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Potato Seed Production of Cultivar Kufri Himalini, *In vitro*

Anoop Badoni* and J. S. Chauhan**

Seed Biotechnology Laboratory, Department of Seed Science and Technology
Faculty of Agriculture, H. N. B. Garhwal University,
(Chauras Campus), Srinagar- 246 174, Uttarakhand, India
*Research Scholar and Young Scientist (UCOST) *Assoc. Prof. and Head
*For correspondence E-mail- annabadoni@yahoo.co.in

Abstract: The nodal cuttings of potato cv. Kufri Himalini was cultured in MS medium consisting three different hormonal combinations of GA_3 and NAA (MSH1- 0.25mg/l GA_3+ 0.01 mg/l NAA, MSH2- 0.25mg/l GA_3+ 0.03 mg/l NAA and MSH3- 0.25mg/l GA_3+ 0.04 mg/l NAA) for shoot and root proliferation. After 35-40 days of incubation, shoots in MSH1 (0.25 mg/l GA_3 and 0.01 mg/l NAA) reached 8.28 cm with 9.4 nodes and 11.9 cm root length, higher then all the combinations. For tuberization three concentration of BAP (8 mg/l, 10 mg/l and 12 mg/l) were used with MS liquid medium, the plantlets were shifted to tuberization media and data were reported for the number of microtuber on per original shoot, average weight of microtuber and number of eyes in each microtuber. Formation and development of microtubers were least with 8 mg/l and higher in 10 mg/l BAP concentration, while with the increasing concentration, BAP inhibit the average number, weight and eyes number of microtubers. [Stem Cell, 2010;1(1):7-10] (ISSN 1545-4570).

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Micropropagation of *Hedychium spicatum* Smith using *In Vitro* Shoot Tip

Anoop Badoni[1], Chetna Bisht[2] and J. S. Chauhan[1]

[1]Researcher, Young Scientist (UCOST), Department of Seed Science and Technology, Faculty of Agriculture, H. N. B. Garhwal University, (Chauras Campus), Srinagar- 246 174, Uttarakhand, India
For Correspondence: e-mail- annabadoni@yahoo.co.in
[2]Research Scholar, High Altitude Plant Physiology Research Center, Faculty of Agriculture, H. N. B. Garhwal University, Srinagar- 246 174, Uttarakhand, India
[1]Associate Professor and Head, Department of Seed Science and Technology, Faculty of Agriculture, H. N. B. Garhwal University, (Chauras Campus), Srinagar- 246 174, Uttarakhand, India

Abstract: *Hedychium spicatum* Smith is a medicinally important species of the genus *Hedychium* commonly known as Vanhaldi, Palashi and Kapurkachari. This species occurs in subtropical and temperate Himalayan region between 1500 m and 2700 m altitudes. In the present study MS media supplemented with different growth regulators such as Kinetin and IAA were used for shoot elongation and root formation from *in vitro* shoot tip. Shoot elongation and rooting percentage (80%) was reported highest on medium with 5.0mM/l Kn and 1.0mM/l IAA. After rooting the complete plantlets were transferred to sterilized soil pots for acclimatization. About 40-50% plantlets survived well. [Stem Cell, 2010;1(1):11-13] (ISSN 1545-4570).

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Sorption studies of lead ions onto activated carbon produced from oil-palm fruit fibre

*[1]Olugbenga Solomon Bello, [1]Mary Adelaide Oladipo, [2]Abimbola Modupe Olatunde

*[1]Ladoke Akintola University of Technology, Department of Pure and Applied Chemistry, P. M. B, 4000, Ogbomoso, Oyo State. Nigeria
[2]University of Ibadan, Department of Chemistry, Ibadan, Oyo State. Nigeria.
Corresponding author +2348035685435. E-mail address: osbello@yahoo.com

ABSTRACT: The batch sorption removal of Pb^{2+} from aqueous solution using treated oil palm fruit fibre was studied. The adsorption equilibrium and kinetic studies of Pb^{2+} on such fibre were then

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	examined at 25°C. Adsorption isotherms of Pb^{2+} on the activated carbon produced from treated oil palm fibre were determined and correlated with common isotherm equations. The equilibrium data for Pb^{2+} adsorption fitted well to the Langmuir equation more than the Freundlich equation with maximum monolayer adsorption capacity of 588.24 mg/g. The batch sorption model, based on a pseudo-second-order mechanism, was applied to predict the rate constant of sorption, the equilibrium capacity and the initial sorption rate with the effects of the initial solution pH and fiber dose. The adsorption capacity at equilibrium increases from 75.48 to 439.06 mg g^{-1} with an increase in the initial lead concentration from 100 to 500 mg l^{-1}. Equilibrium concentrations were evaluated with the equilibrium capacity obtained from the pseudo-second-order rate equation. The adsorption data was found to fit the pseudo second order model more than the pseudo first order model. [Stem Cell, 2010;1(1):14-29]. (ISSN 1545-4570).	
5	**Renal Stem Cells Research and Applications** Hongbao Ma, Yan Yang Brookdale University Hospital and Medical Center, One Brookdale Plaza, Brooklyn, New York 11212, USA. Ma8080@gmail.com **Abstract:** This article is to try describing the renal stem cells in animal and to explore the methods to either repair or regenerate a damaged kidney with stem cells. For this purpose, the 3 sections are concerned: (1) Isolation of stem cells from animal embryo and/or other resources (such as adipose and renal tissue). (2) Embryonic stem (ES) cells differentiate into renal stem cells and/or renal progenitor cells. (3) Renal stem and/or progenitor cells differentiated from ES cells are used for renal repair and/or regeneration. The stem cell treatment can be the most hopeful technique on the renal repair and regenerate. [Stem Cell. 2010;1(1):30-51] (ISSN 1545-4570).	Full Text 30-51
6	**Haemocoelom excludes embryonic stem cells and asexual reproduction in invertebrates?*** P. Murugesan [a], T. Balasubramanian [a] and T. J. Pandian[b] [a]C. A. S. in Marine Biology, Annamalai University, Parangipettai – 608 502 [b]C. A. S in Genomics, School of Biological Sciences, Madurai Kamaraj University, Madurai – 625 021. murugesaan74@rediffmail.com **Abstract:** The terms embryonic and adult stem cells are explained. Previous studies on identification, description and isolation of the embryonic stem cells in different invertebrate groups are briefly summarized. Most invertebrates, which reproduce asexually, have retained the embryonic stem cells in their adult body. A hypothesis is proposed for the possible exclusion of embryonic stem cells and thereby asexual mode of reproduction by the coelom in arthropods and molluscs. [Stem Cell. 2010;1(1):52-57] (ISSN 1545-4570).	Full Text 52-57
7	**STRONG HORSE TEA BY ALICE WALKER: *A REVIEW*** Samiya Taskeen[1] and Abida Taskeen[2] [1] Hajvery University Lahore,Pakistan; Email: funnydoll5@yahoo.com [2]Lahore College for Women University Lahore Pakistan; Email: Paristan12@yahoo.com **ABSTRACT:** This review is about the history of those black skinned people who were treated like slaves in the past because it was believed that black skinned people were born to serve others in fact they are slaves who have no right to live like humans. Alice Walker in “STRONG HORSE TEA” focuses how Rannie Toomer lost her only child and how she has become the victim of white men’s exploitation who suffered from superiority complex and they considered themselves torch-bearers and civilized but in reality they are in need to be civilized and they are savage, ignorant, cruel and prejudice. [Stem Cell. 2010;1(1):58-59] (ISSN 1545-4570).	Full Text 58-59

8	**Effect of *Bacillus thuringiensis var. israelensis* (H-14) on Culex, Aedes and Anopheles larvae (Cotonou; Benin)** Gbehou Nounagnon Achille [1,*], Houssou S. Christophe[2], Li Yilian [3] 1. School of Environmental Studies, China University of Geosciences, Hubei province, 388 lumo Road, 430074 Wuhan, P.R China, , 008615927014830 2. University of Benin, (Abomey-Calavi), Dean of the Faculty of Human sciences Box: 526-Abomey- Calavi- Benin 3. China University of Geosciences, Department of Environmental Sciences, 388 Lumo Road, Hongshan District, Wuhan City, Hubei province, 430074, P.R.China *For correspondence: achillegbehou@yahoo.fr **Abstract:** The use of insect-specific toxins from *Bacillus thuringiensis var. israelensis* is forming an increasingly important component of biological control strategies that are either being implemented or planned for use in mosquito control. In terms of morbidity and mortality caused by vector-borne diseases, mosquitoes are the most dangerous animals confronting mankind. They threaten more than 2 billion people and have substantially influenced the development of mankind, not only socio-economically but also politically. In this study, the use of *Bacillus thuringiensis var. israelensis* crystals for controlling insect's larvae was carried out at laboratory scale. Three species of insects were tested. The operational parameters for the most efficient use and monitoring of *Bacillus thuringiensis var. israelensis* toxins against insect's larvae in the laboratory were discussed. [Stem Cell. 2010;1(1):60-68] (ISSN 1545-4570).	Full Text 60-68
9	**Determination Of Thermal Stability Of Oral Polio Vaccine (Opv) At Different Temperature Under Laboratory Conditions** Muhammad T[1], SS Baba[2], LT Zaria[2], AD El-Yuguda[2] And IB Thilza[3] [1]who National Polio Laboratory University Of Maiduguri Teaching Hospital. [2]department Of Veterinary Microbiology And Parasitology University Of Maiduguri. [3]department Of Veterinary Medicine, University Of Maiduguri. muhammadt6@gmail.com **ABSTRACT:** Expanded program on immunization is one of the strategic universally accepted methods for the control of childhood diseases which include poliomyelitis. In Nigeria both monovalent and trivalent oral polio vaccines are routinely used. Thermal stability was determined using 16 vials obtained from different storage facilities, had titres which ranged from $\log_{10}$ 6.5 to 8.4. These values still fell within the normal limits recommended by WHO as minimum accepted values (P1= $\log_{10}$6.0, P2=5.0 and P3= 5.8). It was observed that the storage facilities in all the three tier of vaccination centres had adequate power supply ranging from solar refrigerators, standby generators and the National Electricity supply. Also, polio vaccine vials have vaccine vial monitor (VVM) device which usually indicate change in color when cold-chain is not maintained. This necessitated the change of vaccine carrier when the need arose during the house to house immunization exercise. Adequate potency obtained in this study confirmed ideal storage condition of vaccines in Maiduguri. [Stem Cell. 2010;1(1):69-73] (ISSN 1545-4570).	Full Text 69-73

Conventional *vis -a- vis* Biotechnological Methods of Propagation in Potato: A Review

Anoop Badoni* and J. S. Chauhan**

Seed Biotechnology Laboratory, Department of Seed Science and Technology,
Faculty of Agriculture, Chauras Campus, H. N. B. Garhwal University,
Srinagar- 246 174, Uttarakhand, India
*Research Scholar, Young Scientist (UCOST), **Assoc. Prof. and Head of Department
*For Correspondence: annabadoni@yahoo.co.in

ABSTRACT

The present review is based on the findings on *in vitro* culture of potato. Because of many problems in conventional method of potato cultivation, it is necessary to develop a suitable method of propagation through biotechnology, which is a better alternative. In all potato growing regions the availability of high quality tuber has been the most demanding over the conventional clonal propagation that favors disease build-up, which drastically reduces crop yield. However, the recent advances in tissue culture and the flexibility of organ development in potato, allows alternate methods of propagation through *in vitro* techniques. In the absence of chemical control of viral disease, meristem tip culture is the only effective method available till date to eliminate virus infections from potato cultivars. This technology has ensured greater availability of diseases free seed for cultivation, which ultimately helps in boosting overall potato production in the country. In view of the above, a protocol have developed for sterilization of explants and found the suitable hormonal combination with MS medium for *in vitro* shoot regeneration, multiplication and rooting in potato cv. *Kufri Himalini*. For development of sterilization protocol two important sterilant sodium hypochlorite and mercuric chloride compared with different durations of 2, 5 and 8 minutes. For shoot proliferation and rooting the sterilized explants were cultured on MS medium, supplemented with different hormonal combinations i.e. MSGN1 (0.1 mg/l GA_3 and 0.01 mg/l NAA), MSGN2 (0.1 mg/l GA_3 and 0.03 mg/l NAA), MSGN3 (0.1 mg/l GA_3 and 0.1 mg/l NAA), MSKN1 (0.01 mg/l Kinetin and 0.1 mg/l NAA), MSKN2 (0.001 mg/l Kinetin and 0.1 mg/l NAA) and MSKN3 (1 mg/l Kinetin and 0.1 mg/l NAA). The observations were recorded after 10, 20 and 30 days to observe the non-growing cultures, infected cultures, healthy cultures, length of stem and number of nods on stem. Result showed that amongst the two sterilants i.e. NaOCl and $HgCl_2$, NaOCl was found better for controlling the infection and had not any adverse effect on explants even in long duration. The lower concentration of auxin (0.01 mg/l NAA) with Gibberelic Acid (0.1 mg/l) was found best for the development of complete plantlets and for multiplication from meristem tips. [Stem Cell. 2010;1(1):1-6] (ISSN 1545-4570).

Keywords: Conventional, clonal, *In vitro*, multiplication, sterilization, shoot regeneration

INTRODUCTION

As a crop of high biological value for its protein and a substantial amount of vitamins, minerals and trace elements, potato is undoubtedly a very important crop in the country (Gebre and Sathyanarayana, 2001). The world population is likely to become 3,000 million by 2020 AD and more than 90% of this population would live in the developing countries under condition of extreme poverty and forced hunger (Khurana *et al*., 1998).

Potato in India is now grown over 1.3 million hectares with an annual production of 23.6 million tones (Directorate of Economics and Statistics, Ministry of Agriculture, Govt. of India, 2004-05). With the update of modern potato technologies, India now ranks 4th in area and 3rd in production of potato in the world. The productivity of potato in India is like better than the world average (166.3 quintals/hectare). By 2020 India will have a population of 1.3 billion bringing about a substantial pressure on land to produce more food. It is also estimated that by 2020 worldwide demand for potatoes would increase by 40 per cent as a result of increased urbanization, rise in per capita income and fast expanding tourism.

Conventional Methods for Propagation

Conventional propagation of potato is done vegetatively using seed tubers and ensures uniformity of the crop in terms of growth and yield, but results in degeneration of the crop due to virus infection, the rate of degeneration varies from place to place and cropping to cropping season. The viruses are transmitted through different ways including planting infected tubers. If the seed stock is illmaintained or frequently replaced with fresh ones, the virus infiltration can reach up to 100% in 3 - 4 successive crop seasons resulting in almost half or one third yields. This is the major problem faced by seed producers.

The main problem of growing potato worldwide is economic losses due to late blight, which is caused by *Phytophthora infestans which* can destroy potato plants within two weeks in wet conditions. Blight can survive even under adverse conditions. The pathogen however, invades and infects potato in the field via zoosporangia, which disperse via soil water, rain splash and wind. Potato cultivars are tetraploid vegetatively propagated crop, which poses several problems in seed production. Generally tuber is used as a seed. Due to progressive accommodation of viral disease in seed stock, availability of good quality seed is a major constraint in potato production, which is approximately 50% of the total production cost. Besides high cost of seed potato, propagation is also characterized by low multiplication rate of only 4-6 times.

Selection of Variety

The ICAR has identified *Kufri Himalini* for commercial cultivation in hill regions. Late blight has intensified over the few years, and resistance to the disease has been decreasing in existing varieties such as Kufri Jyoti and Kufri Giriraj. The new variety, with medium maturity of 110-120 days has been recommended for cultivation in the north- west and eastern hills during summer. *Kufri Himalini* provides a yield advantage of 10% over Kufri Jyoti and Kufri Giriraj. In the plains, its keeping quality is better than all the cultivars developed so far for hill regions (The Hindu, 2005).

Why Biotechnological Methods?

To large production of clonal material i.e., to produce the uniform, identical seed material of potato, micro propagation is the better alternative over to conventional propagation of potato. The in vitro propagation method is most suitable alternative to produce Microtuber seed material of potato. By using the technique, which involves low cost components, the large-scale clonal material can be achieved in short time duration. Use of micro propagation for commercial seed production has moved potato from test tubes to field (Wang and Hu, 1982). The advances are the being of the second "Green Revolution" in agriculture and are expected to make farming more efficient, profitable and environmentally safe (Dhingra *et. al.,* 1992). Micro propagation is a sophisticated technique of regenerating plants using small pieces of plants (so called explants) that is proliferated on an artificial medium under sterile conditions. Importance of micro propagation lies in very fast clonal multiplication of vegetable crops. Micro propagation is used mainly for getting disease- free plants of superior vigour and productivity (Singh, 1997).

MATERIALS AND METHODS

(i) Sterilization Protocol of explants

The present study was carried out to standardize the sterilization procedure of explants of potato cv. *Kufri Himalini*. Two different chemicals i.e. Mercuric chloride (0.1%) and Sodium hypochlorite (1%) were used for study with duration of 2, 5 and 8 minutes. For obtaining sprouts, the tubers were cut into pieces and dipped in a solution of 0.1% bavistin for 2-3 minutes and sown in sand filled plastic pots followed with single wash in distilled water. These were grown under poly house following optimum cultural practices. The sprouts were ready for inoculation after 10-12 days of growth.

The sprouts of 0.5-1 cm. were collected from the mother plant of *Kufri Himalini* in water filled beaker and kept under running water prior to sterilization in the laminar airflow cabinet. The explants were surface sterilized with three selected timings of 2, 5 and 8 minutes. To evaluate the response of different chemicals, implantations of sterilized explants were done using without hormones MS medium. The observations were reporded on 10, 20 and 30 days for the non-growing cultures, infected cultures and healthy cultures.

(ii) In vitro shoot proliferation and rooting

The present investigation was carried out with the objective; to study the effect of two hormonal combinations i.e. GA_3+ NAA and Kinetin + NAA with MS medium on shoot regeneration and multiplication using meristem tips of potato cv. *Kufri Himalini*.

For inoculation of explants different media with hormonal combinations were prepared properly. MS media supplemented with different combinations of GA_3+ NAA and Kinetin + NAA (Table-1), were autoclaved at 15 psi for 20 minute. The hot medium was immediately dispensed in to culture flask (30 ml medium in each flask) and covered with autoclaved cotton plug in Laminar Air Flow Cabinet. 12 replicates of each combination were taken for the study. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explants must be sterilized. After inoculation the cultures of different combinations were shifted to culture growth room at $25^0 \pm 1^0$ c and 16 h photoperiod. Best combination of GA_3+ NAA and Kinetin + NAA with MS medium was selected on the basis of cultures growth performance i.e. length of stems and number of nodes on stem, after 35 days. The mean values of culture growth were calculated and the selected combination was used for sub culturing of plantlets also. The best combination of hormones with MS medium was selected and cultures showed higher growth were further sub-cultured on its parent medium by cutting it in to small pieces in a way that each subsection has at least 1-2 nodes.

TABLE-1: Different hormonal combinations used for shoot proliferation

Symbol used	Hormones		Symbol used with MS medium (full strength with 8 gm./l agar and 30 gm./l Sucrose)
	GA_3	NAA	
GN 1	0.1 mg/l	0.01 mg/l	MSGN 1
GN 2	0.1 mg/l	0.03mg/l	MSGN 2
GN 3	0.1 mg/l	0.1 mg/l	MSGN 3
	Kinetin	**NAA**	
KN 1	0.01 mg/l	0.1 mg/l	MSKN 1
KN 2	0.001 mg/l	0.1mg/l	MSKN 2
KN 3	1 mg/l	0.1 mg/l	MSKN 3

RESULT AND DISCUSSION

(i) Sterilization Protocol of explants

In vitro propagation technique for potato involves various steps i.e. selection of explants, its sterilization and establishment and shoot proliferation and production of *in vitro* tubers. Beside the growth regulators, the culture conditions namely temperature, relative humidity and photoperiod also influence the growth and development process of *in vitro* cultures (Hussey and Stacey, 1981). The first condition for the success of a culture is asepsis. The maintenance of aseptic (free from all microorganisms) or sterile conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explants itself must be sterilized. The importance is to keep the air, surface and floor free of dust. All operations should be carried out in laminar airflow sterile cabinet (Chawla, 2003).

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and achieve better survival (CPRI, 1992). Two different chemicals i.e. Mercuric chloride (0.1%) and Sodium hypochlorite (1%) were used for the present study to standardize the best sterilization protocol for *in vitro* culture of potato cv. *Kufri Himalini*.

In the present study NaOCl was always found better than $HgCl_2$ (Table-2; Plate-1). Sodium hypochlorite (NaOCl) for 8 minute (T3) was selected for suitable sterilization chemical after 5 minute of savlon wash, 30-second dip in ethanol and at last washed with double distilled water.

Mercuric chloride is a very strong sterilant (Gopal *et al.*, 1998) disinfected the single nodal cuttings of 22 cultivars with a mixture of 0.1% Mercuric chloride and 0.1% Sodium lauryl sulfate for 5 minutes. Calcium hypochlorite being a mild sterilant has been used for potato. Nozeram *et al.*, (1977) sterilized potato sprouts by dipping them in alcohol and a few drops of Teepol and then placed them in Calcium hypochlorite solution for 15-25 minutes. Sodium hypochlorite has turned out to be a better sterilant than calcium hypochlorite due to bleaching effects of the later and hence has been extensively used for potato sterilization. Amongst the two sterilants i.e. NaOCl and $HgCl_2$, NaOCl was found better for controlling the infection and it had not any adverse effect on explants even in long duration.

(ii) In vitro shoot proliferation and rooting

Micro propagation is one of the finest ways of plant multiplication by in vitro techniques of plant tissue culture. Micro propagated plants are true to type and often show improved vigor and quality. Micro propagation is the alternative to conventional propagation of potatoes (Chandra and Birhwan, 1994, Naik and Chandra, 1994). *In vitro* propagation methods using meristem tips, nodal cuttings and micro tubers are more reliable for maintaining genetic integrity of the multiplied clones since de-differentiation and the subsequent organogenesis/ embryo genesis with the accompanying genetic changes have been reported (Wang and Hu, 1983).

