MI) on the slides were pooled after t-testing and the statistical differences yielding $p < 0.05$ were considered significant. Data analysis was performed by SPSS (version 16, Inc. USA) software.

Results

This series of 175 normal individual blood donors could be grouped into two experiments (I and II) to replicate categories according to the nature of two different mitogen cell division treatments. Two different mitogens, *viz*. 'self-made' indicated as Exp-I, and 'commercial imported' designated as Exp-II, were used simultaneously for cell division.

Slides for both experiments were coded and randomized prior to scoring MI. Consecutive metaphases on blind selected slides per replicated preparation were scored, whether or not they were suitable for the

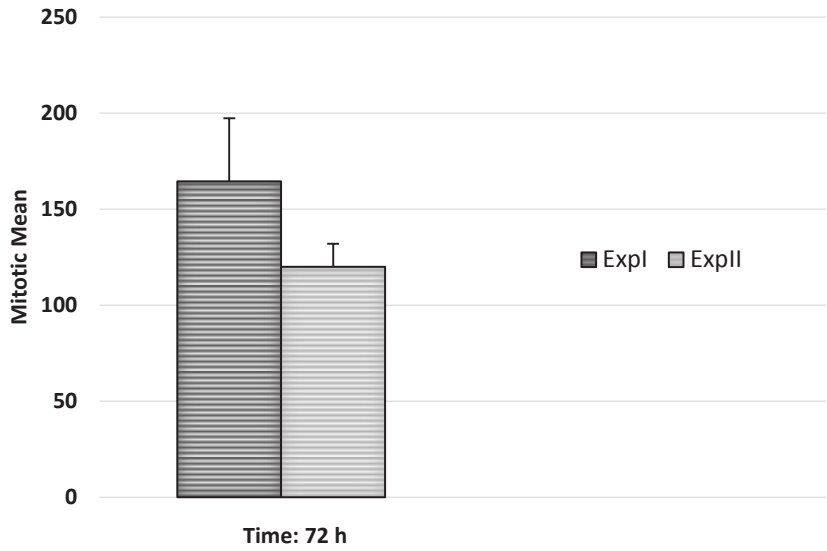

Figure 3. Comparison of mitotic index (MI) in Exp-I and Exp-II with 72 h culture period ($p < 0.001$).

full analysis. Well metaphases, spread on two to five slides from each of the two experiments of cell culture (replicate) with different stimulated cell division were scored. In eight cases, no outgrowth was obtained, hence another five normal blood samples (cases) was prepared with fresh simultaneous mitogen treatments. Generally, chromosome preparation obtained from Exp-I exhibited a higher mitotic activity than the Exp-II individual culture preparation of commercial mitogen material. This indicated that no consistent differences for MI were observed between Exp-I and Exp-II for eight (8%) cases. A total of 73 (77%) cell culture preparations were harvested after three days (72 h), while the remaining 27 (23%) cell culture preparations were arrested at 48 hours. This application of alternative treatment has been followed by a standard and conventional method in accordance with ISCN to arrive at a firm conclusion.

Results revealed some differences in population percentages with various MI between the two experiments (Table 1). It was observed that the mean value of the MI in the Exp-I was significantly greater than that obtained in Exp-II (135.77 vs 102.16, $p < 0.001$) (Fig. 1). The difference significantly increased when applying 72 h with respect to 48 h for both Exp-I and II ($p < 0.001$) (Fig. 2), whereas the difference was not found to be significant, considering the distribution of gender between the two experiments ($p < 0.007$). Figures 3 and 4 illustrate the effect of culture periods on the MI in Exp-I and Exp-II, respectively, and as can be seen, the difference was significant only at 72 h ($p < 0.001$) in comparison with 48 h culture period ($p = 0.510$).

Discussion

The results of this research show that the extracted PHA can be used in the leucocyte cultures for the cytogenetic studies. In developing countries like Iran, where cytogenetic tests are becoming routine in hospitals and in research, the extracted PHA may be utilized, which is less expensive and has a shelf life of more than one year when stored at −20 °C.