Meristem culture provides a reproducible and economically viable method for producing pathogen free plants. As meristem tips are free from viruses, elimation and generation of virus free plants are possible through meristem culture (Jha and Ghosh; 2005). Lam (1977) studied the effect of auxin: Kinetin ratio in the nutrient medium for proliferation of tuber discs of cv. *spunta* and found that the addition of 0.2 mg/l NAA to the medium appeared to adjust the ratio to the points where normal plantlets with both shoots and roots were produced in a single step.

Different combinations of GA_3+ NAA and Kinetin + NAA with MS medium influenced *in vitro* shoot regeneration from meristem tip culture. Data for length of stem and number of nodes on stem were recorded after 35 days of growth in all the combinations. Shoot length in M.S. medium with GA_3 and NAA combination showed better result in comparison to M.S. medium with Kinetin and NAA (Table- 3; Fig.1; Plate-1).

The combination of Kinetin and NAA had consistently given good result for improving shoot length. The MSKN2 (0.001 mg/l Kinetin and 0.1 mg/l NAA) having low concentration of Kinetin and NAA and

MSKN3 (1 mg/l Kinetin and 0.1 mg/l NAA) combinations having higher concentration of Kinetin (1 mg/l) and low concentration of NAA, responded the least mean shoot length and number of nodes. Low concentration of Auxin (0.1 mg/l NAA) plus moderate concentration of Cytokinine (0.01 mg/l Kinetin) showed good development of complete plantlets from meristem tips.

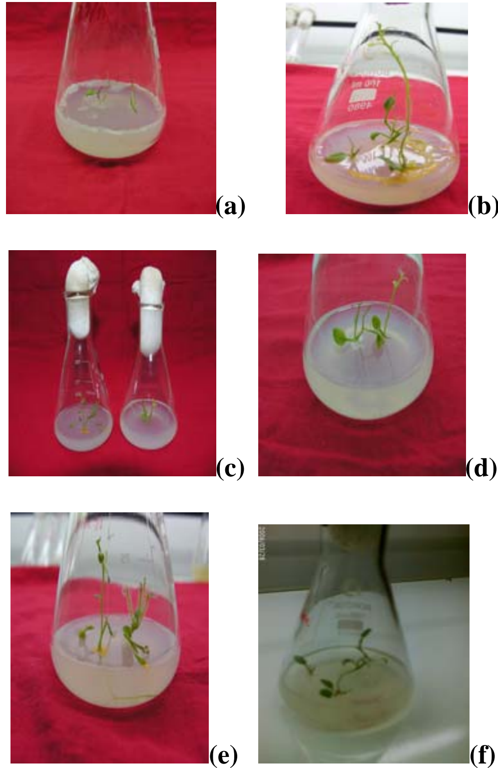

Plate1: Tissue culture study on potato cv. *Kufri Himalini*; **(a) and (b):** infected shoot tips cultured on MSKN3 and MSKN1 media respectively **(c)** selected best plantlets of NaOCl chemical with 8 minute cultured on MSKN2 media **(d)** healthy culture of potato cultured on MSGN2 media **(e)** and **(f)** cultures on MSGN1 and MSGN3 media respectively.

The combination of GA_3 + NAA showed best result for improving shoot length. The MSGN2 (0.1 mg/l GA_3 and 0.03 mg/l NAA) and MSGN3 (0.1 mg/l GA_3 and 0.1 mg/l NAA) combinations respectively having higher concentration of NAA (0.1 mg/l) responded the least mean shoot length and number of nodes. This could be attributed to the fact that higher concentration of NAA inhibit root and shoot growth (Pennazio and Vecchiati, 1976). Result showed that lower concentration of auxin (0.01 mg/l NAA) with Gibberelic Acid (0.1 mg/l GA_3) is best for development of complete plantlets from meristem tips with avoiding callus and satisfactory root formation. It can be concluded from the present findings that GA_3 + NAA (MSGN1) combination is best for shoot regeneration and multiplication of potato cv. *Kufri Himalini* in comparison to the combination Kinetin + NAA with M. S. medium.

Using the tissue culture technique of micro propagation, it is possible not only to reduce the number of field exposures but also to increase the rate of multiplication several times. Plant tissue culture comprises a set of in vitro techniques, methods and strategies that are part of the group of technologies called plant biotechnology. Tissue culture has been exploited to create genetic variability from which crop plants can be improved, to improve the state of health of the planted material and to increase the number of desirable germplasms available to the plant breeder. The culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars. Large-scale micro propagation laboratories are providing millions of plants for the commercial ornamental market and the agricultural, clonally propagated crop market. According to the present study conclusion is that NaOCl for 8 minute was a best sterilant and for shoot proliferation and root formation the combination of GA_3 (0.1 mg/l)+NAA (0.01 mg/l) was found to be better.

TABLE-2 Observations of sterilization procedure

Observations	Sterilents and Duration (in Minutes)					
	Sodium hypochlorite			Mercuric chloride		
	T1	T2	T3	T1	T2	T3
Non-growing cultures	8±2	4±2	5.6±1.5	5.66±1.1	8.66±0.5	8.66±1.5
Infected cultures	3.66±2.5	2.33±0.5	1.33±0.5	3.33±1.5	3.66±0.5	3.33±1.5
Healthy cultures	2±2	4.33±1.5	6±2	1.33±1.5	1.33±0.5	4.33±1.1

TABLE-3: Effect of different hormonal combinations on stem length and number of nodes

Hormonal Combination	Length of stem (cm.)	Number of nodes on stem
MSGN 1	6.8 cm. ± 0.5	5.5 ± 0.5
MSGN 2	6.3 cm. ± 0.5	5.2 ± 0.6
MSGN 3	4.4 cm. ± 0.6	3.0 ± 0.7
MSKN 1	6.4 cm. ± 0.6	5.0 ± 0.7
MSKN 2	5.3 cm. ± 1.2	4.2 ± 0.8
MSKN 3	4.0 cm. ± 0.6	2.7 ± 0.7

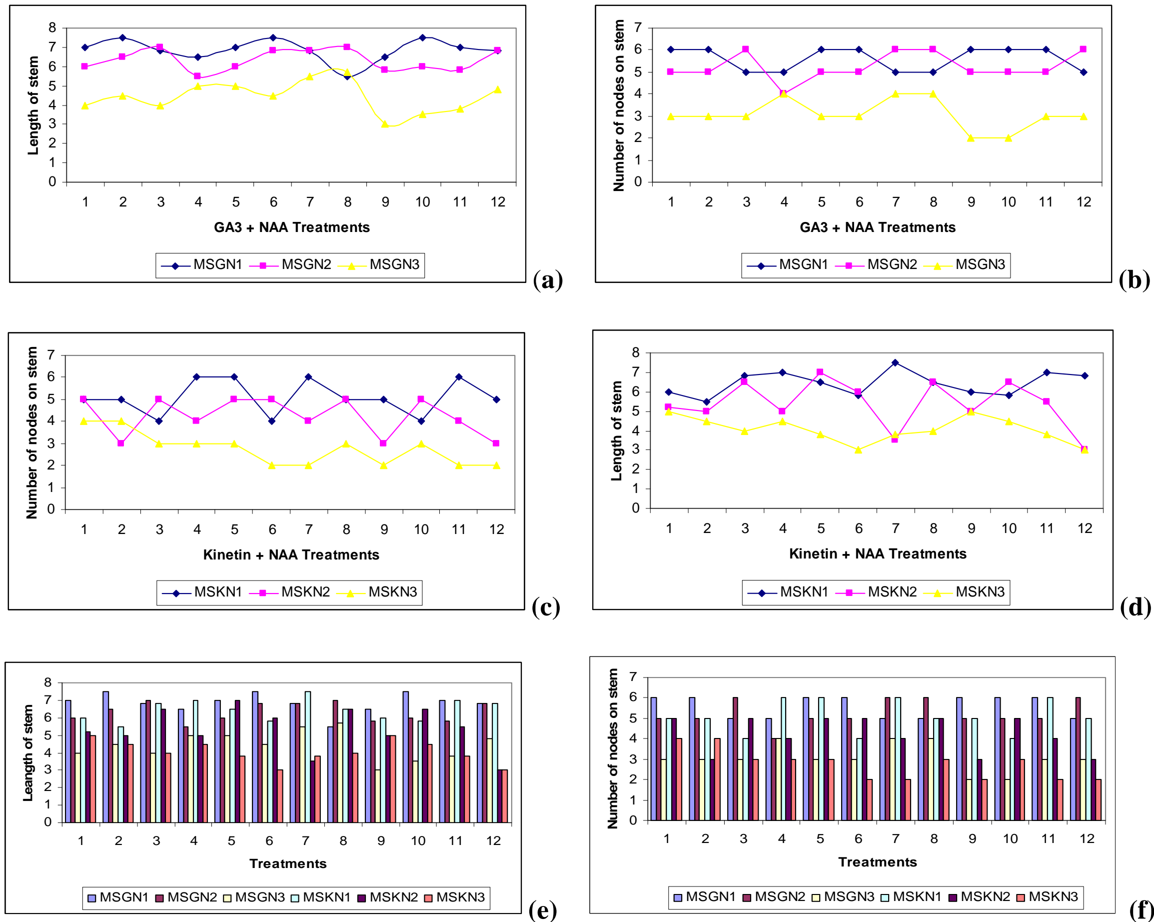

Fig. 1 **(a)** Length of stem, **(b)** Number of nodes, on GA_3+ NAA treatments, **(c)** Length of stem **(d)** Number of nodes on, Kinetin + NAA treatments, **(e)** Length of stem and **(f)** number of nodes on stem; Comparison between deferent treatments.

Correspondence to:
Anoop Badoni
annabadoni@yahoo.co.in

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3/27/2009

Potato Seed Production of Cultivar Kufri Himalini, *In vitro*

Anoop Badoni* and J. S. Chauhan**

Seed Biotechnology Laboratory, Department of Seed Science and Technology
Faculty of Agriculture, H. N. B. Garhwal University,
(Chauras Campus), Srinagar- 246 174, Uttarakhand, India
*Research Scholar and Young Scientist (UCOST) *Assoc. Prof. and Head
*For correspondence E-mail- annabadoni@yahoo.co.in

Abstract

The nodal cuttings of potato cv. Kufri Himalini was cultured in MS medium consisting three different hormonal combinations of GA_3 and NAA (MSH1- 0.25mg/l GA_3+ 0.01 mg/l NAA, MSH2- 0.25mg/l GA_3+ 0.03 mg/l NAA and MSH3- 0.25mg/l GA_3+ 0.04 mg/l NAA) for shoot and root proliferation. After 35-40 days of incubation, shoots in MSH1 (0.25 mg/l GA_3 and 0.01 mg/l NAA) reached 8.28 cm with 9.4 nodes and 11.9 cm root length, higher then all the combinations. For tuberization three concentration of BAP (8 mg/l, 10 mg/l and 12 mg/l) were used with MS liquid medium, the plantlets were shifted to tuberization media and data were reported for the number of microtuber on per original shoot, average weight of microtuber and number of eyes in each microtuber. Formation and development of microtubers were least with 8 mg/l and higher in 10 mg/l BAP concentration, while with the increasing concentration, BAP inhibit the average number, weight and eyes number of microtubers. [Stem Cell, 2010;1(1):7-10] (ISSN 1545-4570).

Key words: Tuberization, microtuber, Kufri Himalini, hormonal combination

Introduction

Potato is one of the most important crops in the world today. It produces more protein and calories per unit area per unit time and per unit of water than any other major plant food. In all potato growing regions the availability of high quality clean seed tuber has been the most limiting owing to the conventional clonal propagation that favors disease build-up that drastically reduces yield (Gebre and Sathyanarayana, 2001). Conventional propagation of potato is done vegetatively using seed tubers and ensures uniformity of the crop in terms of growth and yield, but results in degeneration of the crop due to virus infection, the rate of degeneration varying from place to place and from cropping season to cropping season (Tadesse, 2000). The viruses are transmitted through different ways including through planting infected tubers. If the seed stock is not maintained well or frequently replaced with fresh ones, the virus infiltration can reach up to 100% in 3 - 4 successive crop seasons resulting in almost half or one third yields (Khurana et al. 2001). This is the major problem faced by seed producers. Conventional seed multiplication methods take a long time and are prone to virus problems (Biniam and Tadesse, 2008).

In the rapid multiplication of clean material *in vitro,* the use of single nodal cutting is the most preferred method of propagation since it ensures higher propagation rates with maximum genetic uniformity in potato (Chandra and Naik, 1993). The major factors limiting the rates of multiplication in nodal culture are the short height of the plantlets and low number of nodes on plantlets obtained. Improvement has been made possible by addition of growth regulators to the medium. Gas stimulated development of nodal cutting on MS but at high concentration it produced narrow and elongated shoots (Novak *et al.,* 1980) depending on genotype. Longest main shoot and highest node numbers are reported to be obtained in medium containing NAA and BAP (Yousef *et al.,* 1997). Among these methods, the direct use of microtubers has gained a considerable interest owing to their ease of handling, storage and transport of germplasm and reduced period to produce seed tubers (Jones, 1994). Media conditions such as N concentration, sucrose or osmolarity of the medium have either a direct or indirect effect on induction or development processes of *in vitro* produced microtubers (Garner and Blake, 1989; Khuri and Moorby, 1995).

However, there are limitations both in shoot regeneration and microtuber production. The limitations in many ways are ascribed to the components of the culture environment and to the low photosynthetic ability of the explants or plantlet. Most current systems of microtubers production have problems of obtaining sufficient number and size of microtubers produced per cycle. Thus both shoot and microtuber production systems are still less competitive and economical compared with *in vitro* rapid multiplication (Gebre and Sathyanarayana, 2001). The aim of present study was to produce the microtuber seed material of potato cv. Kufri Himalini for farmers of Uttarakhand Hills, in different concentrations of BAP with MS media.

Material and Methods

Shoot proliferation: The shoot proliferation study was done using potato cv. Kufri Himalini, obtained nodal cutting of about 2-4 cm. The medium was prepared using MS (full strength) salts dissolved in double distilled water and consisted 0f 3% sucrose. The pH was adjusted to 5.8 before boiling the medium. Agar was maintained at the standard concentration (8 gm/l). The nodal cutting as explants was cultured in MS medium consisting three different hormonal combinations of GA_3 and NAA (MSH1- 0.25mg/l GA_3+ 0.01 mg/l NAA, MSH2- 0.25mg/l GA_3+ 0.03 mg/l NAA and MSH3- 0.25mg/l GA_3+ 0.04 mg/l NAA). Cultures were than shifted to culture growth room at $25^0 \pm 1^0$ c and 16/8 hr photoperiod.

In vitro Tuberization: After shoot development for further proliferation the cultures were cut to size and approx. 5-8 propagules were inoculated in each 250 ml flask containing 50 ml of pre-tuberization media (without agar) and kept with 16/8 hr photoperiod for 25-30 days. For tuberization three concentration of BAP (8 mg/l, 10 mg/l and 12 mg/l) were used with MS liquid medium, the plantlets from pre-tuberization media were shifted to tuberization media and kept at $18^0 \pm 1^0$ C temperature under complete darkness for the duration of 60-80 days depending on the growth of microtubers.

Results

Shoot proliferation: After 35-40 days of incubation, shoots in MSH1 (0.25 mg/l GA_3 and 0.01 mg/l NAA) reached 8.28 cm with 9.4 nodes and 11.9 cm root length (Table-1). The MSH2 (0.25 mg/l GA_3 and 0.03 mg/l NAA) and MSH3 (0.25 mg/l GA_3 and 0.04 mg/l NAA) combinations respectively having higher concentration of NAA responded the least mean shoot height and number of nodes. In MSH2 shoot height reached 7.1 cm with 8.2 node number and 10.6 cm root length and in MSH3 shoot height reached 6.1 cm with 6.3 node number and 9.4 cm root length.

Table-1: Effect of GA_3+NAA concentrations with MS media on shoot height, node number, and root length

Growth regulators (mg/l)			Shoot height (cm)	Node number	Root length (cm)
GA_3	NAA	Symbol used			
0.25	0.01	MSH 1	8.2 ± 0.5	9.4 ± 1.0	11.9 ± 1.1
0.25	0.03	MSH 2	7.1 ± 0.5	8.2 ± 1.0	10.6 ± 1.0
0.25	0.04	MSH 3	6.1 ± 0.6	6.3 ± 0.9	9.4 ± 1.0

In vitro Tuberization: *In vitro* tuberization was obtained after proliferating the culture in pre-tuberization medium (liquid propagation medium) where cultures grew profusely (Plate-1-b). The effect of BAP concentrations with MS medium was studied for microtuber formation and development (Table-2). The perusal of data in Table-2 indicates that the number of microtuber on per original shoot, average weight of microtuber and number of eyes in each microtuber were least with 8 mg/l and higher in 10 mg/l BAP concentration, while with the increasing concentration, BAP inhibit the average number, weight and eyes number of microtubers.

Table-2: Effect of BAP concentration with MS media on *in vitro* tuberization

Growth regulator	No. of eyes in each microtuber	Average weight of microtuber (mg)	Microtuber no. per original shoot
BAP (mg/l)			
8	14 (±1.4)	0.342 (±0.02)	4 (±0.7)
10	19.6 (±1.5)	0.450 (±0.02)	6.6 (±0.5)
12	14.4 (±1.1)	0.410 (±0.01)	5.2 (±0.8)

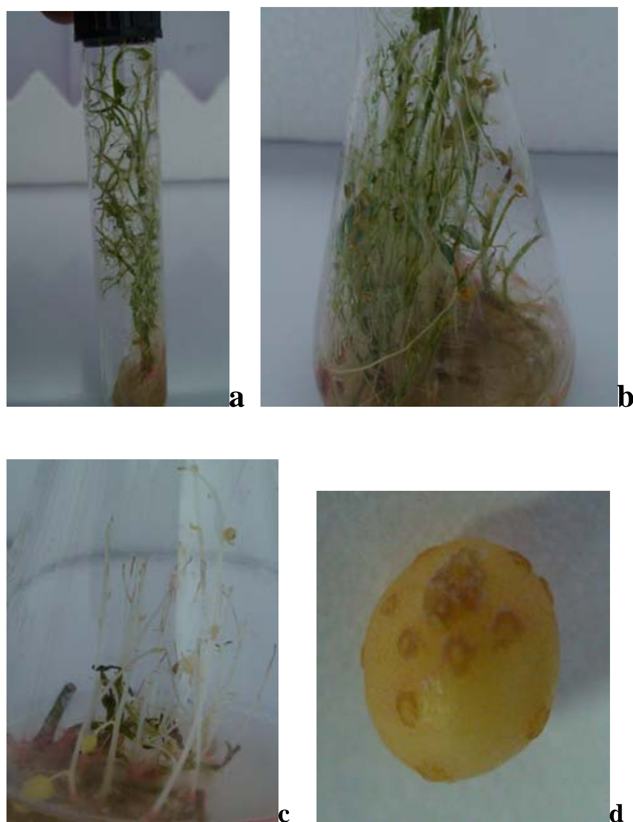

Plate-1: Seed production of potato cv. Kufri Himalini; **(a)** Shoot proliferation, **(b)** pre-tuberaization stage, **(c)** *in vitro* tuberization and **(d)** harvested microtuber

Discussion

The results of shoot proliferation in the present study are comparable or even better than the most rapid node production (x8 to x10 per month) reported earlier by Hussey and Stacey (1981). The MSH2 (0.25 mg/l GA_3 and 0.03 mg/l NAA) and MSH3 (0.25 mg/l GA_3 and 0.04 mg/l NAA) combinations respectively having higher concentration of NAA responded the least mean shoot height and number of nodes. This could be attributed to the fact that higher concentration of NAA inhibit root and shoot growth (Pennazio and Vecchiati, 1976). The cultures proliferating in MS media were maintained separately for tuber induction.

Pre-tuberization medium composed of MS nutrients, GA_3 and NAA was used following the procedure of Naik and Chandra (1993). Shifting the cultures from pre-tuberization to tuberization stage, cultures were exposed to a major change from vegetative growth phase to reproductive phase leading to tuber development. GA was integral component of pre-tuberization medium but during tuber induction stages, GA was withdrawn as it canalizes all the carbohydrates towards shoot development during pre-tuberization and decrease in GA promotes partitioning of biomass to the tubers (Krauss, 1978). Hence tuber induction could be achieved by withdrawal of GA and addition of GA inhibitor. BA as a GA inhibitor, has been used in varying concentration from 2 mg/l to 10 mg/l and due to its GA inhibitory role and the presence of BA canalize all the resources of plants towards tuberization, i.e anabolic activity rather than elongation of stem, with this idea of GA inhibitory metabolites, triazoles have come up as a suitable tuber inducing substances (Harvey, 1990; Simko, 1994).

The present study confirmed that lower concentration of auxin (0.01 mg/l NAA) with Gibberelic Acid (0.25 mg/l GA_3) is best for development of complete plantlets and 10 mg/l concentration of BAP with MS media was best for microtuber development.

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Micropropagation of *Hedychium spicatum* Smith using *In Vitro* Shoot Tip

Anoop Badoni[1], Chetna Bisht[2] and J. S. Chauhan[1]

[1]Researcher, Young Scientist (UCOST), Department of Seed Science and Technology, Faculty of Agriculture, H. N. B. Garhwal University, (Chauras Campus), Srinagar- 246 174, Uttarakhand, India
For Correspondence: e-mail- annabadoni@yahoo.co.in

[2]Research Scholar, High Altitude Plant Physiology Research Center, Faculty of Agriculture, H. N. B. Garhwal University, Srinagar- 246 174, Uttarakhand, India

[1]Associate Professor and Head, Department of Seed Science and Technology, Faculty of Agriculture, H. N. B. Garhwal University, (Chauras Campus), Srinagar- 246 174, Uttarakhand, India

Abstract

Hedychium spicatum Smith is a medicinally important species of the genus *Hedychium* commonly known as Vanhaldi, Palashi and Kapurkachari. This species occurs in subtropical and temperate Himalayan region between 1500 m and 2700 m altitudes. In the present study MS media supplemented with different growth regulators such as Kinetin and IAA were used for shoot elongation and root formation from *in vitro* shoot tip. Shoot elongation and rooting percentage (80%) was reported highest on medium with 5.0mM/l Kn and 1.0mM/l IAA. After rooting the complete plantlets were transferred to sterilized soil pots for acclimatization. About 40-50% plantlets survived well. [Stem Cell, 2010;1(1):11-13] (ISSN 1545-4570).

Key words: Shoot regeneration, rooting and acclimatization

Introduction

Various herbs of medicinal value growing naturally in the higher reaches of Himalayas are under indiscriminate exploitation pressure by traders. In the recent past, the uncontrolled and excessive extraction of Himalayan herbs has gone up to the extent that serious threats are now being feared for the long term availability of many of these species. It is therefore, prime time to recognize the problem and to develop strategies for the conservation and rational exploitation of these herbs (Rawat, 1989). As many of the medicinal species growing at high altitudes have slow growth and poor seedling establishment due to harsh environmental conditions, conventional methods of propagation are not sufficient, and especially for endangered species, attempts for conservation using both *in situ* and *ex situ* methods are immediately needed. In spite of this fact, the conservation measures for Himalayan plant species did not start until recently. It is, however, quite encouraging that in the past few years, there has been a growing interest in the conservation and multiplication of threatened species from the Himalayan region using tissue culture methods (Hemant lata, 1997).

Hedychium spicatum Smith is one of the medicinally important species of the genus *Hedychium* commonly known as Vanhaldi, Palashi and Kapurkachari. This species occurs in subtropical and sub-temperate Himalayan region in oak (Quercus spp.) and deodar (Cedrus deodara) forests on slopes between 1500 m and 2700 m altitudes (Nautiyal *et al.,* 2004; NMPB, 2008). Leaves of the plant are glabrous underneath, broadly lanceolate ending in a tail-like tip. Flowers fragment white with an orange-red base in a dense terminal spike borne on a robust leafy stem. Seeds are black with a red aril (Naithani, 1984; 1985). This species is widely used as Kapurkachari in Ayurvedic preparations. Aromatic rootstock contains essential oil, saccharin, albumin, starch and mucilage. The rhizomes are stomachic carminative, stimulant and tonic, and are used for the treatment of dyspepsia, asthma and bronchitis (Singh, 1983). Rhizome powder is sprinkled as an antiseptic agent and also used as a poultice for various aches and pains (Thakur *et al.,* 1989). Locally rhizomes are boiled and eaten with salt, and roasted powder is given for asthma and decoction of rhizome with Deodar sawdust is taken for tuberculosis (Gaur, 1999).

Material and Methods

The seeds were collected from Valley of Flower, Nanda Devi Biosphere Reserve, district Chamoli of Uttarakhand. The seeds were washed thoroughly in running tap water and surface sterilized with Tween-20 for 10 minutes. Sterilized seeds were rinsed with sterile double distilled water for 3-4 times. These seeds were treated with 0.5% (4% concentrated sodium hypochloride, qualigence) for 5 minutes and finally rinsed with sterilized double distilled water for 3-4 times to remove the traces of sterilants. Sterilized seeds were cultured on agar and

sucrose based medium for germination. After germination, the root portion of the seedlings was removed and shoot tip was used as explants. Shoot tip regenerated from seeds were shifted to MS media (1962) supplemented with different combinations of Kinetin and IAA concentrations (1.0 mM/l Kn + 0.2 mM/l IAA; 3.0 mM/l Kn + 5.0 mM/l IAA and 5.0 mM/l Kn + 1.0 mM/l IAA) for shoot elongation and root formation. After rooting the complete plantlets were transferred to sterilized soil field pots for acclimatization.

Result and Discussion

The results for shoot elongation and rooting indicates (Table-1; Plate-1-a and b) that MS media with higher concentration of Kn and IAA (5.0 mM/l Kn+ 1.0 mM/l IAA) showed higher growth of shoots and rooting percentage (80%). Similar type of shoot growth and rooting from rhizomes of *Hedychium spicatum* showed 80% establishment in MS medium supplemented with Kinetin (5.0 mM) and IAA (1.0 mM) (Bhatt *et al.,* 2008). The lower concentrations of Kn and IAA (1.0 mM/l Kn+0.2 mM/l IAA and 3.0 mM/l Kn+0.5 mM/l IAA) showed slow growth of shoots and low rooting percentage (10% and 40% respectively). Published information on the micropropagation using *in vitro* shoot tip explants of *Hedychium spicatum* is not found earlier. Hardening of the well rooted plantlets was done in the potting mixtures of soil sand and vermi compost (Plate-1-c) and kept under poly house condition for survival and growth. The plantlets were survived well as about 40-50%.

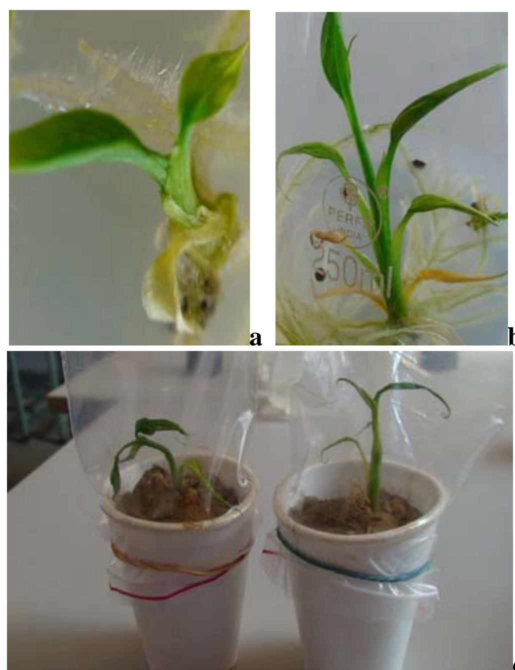

Plate-1 Micro-propagation of *Hedychium spicatum*: (a) and **(b):** Shoot regeneration, elongation and rooting on shoots and **(c)** Hardening of plantlets

The procedure will not only help in developing cultivation packages of the species but will also help in formulating appropriate strategies for conservation and utilization of rare and endemic medicinal plants of Himalayas.

Table-1 Effect of Kn and IAA on shoot elongation and rooting of shoots

Hormones concentrations with MS media (mM/l)		Average shoot length (cm.)	Rooting percentage	Average root length (cm.)
Kn	IAA			
1.0	0.2	3.5±0.5	10	5.5±0.2
3.0	0.5	4.5.6±0.2	40	6.2±0.5
5.0	1.0	6.8±0.2	80	8.5±0.5

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Correspondence to

Anoop Badoni
Researcher and Young Scientist (UCOST)
Department of Seed Science and Technology
H. N. B. Garhwal University, (Chauras Campus),
Srinagar- 246 174, Uttarakhand, India
E-mail- annabadoni@yahoo.co.in

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Sorption studies of lead ions onto activated carbon produced from oil-palm fruit fibre

*[1]Olugbenga Solomon Bello, [1]Mary Adelaide Oladipo, [2]Abimbola Modupe Olatunde

*[1]*Ladoke Akintola University of Technology, Department of Pure and Applied Chemistry, P. M. B, 4000, Ogbomoso, Oyo State. Nigeria*

[2]*University of Ibadan, Department of Chemistry, Ibadan, Oyo State. Nigeria.*

Corresponding author +2348035685435.

E-mail address: osbello@yahoo.com

ABSTRACT: The batch sorption removal of Pb^{2+} from aqueous solution using treated oil palm fruit fibre was studied. The adsorption equilibrium and kinetic studies of Pb^{2+} on such fibre were then examined at 25°C. Adsorption isotherms of Pb^{2+} on the activated carbon produced from treated oil palm fibre were determined and correlated with common isotherm equations. The equilibrium data for Pb^{2+} adsorption fitted well to the Langmuir equation more than the Freundlich equation with maximum monolayer adsorption capacity of 588.24 mg/g. The batch sorption model, based on a pseudo-second-order mechanism, was applied to predict the rate constant of sorption, the equilibrium capacity and the initial sorption rate with the effects of the initial solution pH and fiber dose. The adsorption capacity at equilibrium increases from 75.48 to 439.06 mg g^{-1} with an increase in the initial lead concentration from 100 to 500 mg l^{-1}. Equilibrium concentrations were evaluated with the equilibrium capacity obtained from the pseudo-second-order rate equation. The adsorption data was found to fit the pseudo second order model more than the pseudo first order model. [Stem Cell, 2010;1(1):14-29]. (ISSN 1545-4570).

Keywords: Oil palm fruit fibre; Adsorption; Isotherm; Kinetics. Sorption

1.0. Introduction

The use of activated carbon to remove heavy metal ions from wastewater by adsorption is a well-established process. The economics of this process depends mainly on the cost of the adsorbent material. As such, low cost adsorbents are becoming the focus of many investigations. Low cost adsorbents could be produced from many raw materials such as agriculture and industrial wastes. On the use of low cost adsorbents in the removal of dyes, we have investigated the adsorption of methylene blue onto activated carbon derived from periwinkle shells in recent time (Bello et al. 2008). In Nigeria, the palm oil industry generates huge amounts of palm shell. Some of this solid waste is usually used as fuel to produce process steam and/or electricity in palm oil mills. However, a large portion of it is either burned in open air or dumped in areas adjacent to the mill, which creates huge environmental and disposal problems. In recent years, the interest to palm shell has increased mainly due to the fact that this material was shown to be an excellent source of high quality and low cost activated carbon. However, most of the research on palm shell carbon is focused on the processes of carbonization and activation (Hussein et al. 1996, Lua and Guo 1998, Guo 2002, Daud et al. 2002, Daud and Ali 2004). Application of palm shell activated carbon as an adsorbent offers highly effective technological means dealing with heavy metal pollution of the aqua- environment with minimum investment requirements. The adsorption of cadmium and lead on the modified oil palm shell was investigated (Othman et al. 1994). The capacity of activated carbons obtained from the different parts of oil palm and the modified palm shell activated carbon to remove phenols from wastewaters has also been investigated (Abu Bakar 1999, Salim et al. 2002). The application of palm oil fuel ash in the removal of chromium and zinc from aqueous solutions has been reported (Chu and Hashim 2002a, b). Information on the performance of oil palm fiber based activated carbon for the adsorption of heavy metals from aqueous solutions is scanty. The advantage of using inexpensive natural resource as raw materials for manufacturing activated carbon is that these raw materials are renewable and potentially less expensive to manufacture. The aim of the present study is to explore the use of activated carbon produced from oil palm fibre for the removal of Pb^{2+} from aqueous solution. It is also aimed to determine which isotherm and model fit the adsorption process most.

2.0. Materials and Methods.

2.1. Preparation and Characterization of Adsorbent

The oil palm fruit fibre was obtained from a local oil palm mill in YOACO Area, Ogbomoso, Oyo State, Nigeria. The fibre collected was deoiled by soaking it in hot deionized water and with detergent for 24 hours. It was rinsed thoroughly in hot deionized water to remove all debris and then air dried. The air dried oil palm fruit fibre was grounded using a medium size mortal and pestle. The pretreated material was then carbonized at 700°C under nitrogen atmosphere for 1 h (first pyrolysis). A certain amount of produced char was then soaked with potassium hydroxide (KOH) at impregnation ratio of 1:1 (KOH pellets: char). The mixture was dehydrated in an oven overnight at 105°C; then pyrolysed in a stainless steel vertical tubular reactor placed in a tube furnace under high purity nitrogen (99.995%) flow of 150 $cm^3 min^{-1}$ (second pyrolysis) to a final temperature of 850°C and activated for 2 h. Once the final temperature was reached, the gas flow was switched to carbon dioxide and activation was continued for 2 h. The activated product was then cooled to room temperature under nitrogen flow and washed with deionised water to remove the remaining chemical. Subsequently, the sample was transferred to a beaker containing a 250 ml solution of hydrochloric acid (about 0.1 mol l^{-1}), stirred for 1 h, and then washed with hot deionised water until the pH of the washing solution reached 6 –7. Textural characterization of the activated carbon (AC) was carried out by N_2 adsorption at 77K using Autosorb I, supplied by Quantachrome Corporation, USA. The BET (N_2, 77K) is the most usual standard procedure used when characterizing an activated carbon (Sing *et al.,* 1985). It was found that the BET surface area, total pore volume, average pore diameter and pH point of zero charge (pHpzc) of the activated carbon were 1654m^2 g^{-1}, 1.115 cm^3 g^{-1}, 2.54 nm and 8.0, respectively. The pH point of zero charge (pHpzc) of activated carbon prepared from oil palm fibre shows that there exists a relationship between pHpzc and adsorption capacity of the adsorbent used. The result shows that cation adsorption will be favourable at pH value higher than pHpzc. While anion adsorption will be favoured at pH values lower than their adsorbent pHpzc (Nomanbhay and Palanisamy, 2005).

The treated oil palm fibre sample (10 mg) was ground with 200 mg of KBr (spectroscopic grade) in a mortar pressed into 10 mm diameter disks under 10 tonnes of pressure and high vacuum for 10 min. FTIR spectra were obtained on a JASCO FTIR-3500 spectrometer. The analysis conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 and 4000 cm^{-1}. The FTIR spectra of oil palm fibre, treated oil palm fibre and the oil palm fibre after adsorption are shown in Figure 1. The FTIR spectra of oil palm fibre showed peaks at 3240, 3015, 1650, 1540, 1450, 1420 1250 and 1160 cm^{-1} which may be assigned to OH group, aliphatic C–H group, unsaturated groups like alkene, amide, CH deformation, OH deformation, aromaticity and OH stretch, respectively. The intensity of the peaks were either minimized or shifted slightly in case of treated and adsorbed oil palm fibre, respectively. These results are similar to the ones reported for sawdust (Huang et al. 2005).

2.2. Preparation of Lead Nitrate Solution

All the reagents used were of analytical grade and doubly deionized water was used in sample preparation. 1000 mg l^{-1} stock solution of Pb^{2+} was prepared from $Pb(NO_3)_2$. From the stock solution, working solutions with different initial concentrations (ranging from 100 to 500 mg l^{-1}) were prepared by serial dilution.

2.3. Batch Equilibrium Studies

Adsorption isotherms were performed in a set of 30 Erlenmeyer flasks (250 ml) where solutions of lead (200 ml) with different initial concentrations (100–500 mg l^{-1}) were placed in these flasks. Equal mass of 0.2 g of particle size (225 μm) activated carbon produced from treated oil palm fibre was added to lead solutions and kept in an isothermal shaker (25 ± 1^0C) for 48 h to reach equilibrium of the solid-solution mixture. Similar procedure was followed for another set of Erlenmeyer flask containing the same lead concentration without activated carbon to be used as a control. The pH was adjusted to 7 by adding either few drops of diluted hydrochloric acid or sodium hydroxide (0.1 mol l^{-1}). The flasks were then removed from the shaker and the final concentration of lead in the solution was analyzed using atomic absorption spectrophotometer.

The samples were filtered prior to analysis in order to minimize interference of the carbon fines with the analysis. Each experiment was duplicated under identical conditions. The amount of adsorption at equilibrium, q_e (mg g^{-1}), was calculated by

$$q_e = \frac{(C_o - C_e)V}{W}$$ …………… (1)

where C_o and C_e (mg l^{-1}) are the liquid-phase concentrations of lead at initial and equilibrium, respectively. V is the volume of the solution (litre), and W is the mass of dry adsorbent used (g).

2.4. Batch Kinetic Studies

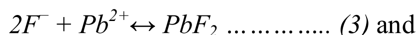
The procedures of kinetic experiments were basically identical to those of equilibrium tests. The aqueous samples were taken at preset time intervals, and the concentrations of lead were similarly measured. The amount of adsorption at time t, q_t (mg g^{-1}), was calculated by:

$$q_t = \frac{(C_o - C_t)V}{W}$$ ……………………………… (2)

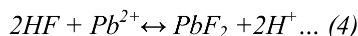
where C_o and C_t (mg l^{-1}) are the liquid-phase concentrations of lead at initial and any time t, respectively. V is the volume of the solution (litre), and W is the mass of dry adsorbent used (g).

2.5. Adsorption Dynamics

The order of a reaction and rate constant must be determined by experiments. A pseudo-second-order rate law expression was applied, which demonstrated how the rate depended on the sorption capacity but not the concentration of the sorbate (Ho and McKay 2000). Oil palm fibre, a cellulose-based sorbent, contains polar functional groups that can be involved in chemical bonding and are responsible for the cation exchange capacity of the oil palm fibre. Thus, the oil palm fibre and lead reaction may be represented in two ways (Ho and McKay 1998, 1999 and 2000).



$2F^- + Pb^{2+} \leftrightarrow PbF_2$ ………….. (3) and



$2HF + Pb^{2+} \leftrightarrow PbF_2 + 2H^+$ … (4)

where F^- and HF are polar sites on the oil palm fibre surface. The rate of sorption to the surface should be proportional to a driving force times an area. The rate of the pseudo-second-order reaction may be dependent on the amount of solute sorbed on the surface of the oil palm fibre at any time and the amount sorbed at equilibrium. The rate expression for the sorption described is:

$$\frac{dF_t}{dt} = k[F_e - F_t]^2$$ …….. (5)

$$\frac{d[HF]_t}{dt} = k[(HF)_e - (HF)_t]^2;$$ (6)

where F_t and $(HF)_t$ are the number of active sites occupied on the oil palm fibre at any time t, and F_e and $(HF)_e$ are the number of equilibrium sites available on the oil palm fibre. The driving force is related to $q_e - q_t$. Thus, the kinetic rate equations can be rewritten as follows:

$$\frac{dq_t}{dt} = k(q_e - q_t)^2;$$ ………… (7)

where k is the rate constant of sorption (g/(mg min)), q_e the amount of lead ions sorbed at equilibrium (mg/g), and q_t is the amount of lead ions sorbed on the surface of the oil palm fibre at any time t (mg/g). Separating the variables in the equation above gives:

$$\frac{dq_t}{(q_e - q_t)^2} = k\,dt;$$ ……… (8)

and integrating this for the boundary conditions $t = 0 - t$ and $q_t = 0 - q_t$ gives:

$$\frac{1}{q_e - q_t} = \frac{1}{q_e} + kt$$ ……. (9)

which is the integrated rate law for a pseudo-second-order reaction. This and Eq. (9) can be rearranged to obtain:

$$q_t = \frac{t}{(1/kq_e^2) + (t/q_e)}$$ …(10)

which has a linear form:

$$\frac{t}{q_t} = \frac{1}{kq_e^2} + \frac{1}{q_e}t$$ ………… (11)

or

$$\frac{q_t}{t} = \frac{h}{1 + kq_e t};$$ …... (12)

where h can be regarded as the initial sorption rate as q_t/t, when t approaches 0. Hence,

$$h = kq_e^2.$$ ………………………….. (13)

Thus, a plot of t/q_t against t of Eq. (9) should give a linear relationship with a slope of $1/q_e$ and an intercept of $1/kq_e^2$. In order to investigate the mechanism of the sorption of lead

ions onto oil palm fibre, a pseudo-second-order mechanism was studied. For the sorption of metal ions, which are small compared with dye molecules, and with a short contact time to equilibrium, the pseudo-second-order kinetic expression was considered likely to be more appropriate (Ho et al. 2000).

3.0 Results and Discussion

3.1. Effect of Agitation Time and Concentration of Lead on Adsorption.

A series of contact time experiments for lead was carried out at different initial concentrations (100–500 mg l^{-1}) and at temperature of 25^0C. Figure 2 shows the contact time necessary for lead with initial concentrations of 100–300 mg l^{-1} to reach equilibrium is 6 h. However, for lead with higher initial concentrations (400–500 mg l^{-1}), longer equilibrium time of 24 h is needed. As can be seen from Figure 2, the amount of the adsorbed lead onto activated carbon produced from treated oil palm fibre increases with time and, at some point in time, reaches a constant value beyond which no more is removed from solution. At this point, the amount of the lead desorbing from the adsorbent is in a state of dynamic equilibrium with the amount of the lead being adsorbed onto the activated carbon. The time required to attain this state of equilibrium is termed the equilibrium time, and the amount of lead adsorbed at the equilibrium time reflects the maximum adsorption capacity of the adsorbent under those operating conditions. The adsorption capacity at equilibrium increases from 75.48 to 439.06 mg g^{-1} with an increase in the initial lead concentration from 100 to 500 mg l^{-1}. It is evident that the activated carbon produced from treated oil palm fibre is efficient in adsorbing lead from aqueous solution, the process attaining equilibrium gradually.

3.2. Adsorption Kinetics

The rate constant of adsorption is determined from the pseudo first-order equation given by Langergren and Svenska (Lagergren and Svenska 1898):

$$\ln (q_e - q_t) = \ln q_e - k_1 t \quad \ldots\ldots\ldots\ldots\ldots. (14)$$

where q_e and q_t are the amounts of lead adsorbed (mg g^{-1}) at equilibrium and at time t (min), respectively, and k_1 the rate constant adsorption (h^{-1}). Values of k_1 were calculated from the plots of ln (q_e -q_t) versus t for different concentrations of lead (Figure 3). Although the correlation coefficient values at high concentration are higher than 0.90, the experimental q_e values do not agree with the calculated ones, obtained from the linear plots (Table 1). This shows that the adsorption of lead onto activated carbon produced from oil palm fibre is not first-order kinetics.