Numerous evidences have demonstrated the wide variety biological activities of PHA, such as the mitogenetic stimulation of lymphocytes and agglutination of cancer cells. The role of various glycosyltransferase in the pathogenicity of cancer cells was also indicated (Kim et al., 2008) and the fusion of mammary gland epithelial cells into enucleated oocytes in caprine, bovine, goats have been exhibited (Tesarik et al., 2000; Begin et al., 2003; Hong et al., 2005). Lectin precipitations using PHA are considered as candidate biomarkers for colorectal cancer discovery (Kim et al., 2008). A PHA skin test is an easy and reliable method of providing an impression of the immune response of surgical patients; it has many advantages above the commonly employed primary and secondary antigens (Meijer et al., 1984). Insulin, like Growth Factor-1, promotes cord blood T cell mutation and inhibits its spontaneous and PHA-induced apoptosis through the down regulation of Fas expression (Tu et al., 2000).

The present work confirmed and extended our previous findings, that the most normal control cases revealed a well-spread MI when a self-made mitogenic product was used, compared with the mitotic index in malignant diseases.

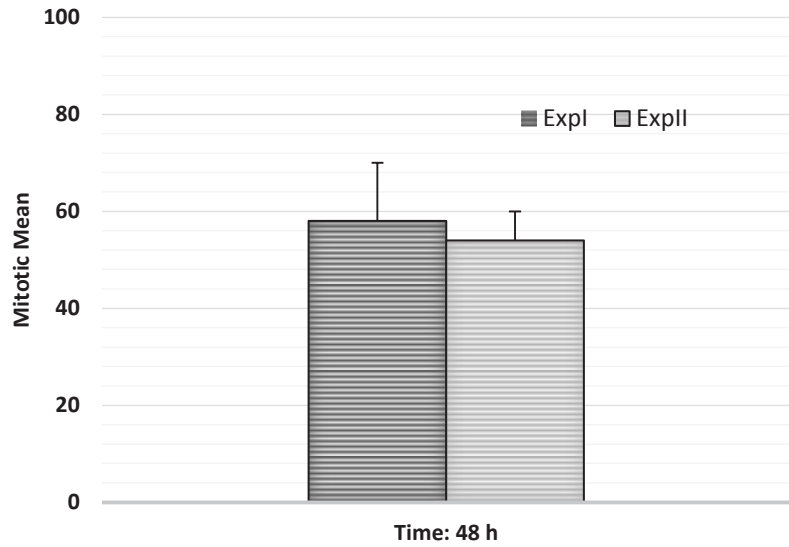

Figure 4. Comparison of mitotic index (MI) in Exp-I and Exp-II with 48 h culture period ($p = 0.510$).

Cytogenetic findings in hematologic malignancies are useful in diagnosis and prognostic approaches. Generally, those samples with low cell counts fail to produce sufficient metaphases for accurate diagnosis. Even when cultures were successful, the chromosomes were often highly contracted, and the metaphases spread showed low quality (Misawa et al., 1988). Based on the present investigation, significant differences ($P<0.001$) between Exp-I and II for MI were found by applying simultaneous treatments of both mitogen reagents. It is reasonable to state that this variation could be a sufficiently significant reason for using the application of an indigenous product rather than an imported commercial PHA mitogen. Moreover, with the improved MI obtained from the procedure presented here, a more precise identification of chromosomal abnormalities in routine laboratory diagnosis of neoplasia would be possible.

Furthermore, it is reasonable to state that lectins could act as modulations of cell substratum interactions and to be essential for the normal differentiation and growth of all multicellular animals. They are capable of inducing cell proliferation, cell arrest, or apoptosis (physiological cell death) and have been implicated in organ morphogenesis, tumor cell metastasis, leukocyte trafficking, skin test for immunological assessment response, and inflammation, as well as the recognition of extracellular matrix (Sharon and Lis, 2004).

Conclusion

Our attempts to develop a self-prepared PHA have led us to the following conclusions; The fresh preparations of indigenous PHA mitogen, have used in order to identify chromosome preparation for normal, as well as other, malignancies are superior to other imported commercial products. This is due to the product being indigenous, has more metaphases scores, freshness, cost-effectiveness, and has easy access, as it is a local product. Briefly, in our country the challenge for the biotechnology and therapeutic product as a profession is in its initial stages compared to the developing countries, although the results of this study may reveal a high demand for this product among health care and clinical dimensions. However, additional experiments are necessary to establish the most appropriate condition of using different mitogens for the metaphase preparation.

Table 1. Population percentage with different MI.

Number of individuals				
Experiment	**MI > 300**	**200 < MI < 300**	**100 < MI < 200**	**MI < 100**
Exp-I (percentage)	11 (8 %)	7 (6 %)	7 (6 %)	25 (24 %)
Exp-II (percentage)	0 (0 %)	10 (10 %)	12 (12 %)	27 (27 %)

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Competing Interests

The authors declare that they have no conflict of interests.

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