On the other hand, a pseudo second-order equation based on equilibrium adsorption (Ho and McKay 1998) is expressed as:

$$1/q_t = 1/k_2 q_e^2 + (1/q_t)\, t \quad \ldots\ldots\ldots\ldots\ldots\ldots (15)$$

where k_2 (g/mg h) is the rate constant of second-order adsorption. If second-order kinetics is applicable, the plot of t/q versus t should show a linear relationship. There is no need to know any parameter beforehand and q_e and k_2 can be determined from the slope and intercept of the plot. Also, this procedure is more likely to predict the behaviour over the whole range of adsorption. The linear plots of t/q versus t (Figure 4) show a good agreement between experimental and calculated q_e values (Table 1).

The correlation coefficients for the second-order kinetic model are greater than 0.99 indicating the applicability of this kinetic equation and the second-order nature of the adsorption process of lead on treated oil palm fibre.

3.3. Test of Kinetic Models

Besides the value of R^2, the applicability of both kinetic models are verified through the sum of error squares (SSE, %). The adsorption kinetics of lead on activated carbon derived from activated carbon produced from oil palm fibre was tested at different initial concentrations. The validity of each model was determined by the sum of error squares (SSE, %) given by:

$$SSE(\%) = \sqrt{\frac{\Sigma(q_e, \exp - q_e, cal)^2}{N}} \quad \ldots.. (16)$$

where N is the number of data points. The higher is the value of R^2 and the lower is the value of SSE; the better will be the goodness of fit. Table 1 lists the calculated values. It was found that the adsorption of lead on activated carbon produced from oil palm fibre can be best described by the second-order kinetic model. This finding was similar to other studies on the adsorption of lead by low-cost adsorbents. For instance, pseudo-second-order kinetic was also observed in the adsorption of cadmium and lead by spent grain (Low et al. 2000) and rubber (*Hevea brasiliensis*) leaf powder (Hanafiah and Ngah 2006, Hanafiah and Shafiei 2006).

3.4. Adsorption isotherms

The adsorption isotherm indicates how the adsorption molecules distribute between the liquid phase and the solid phase when the adsorption process reaches an equilibrium state.

The analysis of equilibrium adsorption data by fitting them to different isotherm models is an important step to find the suitable model that can be used for design purpose (Haghseresht and Lu 1998). Adsorption isotherm study is carried out on two well-known isotherms, Langmuir and Freundlich. Langmuir isotherm assumes monolayer adsorption onto a surface containing a finite number of adsorption sites of uniform strategies of adsorption with no transmigration of adsorbate in the plane of surface (Fytianos 2000). While, Freundlich isotherm model assumes heterogeneous surface energies, in which the energy term in Langmuir equation varies as a function of the surface coverage (Weber and Chakkravorti 1974). The applicability of the isotherm equation is compared by judging the correlation coefficients, R^2.

3.5. Langmuir isotherm

The linear form of Langmuir's isotherm model is given by the following equation:

$C_e/q_e = 1/Q_ob + (1/Q_o)\ C_e$………………. (17)

where C_e is the equilibrium concentration of the adsorbate (lead) (mg/l), q_e, the amount of adsorbate adsorbed per unit mass of adsorbate (mg g^{-1}), and Q_o and b are Langmuir constants related to monolayer adsorption capacity and affinity of adsorbent towards adsorbate, respectively. When C_e/q_e was plotted against C_e, straight line with slope $1/Q_o$ was obtained (Figure5), indicating that the adsorption of lead on activated carbon produced from activated carbon produced from oil palm fibre follows the Langmuir isotherm. The Langmuir constants 'b' and 'Q_o' were calculated from this isotherm and their values are given in Table 2.

The essential characteristics of the Langmuir isotherm can be expressed in terms of a dimensionless equilibrium parameter (R_L) (Hall et al. 1966, Adamson 2001), which is defined by:

$R_L = 1/\ (1 + bC_0)$…………… …….. (18)

where b is the Langmuir constant and C_0 the highest lead concentration (mg l^{-1}). The value of R_L indicates the type of the isotherm to be either unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$) or irreversible ($R_L = 0$). Value of R_L was found to be 0.04 and confirmed that the activated carbon is favorable for adsorption of lead under conditions used in this study.

3.6. Freundlich Isotherm

The well-known logarithmic form of Freundlich model is given by the following equation:

$\log q_e = \log K_F + (1/n) \log C_e$ (19)

where q_e is the amount adsorbed at equilibrium (mg g^{-1}), C_e the equilibrium concentration of the adsorbate (lead) and K_F and n are Freundlich constants, n giving an indication of how favorable the adsorption process and K_F (mg g^{-1}(l mg^{-1})^n) is the adsorption capacity of the adsorbent. K_F can be defined as the adsorption or distribution coefficient and represents the quantity of lead adsorbed onto activated carbon adsorbent for a unit equilibrium concentration.

The slope $1/n$ ranging between 0 and 1 is a measure of adsorption intensity or surface heterogeneity, becoming more heterogeneous as its value gets closer to zero (Haghseresht and Lu 1998). A value for $1/n$ below one indicates a normal Langmuir isotherm while $1/n$ above one is indicative of cooperative adsorption (Fytianos 2000). The plot of log q_e versus log C_e gives straight lines with slope '$1/n$' (Figure 6), which shows that the adsorption of lead also follows the Freundlich isotherm. Accordingly, Freundlich constants (K_F and n) were calculated and recorded in Table 2.

Table 2 shows the values of the parameters of the two isotherms and the related correlation coefficients. As seen from Table 2, the Langmuir model yields a somewhat better fit ($R^2 = 0.975$) than the Freundlich model ($R^2 = 0.922$). As also illustrated in Table 2, the value of $1/n$ is 0.466, which indicates favorable adsorption (Adamson 2001).

3.7. Effect of pH

Variations in the amount of lead (II) sorbed as a function of pH was studied, the pH of the lead(II) solution was set to pH 3 – 6, and results are presented in Figure 7. In this experiment, the agitation speed was 200 rpm, the fiber dose was 1.5 g/dm^3, the initial lead concentration was 120 mg/dm^3. It was observed that there was a rapid increase in the amount of lead sorbed from 2 to 70 % between pH 2 - 4. The percentage sorbed increased slightly to 75 % between pH 4- 5. Thereafter, the amount sorbed remains fairly constant above pH 5. The results obtained were analyzed using the pseudo-second-order model (Figure8). The coefficient of determination, R^2, the rate constant, k, the initial sorption rate, h, and the sorption capacity, q_e, were obtained from the slope and intercept of Eq. (11). Individual values are presented in Table 3. The amount of metal ions removal from solution was shown to be strongly affected by the pH of the solution, because it affects the surface charge, degree of ionization and speciation of the metal. The adsorption of lead (II) onto sphagnum moss

peat, for example, was found to vary with pH in the range 4 – 6, and the pH of maximum lead (II) uptake was found to be pH 5 (Ho et al, 1996). Srivastava et al. also obtained similar results for lead (II) uptake in the pH range 4 – 6 (Srivastava et al. 1989). In this current study, it is observed that the variation in pH values of the lead (II) solution from pH 2 to 5 produced an increase in sorption capacity from 37.31 to 47.39 mg/g and a corresponding increase in the initial sorption rate. It is also observed that as the pH increased from 5 to 6, the sorption capacity decreased. At a low pH, H^+ ions increase in solution and H^+ ions coordinate with OH groups to form $OH_2{}^+$. This gives the sorbent surface a positive character which leads to repulsion of the positively charged lead (II) from the sorbent surface. The coordination of H^+ ion with OH groups also reduces the cation exchange capacity of the sorbent. At higher pH values, however, the OH groups on the sorbent surface ionize to produce negatively charged oxygen (O^-) which enhances the cation exchange capacity.

3.8. Effect of Adsorbent Dose

The dependence of adsorption of lead on oil palm fibre was studied at room temperature (25^0C) at pH 4.0 by varying the amount of adsorbent from 0.10 to 1.5 g while keeping the volume (25 ml) and concentration of the lead aqueous solution constant. The result is shown in Figure 9. The amount of lead adsorbed (mgg^{-1}) was found to decrease with increasing amount of adsorbent. The amount of lead adsorbed decreased from 7.38 to 0.72 for adsorbent amount of 0.10 and 1.5 g, respectively. According to (Shukla *et al.,* 2002), the decrease in adsorption density with increase in adsorbent amount is due to the high number of unsaturated adsorption sites.

Table 1. Comparison of the pseudo first- and second-order adsorption rate constants and calculated and experimental q_e values for different initial lead concentrations at 25°C.

Initial concentration (mg^{-1})	$q_{e,exp}$ (mgg^{-1})	First order kinetic model				Second order kinetic model			
		k_1 (h^{-1})	$q_{e,cal}$ (mgg^{-1})	R^2	SSE(%)	k_2 [$g(mgh)^{-1}$]	$q_{e,cal}$ (mgg^{-1})	R^2	SSE(%)
100	75.48	1.562	137.19	0.92	27.60	0.0148	76.92	0.99	0.64
200	189.36	2.726	303.17	0.93	50.90	0.0093	192.31	0.99	1.74
300	282.64	6.680	404.07	0.95	54.31	0.0061	285.71	0.99	1.37
400	375.09	6.880	503.41	0.93	57.39	0.0058	386.62	0.99	5.77
500	439.06	11.243	745.68	0.92	137.12	0.0032	454.55	0.99	6.92

Table 2: - Langmuir and Freundlich isotherm constants for lead adsorption at 25°C.

Langmuir isotherm	
Q_o (mgg^{-1})	588.24 ± **0.02**
b (L/mg)	0.054 ± **0.03**
R^2	0.975 ± **0.01**
R_L	0.040 ± **0.02**
Freundlich isotherm	
$\frac{1}{n}$	0.466 ± **0.03**
K_F [(mgg^{-1})(mg^{-1})$^{1/n}$]	26.63 ± **0.01**
R^2	0.922 ± **0.02**

All values are means of triplicates ± SD.

Table 3: Pseudo-second-order rate parameters for sorption of lead on treated oil palm fibre at various initial solution pH values at 25°C.

pH	q_e (mg/g)	k (g/(mg min))	h (mg/(g min)	R^2
2	37.31	0.018	25.06	0.989
3	38.02	0.0318	46.08	0.996
4	42.74	0.0349	63.69	0.997
5	47.39	0.0405	90.19	0.998
6	40.63	0.0415	68.51	0.999

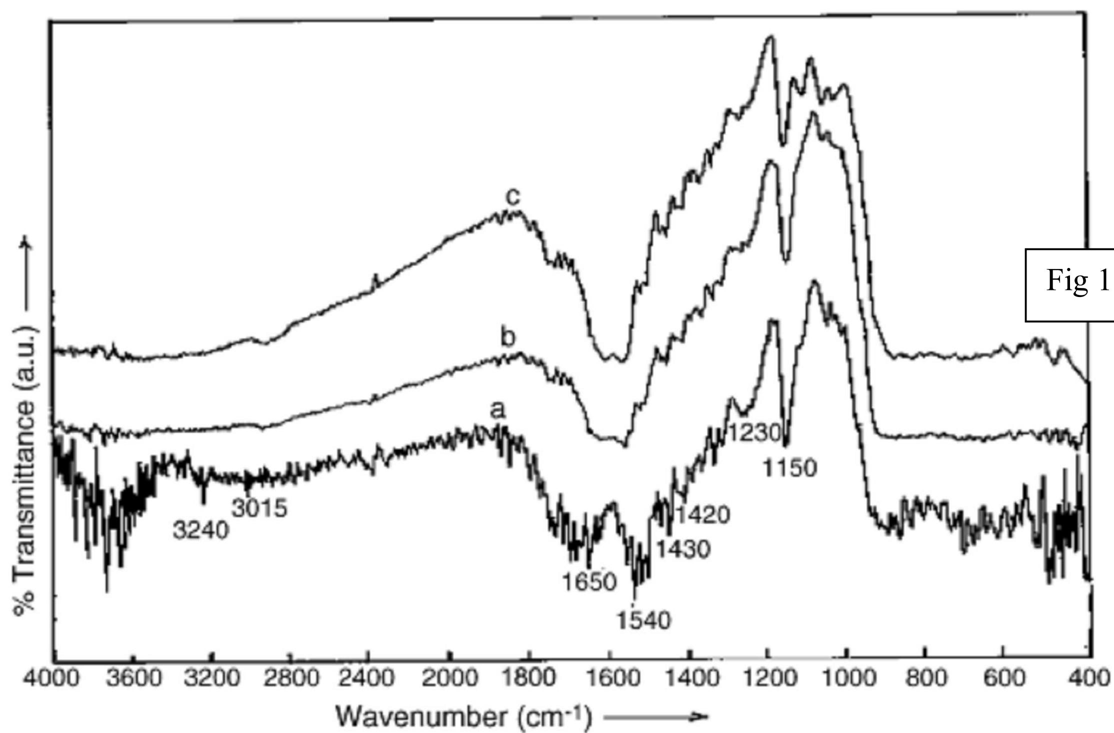


Figure 1. FTIR spectra of (a) untreated oil palm fibre, (b) treated oil palm fibre and (c) treated oil palm fibre after adsorption.

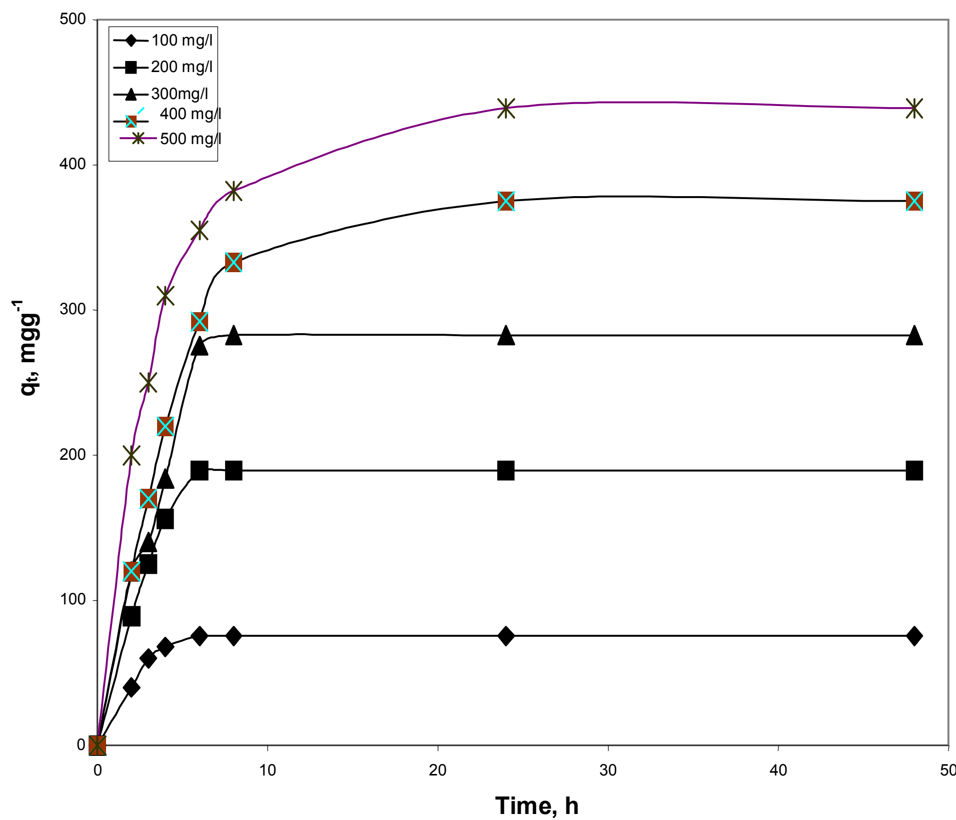


Figure 2. The variation of adsorption capacity with adsorption time at various initial lead concentration at $25^{0}C$ (pH 7, W = 0.2 g).

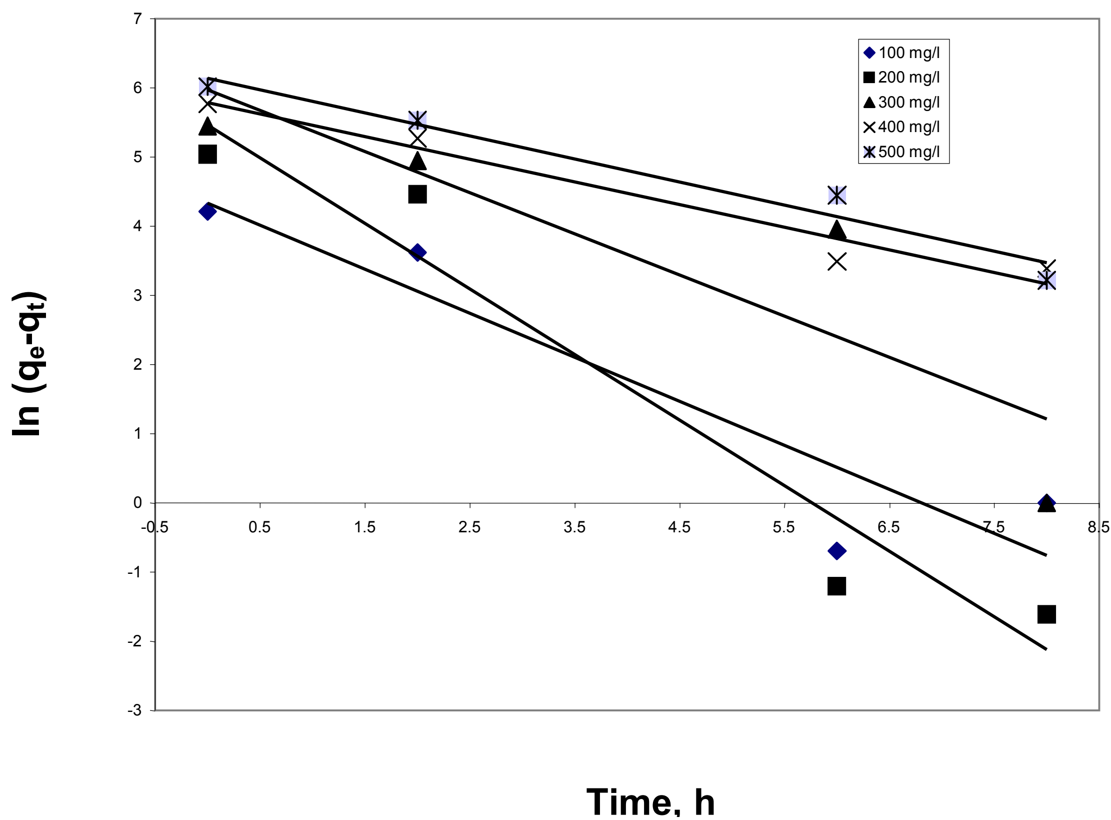


Fig 3

Figure 3. Pseudo-first order kinetics for adsorption of lead by activated carbon produced from treated oil palm fibre at 25^0C.

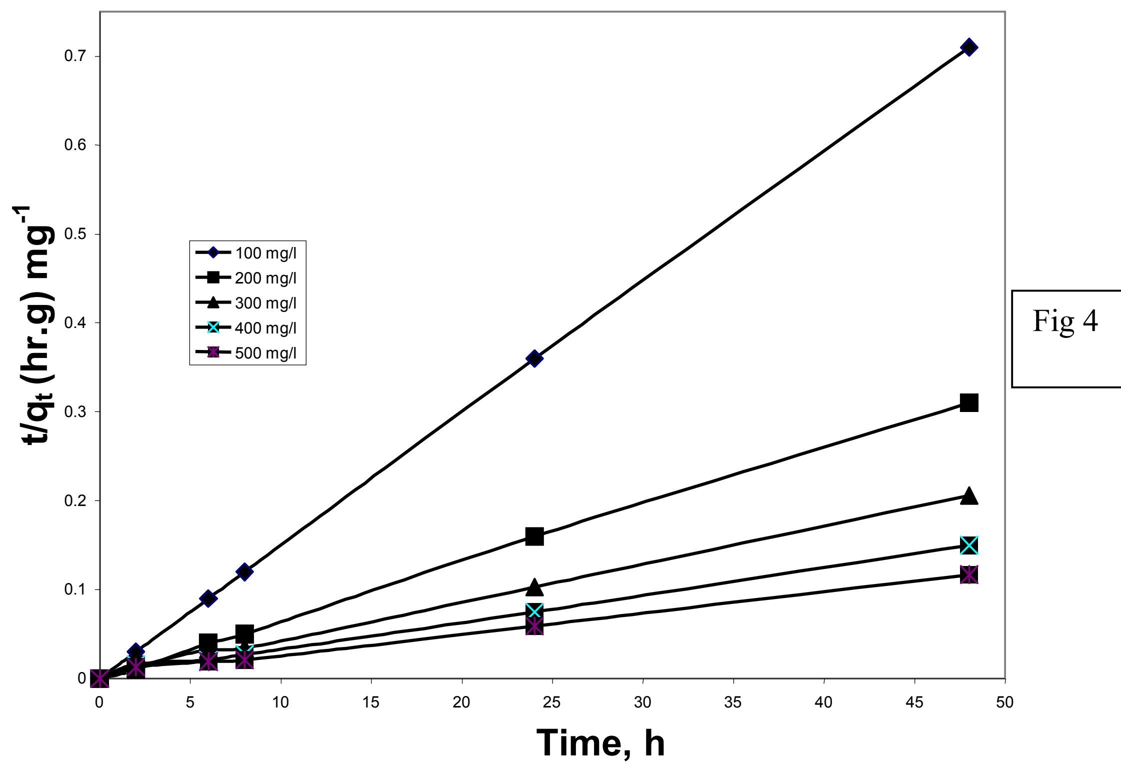


Fig 4

Figure 4. Pseudo-second order kinetics for adsorption of lead by activated carbon produced from treated oil palm fibre at 25^0C.

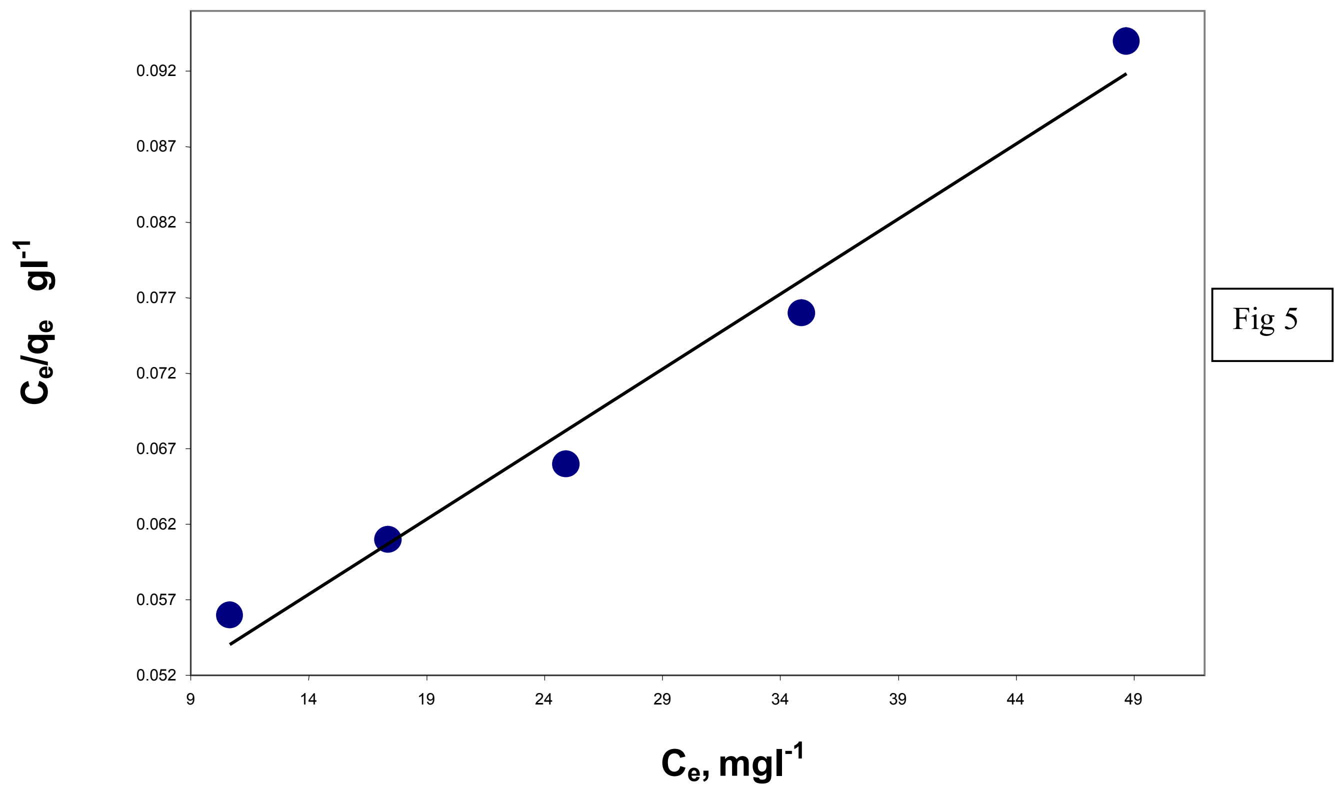


Figure5. Langmuir adsorption isotherm of lead on activated carbon produced from treated oil palm fibre at 25^0C.

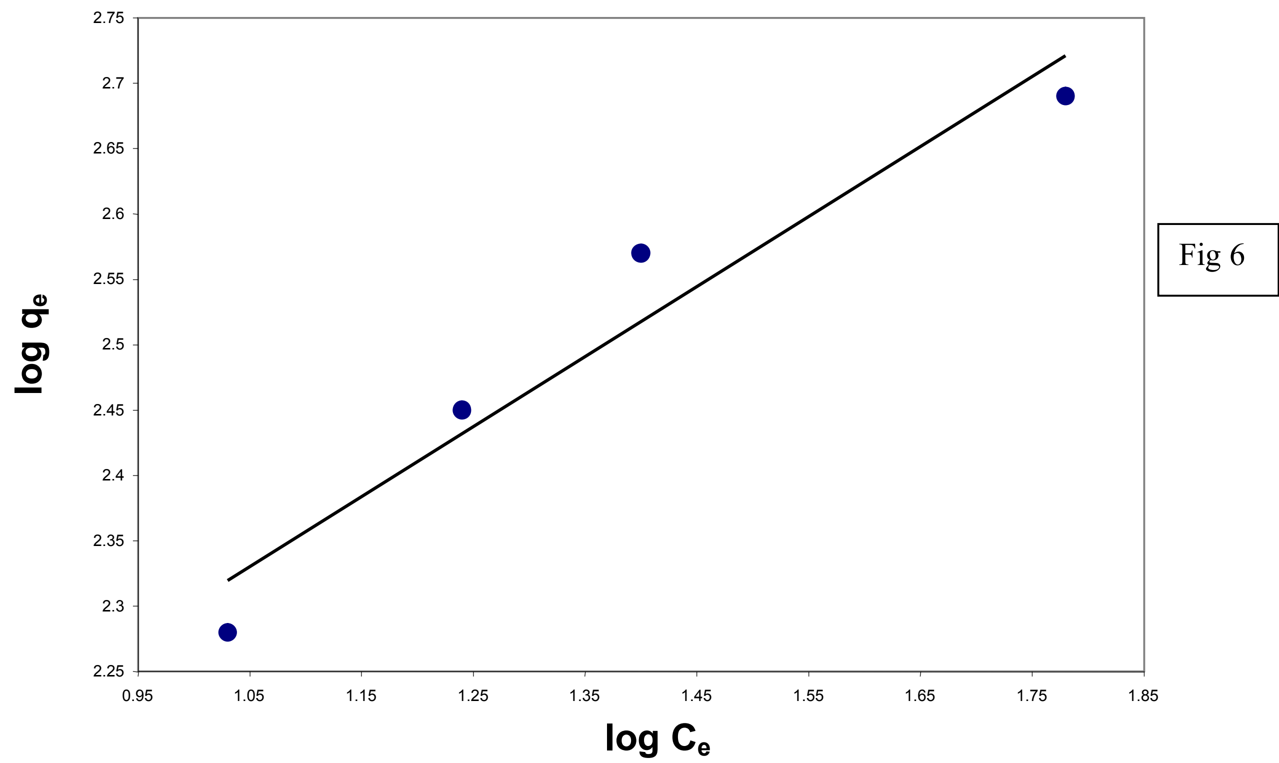


Figure 6. Freundlich adsorption isotherm of lead on activated carbon produced from treated oil palm fibre at 25^0C.

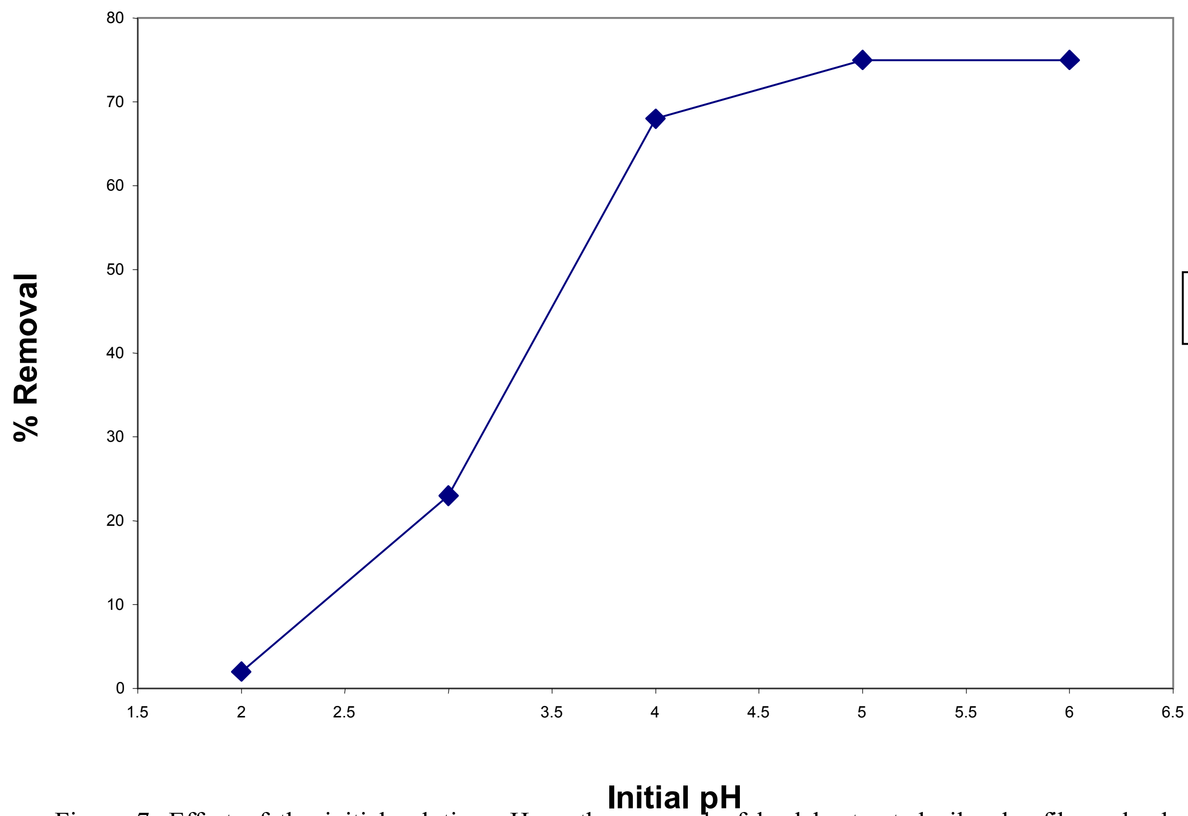


Figure 7. Effect of the initial solution pH on the removal of lead by treated oil palm fibre adsorbent (temperature 25^0C; adsorbent concentration 5 g/L; initial dye concentration 100 mg/L).

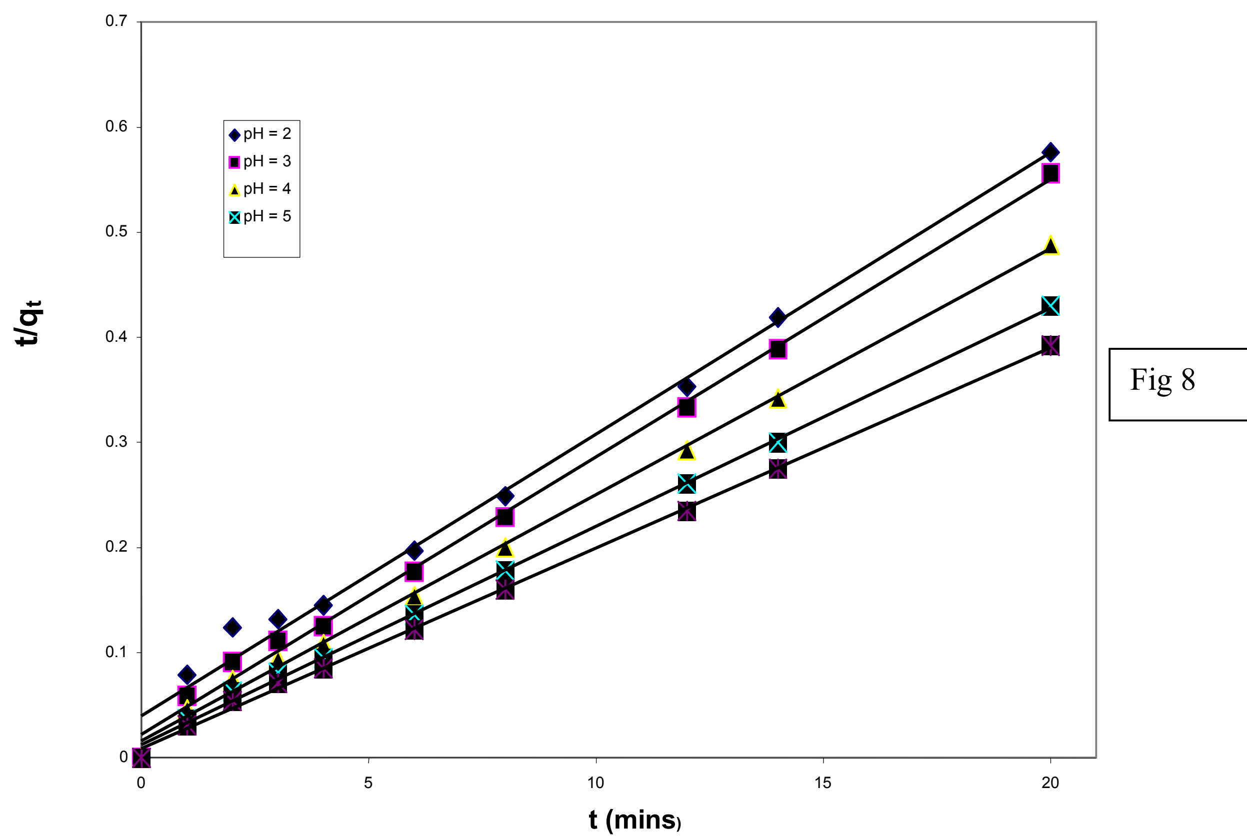


Figure 8. Pseudo-second order kinetics for adsorption of lead adsorption by activated carbon produced from treated oil palm fibre at 25^0C.

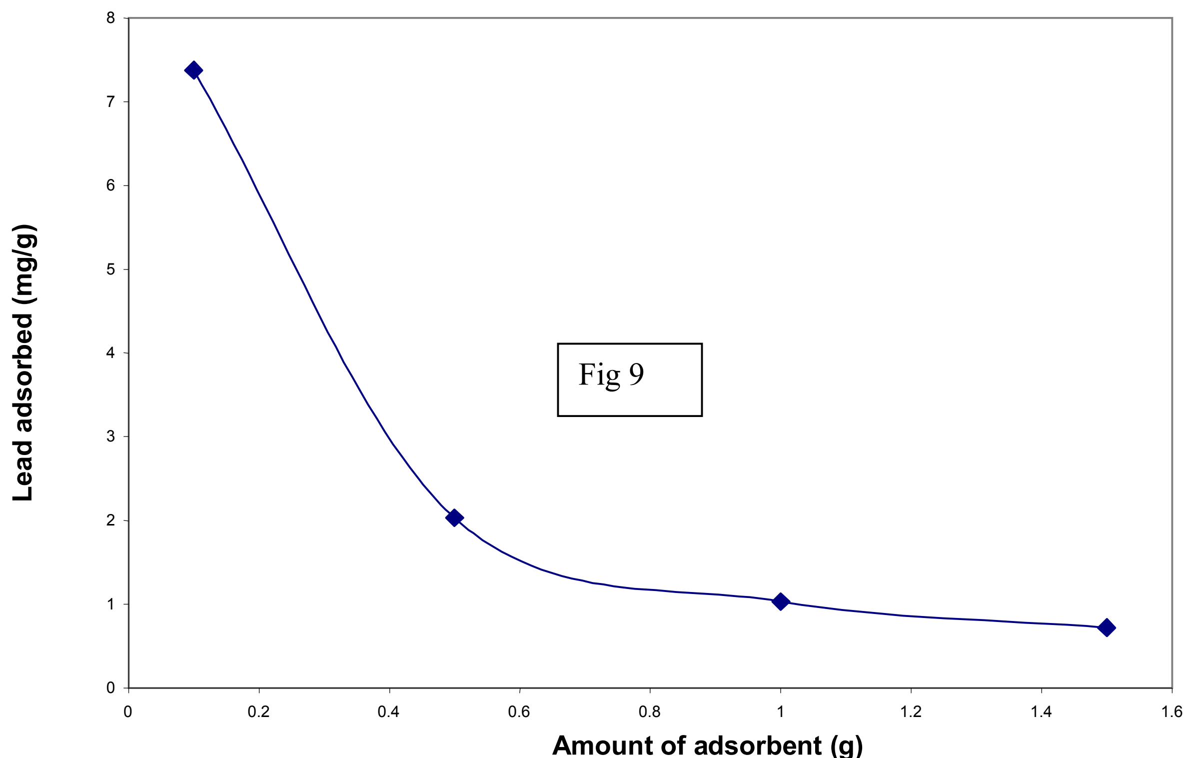


Figure 9. Effect of lead adsorbed on adsorbent dose at 25^{0}C.

4.0 Conclusions

The adsorption of lead onto treated oil palm fibre was investigated in batch experimental system. The following results were obtained:

1. The solution pH played a significant role in influencing the capacity of adsorbents towards the metal ion. An increase in the pH of solution led to a significant increase in the adsorption capacity.
2. The Langmuir isotherm exhibited a little better fit to the lead ion adsorption data by the adsorbent than the Freundlich isotherm.
3. The removal efficiency increased and the adsorption capacity decreased with a rise in adsorbent concentrations.

This work has shown that utilization of treated oil palm fibre will be useful in the treatment of lead ion from industrial waste effluents; it will also eliminate various ecological problems these waste effluents could cause.

Corresponding author:
Olugbenga Solomon Bello*
**Department of P/A Chemistry, P.M.B 4000, LAUTECH, Ogbomoso, Oyo State, Nigeria*
+2348035685435.
E-mail address: osbello@yahoo.com

Mary Adelaide Oladipo
Department of P/A Chemistry, P.M.B 4000, LAUTECH, Ogbomoso, Oyo State, Nigeria.
E-mail address: oladipomary@yahoo.com

Abimbola Modupe Olatunde
University of Ibadan, Department of Chemistry, Ibadan, Oyo State. Nigeria
E-mail address: myjoyng@yahoo.com

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Renal Stem Cells Research and Applications

Hongbao Ma, Yan Yang

Brookdale University Hospital and Medical Center, One Brookdale Plaza
Brooklyn, New York 11212, USA
Ma8080@gmail.com

Abstract: This article is to try describing the renal stem cells in animal and to explore the methods to either repair or regenerate a damaged kidney with stem cells. For this purpose, the 3 sections are concerned: (1) Isolation of stem cells from animal embryo and/or other resources (such as adipose and renal tissue). (2) Embryonic stem (ES) cells differentiate into renal stem cells and/or renal progenitor cells. (3) Renal stem and/or progenitor cells differentiated from ES cells are used for renal repair and/or regeneration. The stem cell treatment can be the most hopeful technique on the renal repair and regenerate. [Stem Cell. 2010;1(1):30-51] (ISSN 1545-4570).

Keywords: renal; kidney; stem cell; animal; repair; regenerate

1. Introduction

Chronic kidney disease is increasing at the rate of 6-8% per year in the Unite States. At present, dialysis and transplantation are the common treatment options. However, it is possible to use stem cells and regenerative medicine as the additional choices for kidney disease treatment. Such new treatments might involve induction of repair using endogenous or exogenous stem cells or the reprogramming of the organ to reinitiate development (Hopkins et al. 2009).

In the 20th century, an efficient treatment was given to patients with renal failure through the development of kidney dialysis and transplantation. These techniques have proved successful, but are marred by inflammation and limited organ availability and graft survival due to immune rejection. More recently, hope has been placed in the development of stem cell-based therapies, in which the function of the failing organ is restored by injected multipotent cells. Possible sources for these cells include differentiated embryonic stem (ES) cells and adult renal stem cells, and circulating multipotent cells, such as bone marrow-derived stem cells. Using the patient's own stem cells to repair kidney damage could circumvent the problems of immune rejection and organ availability.

Kidneys are possible to regenerate, which varies among species. Some bony and cartilaginous fish continue to form new nephrons during adult life. Adult mammals cannot form new nephrons but, to a certain extent, tubules and glomeruli may recover structure and function after limited injury such as acute tubular necrosis. Severe or prolonged injury results in replacement of functional parenchyma by scar tissue, i.e. fibrosis, which correlates clinically with the development of renal failure.

An effective treatment of renal disease is renal cell regeneration, or replace of damaged renal cells, and discourage fibrosis. The origins for renal parenchymal cells could be: (1) the re-entry into cell cycle of differentiated cells; (2) direct transdifferentiation of one cell type into another, such as tubular cells into interstitial cells or vice versa; (3) differentiation from stem cells of the kidney or the bone marrow.

Embryonic stem cells are different to adult or tissue-specific stem cells. Embryonic stem cells are the stem cell that can be grown in large numbers in the laboratory and retain the ability to grow into any type of cells including renal, nerve, heart muscle, bone and insulin-producing cells. It is difficult for the tissue-specific adult stem cells to grow in a great number, hard to isolate and are difficult to grow outside the body. Adult stem cells, such as skin and bone marrow stem cells, normally grow into a limited number of cell types (Snykers et al. 2008).

The role of embryonic or adult stem cells, in particular bone marrow-derived stem cells, in regenerating the kidney after injury has been the subject of intensive investigation. Bone marrow-derived stem cells have been shown to give rise to small numbers of most renal cell types, including tubular cells, mesangial cells, podocytes, vascular cells and interstitial cells. Injections of bone marrow-derived cells do improve renal function in many animal models of renal disease. Many stages of nephrogenesis can be studied using cultured embryonic kidneys, but there is no efficient technique available to readily knockdown or overexpress transgenes for rapid evaluation of resulting phenotypes. Embryonic stem cells have unlimited developmental potential and can be manipulated at the molecular genetic level by a variety of methods. ES cell

technology may achieve the objective of obtaining a versatile cell culture system in which molecular interventions can be used in vitro and consequences of these perturbations on the normal kidney development program in vivo can be studied (Steenhard et al. 2005).

Stem cells and progenitor cells are necessary for repair and regeneration of injured renal tissue. Infiltrating or resident stem cells can contribute to the replacement of lost or damaged tissue. However, the regulation of circulating progenitor cells is not well understood. Many factors influence the stem cell growth in damaged kidney. For example, low levels of erythropoietin induce mobilization and differentiation of endothelial progenitor cells and erythropoietin ameliorates tissue injury. Full regeneration of renal tissue demands the existence of stem cells and an adequate local milieu, a so-called stem cell niche. It was reported that in the regenerating zone of the shark kidney, stem cells exist that can be induced by loss of renal tissue to form new glomeruli. Stem cell may eventually contribute to novel therapies of the kidney disease (Perin et al. 2008).

Recently researchers used a rat model of chronic renal failure in which one kidney is excised so as to increase the load of the remaining kidney, thus causing a chronic deterioration that resembles the clinical situation of renal failure (Alexandre et al. 2008). In Alexandre's project, **t**he rats were divided into 4 groups: Group 1 were sham operated and both kidneys left in place; Group 2 had a kidney removed but were not administered cells; Group 3 were administered $2x10^6$ lineage negative bone marrow cells on day 15 after one of the kidneys was removed; Group 4 were administered $2x10^6$ lineage negative bone marrow cells on days 15, 30, and 45 after one of the kidneys was removed. They found: (1) Expression of inflammatory cytokines was reduced on day 16 in the kidneys of rats receiving stem cells as compared to rats that were nephrectomized but did not receive cells. (2) On day 60 rats receiving stem cells had decreased proteinuria, glomerulosclerosis, anemia, renal infiltration of immune cells and protein expression of monocyte chemoattractant protein-1, as well as decreased interstitial area. (3) Injured rats had higher numbers of proliferating cells in the kidney, whereas rats receiving stem cells had less. (4) Protein expression of the cyclin-dependent kinase inhibitor p21 and of vascular endothelial growth factor increased after nephrectomy and decreased after stem cell treatment. (5) On day 120, renal function (inulin clearance) was improved in the rats which were administered bone marrow cells compared to controls. This study supports the possibility of using bone marrow cells for various aspects of kidney failure. Other studies have demonstrated that administered stem cells promote kidney repair by secretion of insulin growth factor-1 (Cornelissen et al. 2008).

Bone marrow stromal cells, also known as mesenchymal stem cells or fibroblastic colony-forming units, are multipotent non-hematopoietic stem cells adhering to culture plates (Abdallah and Kassem 2009). Mesenchymal stem cells of the bone marrow have the ability to renew and differentiate themselves into multiple lineages of conjunctive tissues, including bone, cartilage, adipose tissue, tendon, muscle, and bone marrow stroma. Those cells have been first described by Friedenstein et al., who found that mesenchymal stem cells adhere to culture plates, look like in vitro fibroblasts, and build up colonies (Friedenstein et al. 1987).

Bone marrow is the site of hematopoiesis and bone marrow transplant has been successfully used for decades as a means of treating various hematological malignancies in which the recipient hematopoietic compartment is replaced by donor-derived stem cells. Progenitor cells in bone marrow are capable to differentiate into other tissues, such as cardiac tissue. Clinical trials have been conducted demonstrating beneficial effects of bone marrow infusion in cardiac patients. It is believed that injured tissue, whether neural tissue after a stroke, or injured cardiac tissue, has the ability to selectively attract bone marrow stem cells, perhaps to induce regeneration. Bone marrow has therapeutic effect in conditions ranging from liver failure, to peripheral artery disease, and the possibility of using bone marrow stem cells in kidney failure has been relatively understudied (Ma et al. 2009).

Mesenchymal stem cells have been brought to the attention of many researchers, because these cells are of great interest for treating various human diseases. Many studies have isolated mesenchymal stem cells and controlled, in vitro, its differentiation into cartilaginous tissue and bone using specific growth factors, with the objective of using this technology for repairing injured tissues of mesenchymal origin (Xian and Foster 2006; Kurdi and Booz 2007).

2. Stem Cells

2.1 Embryonic Stem Cells (ES cells)

ES cells are pluripotent cells derived from the inner cell mass of blastocysts, and are in theory able to give rise to all the cell types of the body. ES cells can be directed into forming renal progenitor cells, and eventually differentiated renal cells. Ureteric bud epithelial cells and metanephric mesenchymal cells that comprise the metanephric kidney primordium are capable of producing nephrons and collecting ducts through reciprocal inductive interaction. Once these cells are induced from pluripotent ES cells, they have

the potential to become powerful tools in the regeneration of kidney tissues. However, there is risk to use stem cells in clinical practice. In vivo injection of ES cells can give rise to teratomas, which are tumours containing cells of all three lineages (ectoderm, endoderm and mesoderm). ES cell-derived teratomas in vivo, renal primordial structures can be detected histochemically, and genes involved in metanephrogenesis are expressed. the potential of ES cells to produce renal primordial duct structures and provides an insight into the regeneration of kidney tissues (Yamamoto et al. 2006). This same potential was reported when ES cells were injected into embryonic mouse kidneys in vitro, and gave rise to ES cell-derived tubules, in this case without forming teratomas (Steenhard et al. 2005). In vitro, transfection of murine ES cells with renal developmental gene Wnt4, as well as the addition of hepatocyte growth factor and activin-A, both promote the formation of renal tubule-like structures, with expression of tubular marker aquaporin-2. Cultured Wnt4-EBs have an ability to differentiate into renal tubular cells; and second, that Wnt4, HGF, and activin A may promote the differentiation of ES cells to renal tubular cells (Kobayashi et al. 2005). The Wnt4-transfected cells can be transplanted into mouse renal cortex, where they also express aquaporin-2 and formed tubular structures. According to Kim et al reported, murine ES cells primed in vitro with retinoic acid, activin-A and BMP-7 (Kim and Dressler 2005), activin-A alone (Vigneau et al. 2007), or BMP-4, differentiate into cells expressing markers of the intermediate mesoderm, early kidney development and/or renal tubule-specific markers (Bruce et al. 2007). After injection of these primed murine ES cells into embryonic kidney cultures, ES cells are incorporated into developing renal tubules, without cell fusion, or into the nephrogenic zone. The primed cells are enriched for renal progenitor cells by FACS and injected in vivo into the kidneys of newborn mice, where they are integrated as proximal tubular cells, without teratoma formation (Vigneau et al. 2007). Human ES cells differentiate in vitro into WT1- and renin-expressing cells following treatment with a combination of specific growth factors (Schuldiner et al. 2000). However, research of the role for ES cells in renal regeneration is still in its infancy (Roufosse and Cook 2008).

2.2 Native Renal Stem Cells and Renal Regeneration

In the embryo, most types of renal parenchymal cells are derived from metanephric mesenchymal cells, which are multipotent and are in addition self-renewing, making them attractive candidates as the stem cells of the embryonic kidney.

In animal models, embryonic metanephroi transplanted into the abdominal cavity of adult animals are colonized by host vasculature, undergo nephrogenesis and produce urine, even if the operation is carried out across species barriers, and with a surprising lack of rejection (Little 2006). Human and porcine embryonic kidney progenitor cells have been isolated and, when injected into mice, can lead to the formation of miniature kidneys producing urine (Dekel et al. 2003), or protect against acute renal failure (Lazzeri et al. 2007). However, there are ethical issues to deal with human ES cells.

In adult mammals, a range of methods have been used to identify potential multipotent precursor cells, including label retention in slow cycling cells, identification of a side population, and expression of stem cell markers such as CD133. This has led to the identification of several candidate renal stem cells, which, depending on the study, are located amongst the tubular cell population (Dekel et al. 2006; Gupta et al. 2006), in the Bowman's capsule, papillary region or cortical interstitium (Bussolati et al. 2005; Sagrinati et al. 2006; Rad et al. 2008). Of note, other studies have not confirmed the presence of a large pool of precursor cells amongst the tubular population and instead argue that regeneration occurs through proliferation of differentiated tubular cells (Vogetseder et al. 2008; Witzgall 2008). Some of the candidate renal stem cells have been shown to enhance recovery after tubular injury, possibly by integration in the tubular epithelium (Rad et al. 2008).

2.3 Bone Marrow-Derived Stem Cells and Renal Regeneration

Bone marrow stem cells would be an ideal source of multipotent cells: they are easy to harvest and are in theory an unlimited source of expandable autologous cells. They display an unexpected plasticity which has been the subject of extensive research over the last few years. The plasticity has been observed both for the haematopoietic stem cell, which gives rise to all differentiated blood cell types, as well as for the bone marrow mesenchymal stem cells, which provide stromal support for haematopoietic stem cell in the bone marrow, and also give rise to various mesenchymal tissues, such as bone, cartilage and fat.

There are important discrepancies in the literature addressing the role of bone marrow cells in renal regeneration. These are partly explained by the methods involved in this research.

The technique most commonly used to study bone marrow cell plasticity is bone marrow transplantation. The host bone marrow is replaced by donor bone marrow, and after bone marrow chimerism is established, donor cells are tracked down in the kidney. The donor bone marrow cells are distinguished

from host cells by virtue of their chromosome content (male Y chromosome-positive cells in a female host), the expression of a reporter molecule (β-galactosidase, luciferase, enhanced green fluorescent protein), or the performance of a function (re-establishment of a function in a knockout mouse model). The type of host cell that the bone marrow-derived cell has given rise to (tubular, mesangial, etc.) is ascertained most often using immunohistochemistry.

Discrepancies between studies are attributable to several factors: (1) observations in different species (mouse, rat, human); (2) use of different models of renal damage (ischaemia/reperfusion, toxic, immunological); (3) different protocols for bone marrow transplantation (irradiation doses, quantity of cells injected); (4) injection of different subgroups of bone marrow cells (whole bone marrow, haematopoietic stem cell, mesenchymal stem cell); (5) sensitivity and specificity of the detection method for bone marrow cell origin (in situ hybridization for the Y chromosome, detection of reporter molecules, functional assays), and (6) sensitivity and specificity of the detection method of the renal cell type (immunohistochemistry for specific cell types such as tubular cell, mesangial cells, etc.).

Renal failure can be the result of an initial insult directed against the tubular epithelium, the glomerular cells or the vascular compartment. In the search for remedies for these varied renal diseases, studies have therefore addressed potential bone marrow origin for various renal cell types. It is useful to bear in mind these technical variations when analysing results reported in the literature (Roufosse and Cook 2008).

2.4 Tubular Epithelium

Although initial studies suggested a high contribution of bone marrow to tubular regeneration, the current view is that only a small proportion of tubular cells are bone marrow-derived, and there is disagreement over whether mesenchymal stem cells, haematopoietic stem cells or both are contributing (Humphreys and Bonventre 2008). The current consensus view is that the predominant source of tubular regeneration is through the proliferation of differentiated tubular cells (Lin et al. 2005). A few authors have not found any bone marrow cells engrafted in tubules, and propose that positive observations of bone marrow-derived tubular cells are the result of artifact (Bussolati et al. 2009). There may also be a progenitor slow-cycling cell population contributing to tubular repair. Does this abolish the hope of harnessing the regenerative power of bone marrow-derived cells? The answer is not necessarily.

Firstly, under certain circumstances, bone marrow engraftment in tubules can be dramatically increased. Held et al. made use of a transgenic fumarylacetoacetate (FAH)-/- mouse, in which discontinuation of the rescue drug NTBC leads to acute tubular necrosis (Held et al. 2006). After transplanting bone marrow from wild-type mice into FAH-/- mice, a few bone marrow-derived tubular cells are noted. In a subset of the FAH-/- mice, there is, in addition, loss of heterozygosity (LOH) in the liver for homogentistic acid hydrogenase, which induces a more severe, ongoing form of acute tubular necrosis. In FAH-/- animals with additional hepatic LOH, up to 50% of tubular cells are bone marrow-derived cells. Engraftment of these wild-type bone marrow-derived cells leads to morphological resolution of ATN and to disappearance of the aminoaciduria present in control mice. In this model, the bone marrow cells have a strong survival advantage over native tubular cells, due to their ability to metabolise toxic products. It is possible that this strong positive selective pressure is necessary for regeneration to occur through wild-type bone marrow cells. Interestingly, most of the bone marrow-derived tubular cells are derived from cell fusion between bone marrow cells and tubular cells. This is supported by a study by Li et al. in which fusion of bone marrow cells to tubular cells account for part of bone marrow-derived tubular cells after ischaemia/reperfusion (I/R) injury, but not all. In this model without selective pressure, the percentage of bone marrow-derived tubular cells is low (1.8%) (Li et al. 2007b).

Secondly, although there is disagreement concerning the underlying mechanism, injection of bone marrow cells, particularly mesenchymal stem cells, has repeatedly been shown to improve renal function in ATN, whether induced by toxins (cisplatin and glycerol) or I/R (Imai and Iwatani 2007). With the role of actual engraftment of bone marrow cells as tubular cells thought to be minimal or absent, mesenchymal stem cells may exert their beneficial effects through their antiapoptotic, mitogenic, immunomodulatory and angiogenic properties, or through the contribution of the bone marrow cells to endothelial cell replacement in the peritubular capillaries. It is important to know the nature of the mediators involved in these properties, and the mechanisms governing the homing of mesenchymal stem cells to the kidney (Imai and Iwatani 2007). Imberti et al. confirmed the importance of paracrine mechanisms using co-culture of mesenchymal stem cells with tubular cells in a Transwell® culture excluding contact between the two cell types, which led to less cisplatin-induced tubular cell death. mesenchymal stem cells have been shown to produce vascular endothelial growth factor, basic fibroblast growth factor, monocyte chemoattractant protein-1, hepatocyte growth factor, and insulin-like growth

factor, as well as immunomodulators TGF-β and PGE_2 (Imai and Iwatani 2007; Imberti et al. 2007). In a recent study, administration of conditioned medium from cultured stromal cells provided the same renoprotective effects as injection of mesenchymal stem cells, suggesting that systemic administration of the beneficial mediators may be just as good as mesenchymal stem cell injection, and safer (Imberti et al. 2007). It is a concern that there have been a few observations of adipogenesis associated with fibrosis and osteogenesis after injection of mesenchymal stem cells (Imai and Iwatani 2007).

Mesenchymal stem cell homing to the kidney has been linked to interactions between molecules upregulated in the injured kidney (SDF-1, hyaluronic acid and PDGF) and ligands expressed on mesenchymal stem cells (respectively, CXCR4, CD44 and PDGF-R) (Imai and Iwatani 2007). Similar beneficial effects on renal function may be induced by mobilizing bone marrow cells from the patient's own bone marrow by administration of growth factors (GF) such as granulocyte colony-forming factor, granulocyte/monocyte colony-forming factor, monocyte colony-forming factor, and stem cell factor. Possible explanations for improved renal function include increased numbers of bone marrow-derived tubular cells, a decrease in neutrophilic infiltrate, or increased cell proliferation and decreased apoptosis in kidneys of GF-treated mice (Roufosse and Cook 2008).

In summary, most but not all authors agree that a small proportion of tubular cells (at most a few percent) are bone marrow-derived after renal injury. The role these bone marrow-derived tubular cells play in improved renal function is probably insignificant, with intrinsic renal cells, either stem cells or differentiated, more likely to play the predominant role in regeneration. However, administration of bone marrow cells or mobilization of bone marrow cells using GF may be used to protect against renal injury. This may be due to paracrine / immunomodulatory effects or endothelial regeneration. In addition, there may be a therapeutic role for bone marrow-derived cells engineered to replace a defective gene, due to a local strong positive selective pressure. mesenchymal stem cells have emerged as the most promising candidate for stem cell therapy, and appear safe, such that phase I clinical trials of mesenchymal stem cell injection for the treatment of acute kidney injury are scheduled to begin shortly (Imai and Iwatani 2007).

2.5 Mesangial Cells

Mesangial cells are modified smooth muscle cells in the glomerular tuft, and provide structural support for the complex of glomerular capillaries. They may be injured by immune complex deposition, toxins and in diabetes. Although mesangial cells have regenerative potential, persistent mesangial damage can lead to glomerulosclerosis. In cell culture, bone marrow cells treated with PDGF-BB in the presence of collagen IV convert to cells with many mesangial characteristics (Suzuki et al. 2004). In rodent models of bone marrow transplantation, there is also support for partial bone marrow derivation of mesangial cells, whether glomeruli are injured or not. In models where mesangial damage has been induced, infusion of bone marrow cells may be associated with improved function, which has been attributed to mesangial and endothelial regeneration or, in the case of mesenchymal stem cells, paracrine mechanisms. Conversely, a deleterious mesangial phenotype responsible for mesangial sclerosis, such as in Os/- or db/db mice, can be induced by transplanting wild-type mice with transgenic mouse bone marrow. Some studies have further illustrated functionality of the bone marrow-derived mesangial cells by harvesting the bone marrow-derived mesangial cells, growing them in culture, and showing angiotensin-II induced contraction in vitro, a typical mesangial function (Kunter et al. 2006).

2.6 Podocytes

Podocytes are epithelial cells with complex interdigitating foot processes which create the slit diaphragm, and contribute to the synthesis of the glomerular basement membrane. Both the slit diaphragm and the glomerular basement membrane are implicated in creating a filtration barrier between blood and urine. Initial studies identified rare bone marrow-derived cells at the periphery of the glomerular tuft, in the location of podocytes. Two recent studies have suggested integration of bone marrow-derived cells as functional podocytes, with production of matrix protein. These studies involved the use of a mouse model of Alport's disease, in which the animals suffer from defective synthesis of the alpha-3 chain of collagen type IV, with glomerular basement membrane abnormalities and progression to glomerulosclerosis and renal failure. Using whole bone marrow transplantation from wild-type animals, both Prodromidi et al. and Sugimoto et al. showed the presence of bone marrow-derived podocytes and mesangial cells, accompanied by re-expression of the defective collagen chains, and improved renal histology and function. Although the bone marrow-derived cells were not numerous, their presence was sufficient to re-establish synthesis of the defective collagen chain. However, the improvement in renal function was substantial raising the possibility that there may be mechanisms involved other than replacement of podocytes. A similar experiment using mesenchymal stem cells only rather than whole bone marrow also led to a reduction in interstitial fibrosis,

but without engraftment of bone marrow cells in the kidney, and with no beneficial effect on survival or renal function (Sugimoto et al. 2006).

2.7 Vascular Cells

Endothelial cells are present in the glomerular capillaries, in large vessels and in the abundant network of peritubular capillaries. Endothelial cells are attractive candidates for progeny of bone marrow-derived cells in view of their immediate contact with circulating cells, the existence of known circulating endothelial precursors, and the existence of a known endothelial precursor in the bone marrow: the haemangioblast.

In a rat model of glomerulonephritis, where glomerular endothelial cells are injured, culture-modified bone marrow mononuclear cells injected into the renal artery boosted renal regeneration. This was attributed both to incorporation of bone marrow-derived cells into the endothelial lining and to production of angiogenic factors by the injected cells. Similarly, following acute tubular necrosis, the peritubular capillaries are damaged. The return of blood flow, which depends on endothelial cell integrity, is essential for renal recovery. Duffield et al. contend that bone marrow cells boost renal function after I/R by participating in endothelial cell regeneration. Li et al. observed bone marrow-derived vWF+ and CD31+ endothelial cells in a mouse model of adriamycin-induced nephrosis with subsequent renal fibrosis (Li et al. 2007a).

2.8 Interstitial Cells

The kidney contains a complex population of interstitial cells serving several functions, such as providing a scaffold for renal structure and producing several hormonal substances such as erythropoietin. It may even contain a population of adult native renal stem cells which play a role in renal regeneration. There is also evidence that bone marrow-derived cells could be a source for up to 30% of α-SMA-positive interstitial myofibroblasts, which have been incriminated in the production of extracellular matrix in renal fibrosis. If the bone marrow is indeed a source for such cells, the use of bone marrow cell injections for the treatment of renal failure would run the risk of enhancing fibrosis (Broekema et al. 2007).

2.9 Adipose Stem Cells

Adipose, also known as fat tissue, is the richest and most accessible known source of stem cells. It contains a specialized class of stem cells comprised of multiple cell types that promote healing and repair. Adipose stem cells have been shown to differentiate into multiple cell types including muscle, bone, fat, cartilage and nerve, etc. Beyond differentiation, regenerative cells may provide therapeutic benefit through the release of growth factors and other therapeutic healing mechanisms. The major advantages of adipose tissue as a source of regenerative cells, which distinguish it from alternative cell sources, include: **(1) Yield:** A therapeutic dose of regenerative cells can be isolated in approximately one hour without cell culture. **(2) Safety:** Patients receive their own cells (autologous-use) so there is no risk of immune rejection or transmission. **(3) Versatility:** Stem cells from adipose tissue benefit from multiple mechanisms-of-action.

3. Materials and Methods

3.1 Animal treatment in the research

3.1.1 Partial Ureteral Obstruction (PUO)

To test the effects of stem cells on the animals, rat could be applied. First, the partial ureteal obstruction could be applied as the kidney damage model.

Male mice weighing 25 to 30 g are used in this experiment. The mice are housed in individual metabolic cages, have free access to water, and observed for intake of food and water. After the induction of general anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body body weight, intraperitoneally) and a low midline abdominemal incision is made. All mice are operated the both side proximal ureteral ligation. The ureters are identified through a small suprapubic incision and are traced to insertion in the bladder individually then isolated from the surrounding tissues via an abdominal incision, and mobilized with minimal dissection to preserve the surrounding neurovasculature and retracted with vessel loops. The psoas muscle is split by blunt dissection to create a space which will accommodate two-thirds of the length of the ureter. The ureter is moved into that interstice, after which the muscle is reapproximated with 2 interrupted 4-0 silk sutures at the ureterovesical junction. The abdominal wound is then closed with sutures under aseptic conditions, and the ligation is released after 24 h and the animals, referred to as unilateral ureteral obstruction-treated, allowed to recover. After recovery from anesthesia, the mice are housed individually in plastic cages and given buprenorphine hydrochloride (0.02 mg/kg body weight, i.m.) to relieve postoperative discomfort. Before the surgery and clearance experiments, the mice fast but have free access to water. The sham operation consists of a similar suprapubic incision and identification of the left ureter, but ligation of the ureter is not performed (Ma, et al, 2008). The animals are conducted in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. The study

chould be approved by the insitutional Animal Care and Use Committee.

If use rat, the rats are anesthetized with sodium pentobarbital (50 mg/kg I.P.) and a low midline abdominal incision is made. Right nephrectomy is performed first. The right kidney is mobilized with minimal dissection. Two 2-0 silk ties are placed around the hilar vessels and the kidney is removed. The left ureter is then traced to its insertion in the bladder, mobilized with minimal dissection to preserve the surrounding neurovasculature and retracted with vessel loops. The psoas muscle is split by blunt dissection to create a space which would accommodate two-thirds of the length of the ureter. The left ureter is moved into that interstice, after which the muscle is reapproximated with three interrupted 5-0 silk sutures. The abdominal wound is then closed with sutures. For the sham operation, the rats underwent the same surgical procedure, including right nephrectomy, but the left ureter is diverted into the psoas muscle.

3.1.2 C1earance studies

Renal clearance experiments are carried out in rats with sham-PUO or with PUO (Chou et al. 2003). The rats are anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a heating pad to maintain body temperature. A tracheostomy is performed. Then the jugular vein and carotid artery are cannulated (with PE-50 catheters) for infusion of inulin[/PAH] and monitoring of arterial blood pressure/blood sampling, respectively. 10% inulin/20% PAH (in normal saline) infusion is started at the rate of 30 ul/min for measurement of GFR and effective RPF (starting at t=minus 60 min). A small low midline incision (just large enough to slip the bladder through) is made and the bladder is cannulated for urine collection with a PE-240 catheter which had a mushroom-shaped cap. This is secured in the bladder with two 3-0 silk sutures.

Following the surgical preparation, the inulin/PAH are allowed to equilibrate in the rat for 60 minutes before blood and urine samples are obtained. Thereafter (starting at t=0), urine is separately collected from the bladder for three 30 minute periods. Blood samples are taken at t=45 minutes and t=105 minutes. GFR and renal plasma flow are measured with the standard methods of inulin and PAH clearance, respectively. At the end of the clearance experiments, the rats are sacrificed with an intravenous injection of sodium pentobarbital.

Urine volume is measured gravimetrically. Blood cells are separated from the plasma by centrifugation. Inulin concentrations in urine and plasma are measured by standard spectrophotometry. GFR is calculated by standard inulin clearance techniques.

3.2 Stem cell isolation and cell culture

3.2.1 Isolation of renal stem cells or multipotent renal progenitor cells (MRPC)

Multipotent renal progenitor cells (MRPC) are isolated from adult mouse kidneys using culture conditions (Jiang et al. 2002).

Mouse kidneys are perfused *in vivo* through aorta with cold saline to flush the blood from the kidney, harvested, minced, and partially digested using collagenase in the presence of soybean trypsin inhibitor. The cell suspension is washed and plated in a medium that consisted of 60% DMEM-LG (Life Technologies-BRL, Grand Island, NY, USA), 40% MCDB-201 (Sigma Chemical Co., St. Louis, MO, USA), 1x insulin-transferrin-selenium (Invitrogen, Carlsbad, CA, USA), LA-BSA 1 mg/ml (Sigma Chemical Co., St. Louis, MO, , USA), 0.05 uM dexamethasone (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mM ascorbic acid 2-phosphate (Sigma Chemical Co., St. Louis, MO, USA), 100 U penicillin and 1000 U streptomycin (Life Technologies-BRL, USA) with 2% FCS (Hyclone Laboratories, Logan, UT, USA), 10 ng/ml EGF, 10 ng/ml PDGF-BB, and 10 ng/ml leukemia inhibitory factor (R&D Systems, Minneapolis, MN, USA). The cells are plated on fibronectin coated culture flasks at low density (300 cells/cm^2), to avoid cell–cell contact, and cultured at 37°C in the presence of 5% CO_2. Suppose that the cells could live up to 6 weeks. Single clones of cells are obtained by plating the cells at nontouching density and then using cloning rings to pick individual colonies of cells at the 5- to 10-cell stage.

3.2.2 Embryos

C57BL/6 female mice are injected with pregnant mare serum gonadotropin and human chorionic gonadotropin to collect oocytes. Spermatozoa are collected from the cauda epididymis of mice. In vitro fertilization is performed as described previously (Sugiyama et al., 1992). The fertilized embryos are frozen at the two-cell stage. The embryos developed to blastocyst stage in KSOM medium after thawing. Cell Culture and Chimera Production Brief exposure to acidic Tyrode’s solution is performed to remove the zonae pellucidae from the cultured blastocysts. The denuded embryos are placed on a feeder layer of mitomycin C-inactivated confluent embryonic fibroblasts in four-well plates. The embryonic fibroblasts and blastocysts are cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% KSR (Invitrogen, Carlsbad, CA, USA), 0.1 mM 2-mercaptoethanol, 103 units/ml leukemia inhibitor factor, LIF (ESGRO), nonessential amino acids, and sodium pyruvate. The growing blastocysts

attached to the feeder layer within 48 h. The inner cell mass (ICM) is apparent inside and extended above the flat trophoblast cells spreading from the attached blastocysts. The expanded colonies are dissociated, trypsinized, and seeded onto a new feeder layer 4 days after attachment. This process is repeated several times at intervals of 2 to 4 days; colonies are never allowed to become larger than 400 mm in diameter. The putative ES cells are stocked at passage 7 and 8. We used B6G-2 cells after passage 10 to examine a potential for pluripotency in vitro and in vivo. Host embryos are cultured from the two-cell stage to the morula and blastocyst stages after superovulation and natural mating. The ES-like cells are injected into the cavity of the host blastocyst and the subzonal cavity of the host morula. After injection, the chimeric blastocysts are transferred to the uteri of pseudopregnant recipient mice at 2.5 days postcoitus.

3.2.3 ES cells isolation and cell line establishment

Renal stem cells are isolated from the blastocysts of mouse embryos using the culture conditions. Natural mating between 20 female superovulated and male mice are done to provide blastocysts. The blastocysts are flushed to form uterus by M2 media 5 days after mating. Mouse embryonic fibroblast in a natural cycle or in a superovulated cycle are prepared to form embryos in a midgestation age according to protocols discribed by Shimizukawa (Shimizukawa et al. 2005).

The blastocysts are transfered to 35 mm dishs on the mouse embryonic fibroblast feeder group or mouse embryonic fibroblast feeder layer group which perviously inactivated with mitomycin C (kyowa, Japan) 10μg/ml for 2 h in CO_2 incubator. The ES media containg DMEM high glucose (Sigma Chemical Co., St. Louis, MO, USA) + 20% FBS (Gibco) + LIF 1000 IU/ml (Sigma Chemical Co., St. Louis, MO, USA) + 2-mercaptoethanol 0.1 mMol (Sigma Chemical Co., St. Louis, MO, USA) + L–Glutamin 2 mMol (Sigma Chemical Co., St. Louis, MO, USA) and Penicillin/Streptomycin 100 mg/100 IU/ml (Sigma Chemical Co., St. Louis, MO, USA) for 3 days. Disaggregation is carried out according to method discribed by Bongso et al. (1994) with some modification. Briefely, the outhgrowth ICM are disaggregated mechanically by hand pulled pasteur pipette in different size in 50 μl DMEM media under mineral oil (Sigma Chemical Co., St. Louis, MO, USA). Then, the disaggregated ICM is transfered to one well of 96-well dish (NUNC) and cultured for 3 days. An alternative procedure for disaggregation of ICM is culturing of blastocysts on 96-well dish and trypsonizing the outgrowth ICM in situ with trypsin/EDTA 0.1% /1 mM (Sigma Chemical Co., St. Louis, MO, USA) in PBS. It is possible to trypsonize the cells in 96-well dishs up to 3 more passages every 3 days until the ES colony morphology appear in the expansion stage. It is essential to monitor microscopically the formation of ES colonies in this stage daily. The colony positive dishes have to subculture 2 times more in the colony formation stage untill the cells became confluent enough for passage in 4-well dish (NUNC). Depending to doubelling time the cells must be trypsonized up to 4 further passages every 3 days. Then the confluent cells are passaged into 35 mm dish (NUNC) as the passage number one. The first frozen cells are carried out in passage number two (60 mm dish) using DMSO 10%, FBS 20% and DMEM media. Alkalin phosphatase assessment The ES cells are cultured in 35 mm dish for growing, then the ES colonies are fixed by 4% formalin in PBS buffer and naphtol AS-MX (Sigma Chemical Co., St. Louis, MO, USA) is used according to manufactures instraction for alkalin phosphatase staining.

3.2.4 Cell culture medium

1. **Media for mouse embryonic feeder layer cells:** High-glucose Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 100 unit/l penicillin, 100 unit/l streptomycin, 1% nonessential amino acids. For 1 liter, mix 890 ml DMEM with 90 ml FBS, 10 ml penicillin and streptomycin solution, 10 ml nonessential amino acids.
2. **Medium for mouse stem cell:** High-glucose DMEM, 10% FBS, 100 unit/l penicillin, 100 unit/l streptomycin, 1% nonessential amino acids, 5 ml nucleoside solution, 0.09 mg/l insulin, 1000 unit/ml LIF. For 1 liter, mix 890 ml DMEM with 90 ml FBS, 10 ml penicillin and streptomycin solution, 10 ml nonessential amino acids, 10 ul insulin solution and 1 ml LIF.
3. **Differentiation medium:** RPMI 1640 supplemented with 10% FBS (heat inactivated), 1 mM L-glutamine, 100 unit/l penicillin, 100 unit/l streptomycin. For 1 liter, mix 890 ml RPMI with 90 ml FBS, 10 ml penicillin and streptomycin solution, 10 ml L-glutomine.
4. **Freezing medium**: 9 ml DMEM and 1 ml DMSO.

3.2.5 Mouse ES cells in culture

Isolated ES cells are added to 5 ml DMEM supplemented by 10% FBS (Fetal Bovine Serum, Gibco, UK), 100 U/ml penicillin (Sigma Chemical Co., St. Louis, MO, USA) and 100 U/ml streptomycin (Sigma Chemical Co., St. Louis, MO, USA) and washed by centrifugation at 1200 rpm for 5 min. The cell pellet is collected and cultured in a 75-cm^2 flask in

a DMEM medium supplemented by 10% FBS and antibiotics. The cultures are incubated at 37°C in a 5% CO_2 environment. Four days after primary culture initiation, the culture medium are collected, centrifuged and the resultant cell pellet are replated in a fresh 75-cm^2 flask. These cultures (established from removed medium) are fed twice weekly and upon confluency, the cells are lifted by Tripsin/EDTA (Gibco, UK), counted and passaged at 1:3 ratios (about 1.5×10^6 cell/75-cm^2 flask). Cell passage is performed up to subculture 3 (it should be mentioned that the medium of each passaged culture are contained a few floating cells not attached on culture surface with replating due probably to their non mesenchymal nature). In parallel to the culture established from removed medium, the cultures of marrow, primarily adherent cells, are expanded by three successive passages at 1:3 split ratios. During the cultivation period, time needed by the culture (established either by primarily adherent cells or the cells floating in removed medium) to approach confluence, as an index of cell growth rate, are recorded. At the end, the passaged-3 cells from either group are evaluated in terms of their differentiation potential towards skeletal lineages as bone, cartilage and adipose cells (Saito et al. 2002; Winkler et al. 2008).

1. Mytomycin C treatment of feeder layer cells: When the feeder layer cells reach confluency they are treated with mitomycin C to induce mitotic arrest. The cells are still capable of conditioning the media.
2. Add 5 ml mitomycin C (10 ug/ml) to a 10 ml dish.
3. Incubate the cells for 3 hours.
4. Remove the mitomycin C solution, rinse the dish 5 times with PBS and add fresh medium.

3.2.6 Gelatin coating of tissue culture plastic

1. Coat tissue culture dishses with 1% gelatin solution.
2. Incubate for 4 hours.
3. Wash 3 times with PBS.

3.2.7 Maintenance of mouse ES cells (Nichols and Ying 2006)

Mouse embryo stem cells are isolated from the inner cell mass of 5-day old blastocysts, derived from pregnant rats, and isolated mouse ES cells are grown on mitomycin C-treated feeder layer or on gelatin-coated dishes:

1. Culture mouse embryo stem cells at a moderate density and subculture by splitting no more than 1/10.
2. Routinely, subculture mouse embryo stem cells every 3 days. To prevent differentiation, the rat embryo stem cells should be dissociated into single cells after subculturing.
3. Change the media every day.

3.2.8 Tetraploid ES cell aggregates

Two-cell embryos are prepared from C57BL/6 females that are superovulated and mated naturally. The fusion of blastomeres of the two-cell stage is performed using an ET-3 Embryonic Cell Fusion System (Fujihira, Tokyo) in accordance with the manufacturer's instructions to produce tetraploid embryos. The tetraploid embryos are then cultured in KSOM medium until aggregation. The zonae pellucidae from tetraploid embryos are removed with acidic Tyrode's solution. Two tetraploid embryos are aggregated with ES cells at the eight-cell stage (Nagy et al., 1990, 1993) and transferred to the uteri of pseudopregnant recipient mice at 2.5 days postcoitus.

3.2.9 Characterization of stem cells and multipotent renal progenitor cells (Gupta et al. 2006; Lazzeri et al. 2007)

1. **Cell surface marker analysis:** All staining reactions are performed using 10^5 cells in 100 ul of staining buffer. Rat ES cells for stage-specific embryonic antigen-1 (SSEA-1) or freshly isolated rat bone marrow cells are used as positive control. Unstained cells and corresponding isotype antibodies are used as negative control. Primary antibodies are used. Dead cells are excluded and doublets are excluded on the basis of three hierarchical gates (forward/side scatter area, forward scatter height/width, and side scatter height/width). Antibodies of mouse anti-rat CD90-PerCP, CD11b-FITC, CD45-PE, CD106-PE, CD44H-FITC, RT1B-biotin, RT1A-biotin, CD31-biotin (Becton Dickinson, San Diego, CA, USA) and purified anti-mouse SSEA-1 (MAB4301; Chemicon, Temecula, CA, USA) are used.

2. **Telomere Length and Telomerase Enzyme Assay:** For measurement of telomere length, DNA is prepared from cells by standard methods of proteinase K digestion followed by salt precipitation and digested overnight with Hinf III and RsaI. Fragments are run on a 0.6% agarose gel and vacuum blotted to positively charged nylon. The blot is probed overnight with a digoxigenin-labeled hexamer (TTAGGG) and then incubated with anti–digoxigenin-alkaline phosphatase-labeled antibody for 30 min. Telomere fragments are detected by chemiluminescence. The TRAP protocol adapted by Roche Applied Science

(Indianapolis, IN, USA) is used to assay for telomerase activity.

3. **DNA analysis by FACS:** MRPC are fixed in ice-cold 70% ethanol for 10 min and treated with 1 mg/ml ribonuclease for 5 min at room temperature. Propidium iodide (50 ug/ml) is added to the cell suspension and analyzed using 488 nm excitation, gating out doublets and clumps, using pulse processing and collecting fluorescence above 620 nm on a FACS Calibur (BD Bioscience, San Jose, CA, USA). Data are analyzed using Modfit LT software (Verity Software House, Topsham, ME, USA).

3.2.10 In vitro differentiation

Differentiation into cells of the neuronal lineage in vitro is performed according to Strubring et al.'s (1995) protocol. The putative ES cells are prepared at 400 cells / 20 ml in DMEM medium containing 20% FCS (Hyclone, Logan, UT), 10-7 all-trans retinoic acid (Sigma Chemical Co., St. Louis, MO, USA), 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), nonessential amino acids, and 50 mM 2-mercaptoethanol. The cells are cultured by the hanging drop method for 2 days to form embryo-like aggregates. The embryoid bodies are then collected, washed carefully, and plated into dishes coated with gelatin in the above medium without RA to allow them to attach and differentiate.

For differentiation of MRPC toward a renal cell lineage, cells are grown to confluence on fibronectin-coated four-well chamber slides and incubated with a "nephrogenic cocktail" that contained fibroblast growth factor 2 (FGF2; 50 ng/ml), TGF-beta (4 ng/ml), and leukemia inhibitory factor (20 ng/ml). All differentiation cultures are maintained for 2 weeks except where stated, and medium is renewed every 48 hours. For determination of whether MRPC could differentiate into cells of other germ cell layers, cells are incubated under conditions that promoted differentiation into endothelium (mesoderm), neurons (ectoderm), and hepatocytes (endoderm). Endothelial differentiation is induced by growing MRPC on fibronectin-coated wells (15,000 cells/cm^2) in the presence of 10 ng/ml vascular endothelial growth factor (VEGF). Neuronal differentiation is induced by growing MRPC on fibronectin-coated wells (5000 cells/cm^2) in the presence of 100 ng/ml basic FGF. Hepatocyte differentiation is induced by growing MRPC on Matrigel (20,000 cells/cm^2) in the presence of 10 ng/ml FGF-4 and 20 ng/ml hepatocyte growth factor. Cells are characterized by reverse transcriptase–PCR (RT-PCR) and immunofluorescence as described in the RT-PCR section. For the MRPC that are differentiated into endothelial cells, LDL uptake is examined by incubating the cells with Dil-Ac-LDL (10 ug/ml) at 37°C for 60 min. Undifferentiated MRPC are used as a control (Chen et al. 2008; Wong et al. 2008).

3.2.11 In vivo differentiation

1. **Ischemia reperfusion experiment:** For these experiments, MRPC are transduced using a mouse stem cell virus–enhanced green fluorescence protein (eGFP) retrovirus. These cells expressed eGFP and are referred to as eMRPC. Mice are anesthetized with pentobarbital (50 mg/kg intraperitoneally) and prepared, and using a midline incision, nontraumatic vascular clamps are applied across both renal pedicles for 35 min. Immediately after ischemia, 100 ul (10^6 cells) of an eMRPC cell suspension in PBS is injected directly into the abdominal aorta, above the renal arteries, after application of a vascular clamp to the abdominal aorta below the renal arteries to direct the flow of the injected cells. The kidneys are harvested 10 days later to examine *in vivo* differentiation of the injected cells.
2. **Subcapsular injection experiment:** Mice are anesthetized, the kidneys exposed, and eMRPC (10^6 cells) are injected under the renal capsule. Mice are killed 3 weeks later, and kidneys are harvested for tissue analysis.
3. **Effect of MRPC on renal function after ischemia-reperfusion:** For determination of whether MRPC injection facilitates renal functional recovery, mice undergo 30 min of ischemia induced by bilateral renal artery clamps followed immediately by injection of MRPC. As controls, mice are treated identically except that they receive either the saline vehicle or an MRPC cell suspension (10^6 cells) that have been preincubated for 12 hours with actinomycin D (1 ug/ml) to block transcription in the injected cells. For determination of whether injected MRPC have a deleterious effect on renal function, experiments are performed injecting saline vehicle (n=2) or an MRPC cell suspension (10^6 cells; n=2) after sham operation. Renal function is assessed by serial measurement of serum creatinine and 24-hour creatinine clearance (Hishikawa and Fujita 2008).

3.2.12 Signs of differentiation

Cell surrounding the characteristic colonies, with a flattened morphology and a dark and spiky appearance, are typical for different treated cells. Cells with a clearly visible nucleus and growing within flat

colonies are more likely to have undergone differentiation. For AP staining of embryo stem cells, use the following protocol:

1. Rinse cells thoroughly with PBS.
2. Fix the cells in 10 ml ice-cold methanol for 10 min.
3. Rinse with aqua dest and incubate in fresh distilled water for 1 min.
4. Freshly prepare AP substrate.
5. Incubate for 45 min at room temperature, then rinse with aqua dest.
6. Counter stain nuclei with Hemalum for 5 min.
7. Mount the cells with Kaiser's glycerin gelatin and cover with cover slips.

3.2.13 Formation of EBs and spontaneously differentiation

The ES colonies are cultured for 5 days on 24-well dish (Cellstar) in suspention state by adding 1% trypsine to ES media and removing LIF. Then, the EBs are trypsonized with mild 0.5% /0.5mM trypsin/EDTA (Sigma Chemical Co., St. Louis, MO, USA) in PBS and then the media removed and transfer into centrifuge tube for a few minutes. The sedimentary EBs are transferred on the collagen coated 4-well dish and cultured for 20 days to induce the spontanously differentiation. For detection of hematopoeitic cells, the differentiated cells are fixed by carrnoy's fixative (glacial acetic acid and metanol 1:3) and stained by Wright-Gimsa method.

3.3 Damaged renal repairing and renal regeneration by stem cells

Cultured renal stem cells or MRPC are introduced to the obstructed kidneys of mice. The use of human embryonic ES cells for the treatment of organ dysfunction is associated with legal and ethical issues which society as a whole has yet to decide on. In the meantime, fundamental research aiming to prove that ES cells can be directed into forming renal progenitor cells, and eventually differentiated renal cells, is underway. ES cells are pluripotent cells derived from the inner cell mass of blastocysts, and are in theory able to give rise to all the cell types of the body. ES cell lines have been derived from mice, non-human primates and humans. In vivo injection of ES cells can give rise to teratomas, which are tumours containing cells of all three lineages (ectoderm, endoderm and mesoderm). This tumorigenesis may limit the clinical use of ES cells to treat organ dysfunction. Nevertheless, in murine ES cell-derived teratomas in vivo, renal primordial structures can be detected histochemically, and genes involved in metanephrogenesis are expressed. This same potential is noted when ES cells are injected into embryonic mouse kidneys in vitro, and gave rise to ES cell-derived tubules, in this case without forming teratomas. In vitro, transfection of murine ES cells with renal developmental gene Wnt4, as well as the addition of hepatocyte growth factor and activin-A, both promote the formation of renal tubule-like structures, with expression of tubular marker aquaporin-2. The Wnt4-transfected cells are transplanted into mouse renal cortex, where they also expressed aquaporin-2 and formed tubular structures. Similarly, murine ES cells primed in vitro with retinoic acid, activin-A and BMP-7, activin-A alone, or BMP-4, differentiate into cells expressing markers of the intermediate mesoderm, early kidney development and/or renal tubule-specific markers. After injection of these primed murine ES cells into embryonic kidney cultures, ES cells are incorporated into developing renal tubules, without cell fusion, or into the nephrogenic zone. The primed cells are enriched for renal progenitor cells by FACS and injected in vivo into the kidneys of newborn mice, where they are integrated as proximal tubular cells, without teratoma formation (Wu et al. 2008).

3.4 Analysis of the cultured renal stem cells

3.4.1 RT-PCR

Total RNA is isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA is DNAse 1 treated, and cDNA is synthesized using the Taqman Reverse Transcription Kit (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of 5 mg of total RNA are used for cDNA synthesis using the SuperScript II first-strand synthesis system with oligo(dT) (Invitrogen, Carlsbad, CA, USA). The forward and reverse primers used are considered below. For *Pax2*, the RT2 PCR primer set is used for rat (LOC293992; Superarray Bioscience Corp., Frederick, MD, USA). The BD rat universal reference total RNA is used as a positive control for this reaction (BD Biosciences, USA). Quantitative real-time PCR is performed on a Bio-Rad RT-PCR equipment (Bio-Rad Laboratories, Hercules, CA, USA). Reaction conditions for amplification are as follows: 40 cycles of a two-step PCR (95°C for 15 seconds and 60°C for 60 seconds) after initial denaturation (95°C for 10 minutes) with 1 ul of a cDNA reaction in 1x SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA).

cDNAs are amplified with Taq DNA polymerase (Takara, Tokyo, Japan). The PCR reaction consisted of 25–40 cycles. The sequences of the upstream and downstream primer pairs and amplimer lengths (bp) for each gene are as follows:

Pou5f1(GGCGTTCTCTTTGGAAAGGTGTTC, CTCGAACCACATCCTTCTCTAG, 313 bp),
Pecam1(TGCGATGGTGTATAACGTCA, GCTTGGCAGCGAAACACTAA, 384 bp),

Utf1(GCCAACTCATGGGGCTATTG, CGTGGAAGAACTGAATCTGAGC, 204 bp), Cd9(CAGTGCTTGCTATTGGACTATG, GCCACAGCAGTCCAACGCCATA, 424 bp), Zfp42(CGTGTAACATACACCATCCG, GAAATCCTCTTCCAGAATGG, 123 bp), Spp1(GCAGACACTTTCACTCCAATCG, GCCCTTTCCGTTGTTGTCCTG, 243 bp).

3.4.2 Immunohistochemistry

Kidney tissue sections are fixed in 4% paraformaldehyde and permeabilized with Triton X-100. After blocking with 1% BSA/PBS for 1 hour, sections are incubated with primary antibodies diluted in 0.3% BSA/PBS overnight at 4°C. Slides subsequently are washed in PBS and incubated with secondary fluorochrome-conjugated antibodies for 45 minutes. The following antibodies are used in 1:100 dilution: Anti–von Willebrand factor (anft-vWF; F-3220; Sigma Chemical Co., St. Louis, MO, USA), anti-albumin (55442; ICN/Cappel, Costa Mesa, CA, USA), FITC-conjugated anti-pan cytokeratin (F0397; Sigma Chemical Co., St. Louis, MO, USA), anti-neurofilament 200 (N0142; Sigma Chemical Co., St. Louis, MO, USA), Texas red–conjugated anti-GFP (600-109-215; Rockland, Gilbertsville, PA, USA), anti–zona occludens-1 (anti–ZO-1; 61-7300; Zymed, San Francisco, CA, USA), anti–MHC I (12-5321-81; eBioscience, San Diego, CA, USA), anti–MHC II (12-5999-81; eBioscience, USA), TRITC-conjugated anti-PCNA (SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-THP (CL-1032-A; Cedarlane, Burlington, NC, USA), and anti-vimentin (V4630; Sigma Chemical Co., St. Louis, MO, USA). The following lectins are used in 1:500 dilutions for 45 minutes at room temperature: Rhodamine Peanut Agglutinin (RL-1072; Vector Laboratories, Burlingame, CA, USA) and Rhodamine Phaseolus Vulgaris Erythroagglutinin (RL-1122; Vector Laboratories, USA). For detection of Oct4, 8-um-thick formalin-fixed, paraffin-embedded sections of rat kidney are deparaffinized in xylene for 10 min, followed by hydration through graded ethanol. Endogenous peroxidase is injected for cells. The kidneys are harvested 10 days later to examine *in vivo* differentiation of the injected cells.

3.4.3 Characterization of markers for and regulators of renal progenitors

To define a renal stem cell or drive an ES cell towards a renal fate, it is first critical to define the populations required. Individual projects within Section 1 will involve a series of expression profiling experiments carried out to record the temporal transcriptional program of metanephric development. Each project is aimed at both defining the expression pattern of renal development and identifying secreted proteins and cell surface markers that may be of value to Section 2.

4.5.4 Identification of secreted factors involved in the induction of renal development

Identification of UB tip-specific novel growth factors which induce nephron induction and novel UB tip specific receptors that transduce branching signals from the MM. The former may be useful in experimental induction of stem cells towards a renal fate. The latter may assist in the isolation of UB progenitors. The aim of this project will be to profile ureteric tree versus renal mesenchyme. Profiling will also be performed on tree branch versus tip. These studies will identify growth factors being produced by the tree that may be important in instructing the mesenchyme to form nephrons. Hox7b-GFP transgenic mice will be sued to allow the separation of tree from mesenchyme using FACs and laser microcapture. c-ret antibodies will be used to identify tip from branch of the ureteric tree. Growth factors from mesenchyme that may direct branching will also be identified. Novel factors will be ectopically expressed and their ability to drive mesenchymal differentiation in explants or induce tubule formation from mIMCD3 cells in vitro will be assessed.

3.4.5 Identification of renal progenitor markers to assist in the identification and isolation of renal stem cell populations

In this project, a complete temporal expression analysis of the developing kidney from 10.5 dpc to postnatal will be examined. Upon this temporal framework, spatial information from Project 1 and 3 can be placed. Specific A versus B profiling will also be performed between 10.5 dpc renal mesenchyme and adjacent intermediate mesoderm that will not become kidney. This project particularly seeks to identify transmembrane markers of potential progenitor cell populations in the kidney. As describe previously, the membrane organization of all mammalian genes will be assessed computationally. Genes believed to encode trans-membrane proteins and are found to be expressed in the kidney, based on expression data obtained in projects 1 and 2, will be further analyzed. In situ hybridization will be used to assess the spatial expression pattern of these potential cell surface markers in the kidney. Antibodies will be made to lead markers and these will be used to isolate different cell populations. Finally, the potential of these populations to repair damaged kidneys will be assessed in a variety of explant and engrafting assays.

3.4.6 Expression profiling of renal sub-compartments, including the interstitial cells, tissue

macrophages and podocytes, to identify specific markers of the endpoints of renal differentiation

In addition to the temporal expression profiling of kidney development, a series of profiling experiments are to be undertaken to define expression markers of specific cell types and regions of the kidney. Specific cell types are to be generated by primary culture methods (renal interstital cells) and cell sorting of cell specific GFP-tagged cells form the kidneys of different transgenic mice (renal macrophages, cap condensates, podocytes). This data will provide an expanded set of expression markers for specific cell types and differentiation states for cells that make up the mammalian kidney. It will also provide an excellent recourse for cell specific expression markers that can be used in Project 4.

3.4.7 Examination of the potential for ES cells to be differentiated into the necessary lineages for renal de novo generation or repair

In this project we will attempt to direct murine or human embryonal stem cells towards a renal fate using a variety of inducing conditions. In the case of murine ES cells, this will be an adaptation of the mesodermal induction process used in embryoid body (EB) formation. As tagged murine ES cells can be generated, this will be the most insightful approach. Information from Section 1 will provide i) markers for which progress towards a renal fate can be monitored using wholemount in situ hybridisation, ii) growth factors which may assist in the process and iii) cell surface markers which can facilitate the isolation and enrichment of the desired cell types from mixed progenitor populations. We will also use human embryonal cells to more crudely assess this renal potential. Both of these can be used to test novel growth factors isolated in Projects 1 and 2.

3.4.8 Measuring the mesenchymal stem cells dimensions

Since the cell size can influence the time in which the culture become confluence, we measured the mesenchymal stem cells size from both cultures. For this, the length and width (the broadest part of the cells) of the fibroblastic mesenchymal stem cells from unconfluenced culture are measured using the objective micrometer mounted on the phase contrast inverted microscope.

3.4.9 Adipogenesis

Confluenced passaged-3 cells in 6-well culture plates are used to evaluate the adipogenic ability of the isolated cells. The proliferation medium of the cells is replaced by adipogenic DMEM medium containing 100 nM dexamethazone (Sigma Chemical Co., St. Louis, MO, USA) and 50 mg/ml indomethasine (Sigma Chemical Co., St. Louis, MO, USA). The cultures are then incubated for 21 days in 37ºC, 5%CO_2. The medium is changed 3 times a week. Occurrence of adipogenic differentiation is evaluated by Oil red staining as well as RT-PCR analysis.

3.4.10 Oil red staining

The culture is fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red solution in 99% isopropanol for 15 minute. At the end, the stain solution is removed and the cultures are washed with 70% ethanol before they are observed by light microscopy.

3.4.11 Osteogenesis

Confluenced passaged-3 cells in 6-well plates are used to induce bone differentiation. The proliferation medium of the cultures is replaced by osteogenic medium that is consisted of DMEM supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma Chemical Co., St. Louis, MO, USA), 10 nM dexamethazone (Sigma Chemical Co., St. Louis, MO, USA) and 10 mM ßglycerole phosphate (Sigma Chemical Co., St. Louis, MO, USA). The cultures are incubated at 37°C temprature and 5% CO_2 environment for 21 days with medium replacement of three times a week. Occurrence of differentiation is examined by alizarin red staining and RT-PCR analysis.

3.4.12 Alizarin red staining

Alizarin red staining is used to detect wheatear the mineralized matrix is formed in the cultures. For staining, the cultures are first fixed by methanol for 10 minutes, then subjected to alizarin red solution for 2 minutes, washed by distilled water and observed with light microscope.

3.4.13 Chondrogenesis

To induce the cartilage differentiation, micro mass culture system is used. For this purpose, 2.5×10^5 passaged-3 cells are pelleted under 1200 g for 5 minute and cultured in a chondrogenic medium containing DMEM supplemented by 10 ng/ml transforming growth factor-ß (Sigma Chemical Co., St. Louis, MO, USA), 10 ng/ml bone morphogenetic protein-6 (Sigma Chemical Co., St. Louis, MO, USA), 50 mg/ml insulin/ transferin/selenium+ premix (Sigma Chemical Co., St. Louis, MO, USA) and 1.25 mg bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) and 1% fetal bovine serum (Gibco, UK). The chondrogenic culture is maintained at 37ºC, 5% CO_2 for 21 days with a medium replacement of three times a week. At the end of this period, the cultures are evaluated for cartilage differentiation by specific straining of toloidin blue and RT-PCR analysis.

3.4.14 Toluidin blue staining

To examine cartilage differentiation, the pellets are subjected to the following: fixing in 10% formalin; dehydrating in an ascending ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5 µ by microtome. The sections are then stained in toluidin blue for 30 second at room temperature and viewed by light microscope.

3.4.15 RNA extraction and RT-PCR analysis of gene expression

Total RNA is collected from the cells having been induced to differentiate into osteoblastic, chondrocytic and adipocytic lineages as detailed above, using RNXPlusTM solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples are digested with DNase I (Fermentas) to remove contaminating genomic DNA. The standard reversetranscription reaction is performed with 5 µg total RNA using Oligo (dT) 18 as a primer and RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR is as follows: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25µl reaction Taq DNA polymerase (Fermentas). The primers are utilized to detect differentiations. Amplification conditions are as follows: initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of denaturation at 94˚C for 45 minutes; annealing at 65 (insulin), 57 (GLUT1), 55 (GLUT2), 56 (glucagon), 65 (Oct4) and 60˚C (β-actin) for 45 minutes; extension at 72˚C for 30 minutes; and a final polymerization at 72˚C for 10 minutes. Each PCR is performed in triplicate and under linear conditions. The products are analyzed on 2% agarose gel and visualized by ethidium bromide staining.

3.4.16 Alkaline phosphatase analysis

Activity of the putative ES cells are stained for alkaline phosphatase activity in the cytoplasm using an alkaline phosphatase staining kit (Sigma Chemical Co., St. Louis, MO, USA). Fixation and staining are performed according to the protocol supplied by the manufacturer.

3.5 Analysis of renal repair effects by stem cells

3.5.1 C1earance Studies

The mice are anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a heating pad to maintain body temperature. A tracheostomy is performed. Then the jugular vein and carotid artery are cannulated (with PE-50 catheters) for infusion of inulin/PAH and monitoring of arterial blood pressure/blood sampling, respectively. 10% inulin/20% PAH (in normal saline) infusion is started at the rate of 30 ul/min for measurement of GFR and effective RPF (starting at t=minus 60 min). A small low midline incision (just large enough to slip the bladder through) is made and the bladder is cannulated for urine collection with a PE-240 catheter which has a mushroom-shaped cap. This is secured in the bladder with two 3-0 silk sutures (Chou et al. 2003). Following the surgical preparation, the inulin/PAH are allowed to equilibrate in the rat for 60 minutes before blood and urine samples are obtained. Thereafter (starting at t=0), urine is separately collected from the bladder for three 30 minute periods. Blood samples are taken at t=45 minutes and t=105 minutes. GFR and renal plasma flow are measured with the standard methods of inulin and PAH clearance, respectively. At the end of the clearance experiments, the rats are sacrificed with an intravenous injection of sodium pentobarbital. Urine volume is measured gravimetrically. Blood cells are separated from the plasma by centrifugation. Inulin concentrations in urine and plasma are measured by standard spectrophotometry. GFR is calculated by standard inulin clearance techniques.

3.5.2 Western Blot Analysis of Alpha-smooth Muscle Actin (alpha-SMA)

Accumulation of alpha-SMA, an indicator of tubulointerstitial fibrosis (Bohle A. Strutz F), is measured in the cortex and medulla by Western blotting. Immunoblot analysis of alpha-SMA protein in the cortex and medulla are performed in the sham-operated rats and rats with PUO in 3 groups of rats as described above. After the left kidney is removed through a midline abdominal incision, the cortex and medulla are separated and glass homogenized in lysis buffer on ice for total protein extraction. The homogenates are centrifuged at 12,000 r.p.m. for 20 min at 4°C. The supernatants are stored at -80°C in aliquots until analysis. The total protein concentrations of the samples are determined using BCA Protein Assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Protein extracts containing 100 µg of total protein are used. Western blot analysis is performed according to the procedures previously reported from our laboratory (Chou et al. 2003). Mouse monoclonal anti-alpha-SMA (Sigma Chemical Co., St. Louis, MO, USA) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) are used as the primary and secondary antibody at a dilution of 1:1000. The alpha-SMA immunoblot signals are normalized to the corresponding beta-actin band signals (Miyajima et al. 2000). A monoclonal mouse antibody for the structural protein β-actin (Sigma Chemical Co., St. Louis, MO, USA) is used as a loading control. Membranes are

stripped prior to β-actin analysis with buffer containing 0.2% sodium dodecyl sulphate and 50 mM glycine, adjusted to pH 2.6 with HCl, at room temperature for 2 min. After washing three times for 5 minutes each in 0.1% TBS, steps 3 to 9 above are repeated for β-actin. The immunoblot films are scanned and analyzed using imaging densitometry software (Bio-Rad Laboratories, Hercules, CA, USA). The data for alpha-SMA immunoblot signals are normalized to the corresponding ß-actin band signals.

3.5.3 Histological Studies:

The kidney specimens are embedded in paraffin after overnight fixation in 10% neutral buffered formalin. Sections (5-μm thick) are stained by H&E method and trichrome method with Gomori trichrome kit (Richard-Allen Scientific, Kalamazoo, MI) to demonstrate collagen deposition. Tubular and interstitial changes in each group are graded on a scale from 0-4 under a micrometric ocular grid in accordance with the methods previously described (Remuzzi et al. 1999).

3.5.4 Radiological studies:

Sham-operated rats and rats with PUO are anesthetized with pentobarbital sodium (50 mg/kg i.p.) and a tail vein is cannulated with a 24-gauge catheter. Ioversol (Optiray 300, Mallinckrodt Inc, St. Louis, MO) is injected intravenously at 2 ml/kg BW and x-ray images of the rats are captured at 5 minutes by a portable x-ray machine (General Electric). A nuclear renal scan is also performed.

3.5.5 Blood biochemistry.

Body/organ weight, serum lipids, serum glucose and urine glucose are measured.

3.5.6 Kidney lipid contents.

Determine cholesterol/triglycerol content from kidney tissue (n=4-5 from each group).

3.5.7 Protein expression in kidney.

Use ½ kidney. Western blot for HMGCR, PPAR, SREBP-1, SREBP-2, TNF-α, TGF-β1, TGF-β2, HMG-CoA, PAI-1, nephrin, podocin, ABCA1, α-actin, VEGF, COX-2, and HIF expressions are performed.

3.5.8 Kidney RNA for RT-PCR analysis.

Use the other ½ kidney. Total RNA is ideally extracted with TRIzol and kept in 80% ethanol until PCR. Gene expression (mRNA) is determined for SREBP-1, SREBP-2, TGF-1, TGF-2, HMGCR, ABC-1, ABCA-1, PAI-1, nephrin and podocin (n=4-5 from each group).

3.5.9 Histology study.

¼ of the kidney is used. TRI and HE staining for fibrosis. Microphage infiltration/MCP-1 expression (n=3-4 from each group).

3.5.10 Blood and urine chemistries:

Serum glucose, urine glucose, total cholesterol, and triglycerides are determined by kits (Wako Chemicals USA, Inc., Richmond, VA, USA).

3.5.11 Serum creatinine and BUN:

Serum creatinine and BUN are determined using Autoanalyzer (Beckman Instruments Inc., Fullerton, California, USA). Urine albumin concentration is determined by competitive ELISA via the Albuwell M kit (Exocell, Philadelphia, PA, USA). Urine creatinine concentration is determined by Jaffe's reaction of alkaline picrate with creatinine via the Creatinine Companion kit (Exocell, Philadelphia, PA, USA, catalog number 1012).

3.5.12 RNA isolation and quantitative real-time PCR:

Total RNA is isolated from the cortex of kidney by using TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA is synthesized by using reverse transcript reagents (Bio-Rad iScript cDNA synthesis kit) (Bio-Rad Laboratories, Hercules, CA, USA) after DNase treatment (Invitrogen, Carlsbad, CA, USA). The mRNA level is quantified by using Bio-Rad iCyCler Real Time PCR system. 36B4 is used as internal control and the amount of RNA is calculated by the comparative CT method. All the data are calculated from duplicate reactions.

3.5.13 Homogenate, nuclei and membrane isolation:

Kidneys are homogenized at 4°C in homogenization buffer (20 mM Tris-Cl, pH 7.4, 75 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol), supplemented with a protease inhibitor cocktail consisting 10 mM AEBSF, 0.08 mM Aprotinin, 2 mM Leupeptin, 4 mM Bestatin, 1.5 mM pepstatin A, 1.4 mM E-64 (Sigma Chemical Co., St. Louis, MO, USA). Nuclear extracts are prepared according to the method of Morooka et al [18] with minor modifications as we have previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b).

3.5.14 Protein electrophoresis and Western blotting of nuclear extracts and cortical homogenates:

Equal amount of protein samples are subjected to SDSPAGE (10% wt/vol) and they are then transferred to nitrocellulose membranes. After blockage with 5% fat-free milk powder with 1% Triton X-100 in Tris-buffered saline (20 mM Tris-Cl,

150 mM NaCl, pH 7.4), blots are incubated with antibodies against SREBP-1 (Santa Cruz, 1:1000), SREBP-2 (Santa Cruz, 1:1000), PPAR-β (ABR: 1:1000), TGF-1 (Santa Cruz, 1:1,000), TGF-2, plasminogen activator inhibitor-1 (PAI-1, Santa Cruz, 1:1,000), VEGF (Santa Cruz, 1:1,000), TGFb-1 (Santa Cruz, 1:1,000), type IV collagen (Santa Cruz, 1:1,000), or fibronectin (Sigma Chemical Co., St. Louis, MO, USA, 1:2,000). Corresponding secondary antibodies are visualized using enhanced chemiluminescence (Pierce, Bradford, IL, USA). The signals are quantified with a Phosphor Imager with chemiluminescence detector and the accompanying densitometry software (Bio-Rad Laboratories, Hercules, CA, USA).

3.5.15 Lipid extraction and measurement of lipid composition

Lipids from the renal cortex are extracted by the method of Bligh and Dyer [19]. **A) To determine triglyceride and total cholesterol content:** Totals lipids are extracted from the renal cortex and triglyceride and cholesterol content is measured as we have previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b). **B) To determine the glycosphingolipid composition:** an aliquot of the lipid extract is evaporated to dryness and subjected to alkaline methanolysis. The lipids are chromatographed on high performance thin layer chromatography plates (HPTLC, E. Merck 5641). Glucosylceramide and ganglioside GM3 are separated with a solvent system consisting of chloroform: methanol: water (65:25:4) on plates which are pretreated with 2.5% borax in methanol: water (1:1). The lipid bands are visualized by impregnating the plates with a modified charring reagent (100 g of $CuSO_45H_2O$ in conc. H_3PO_4: water: methanol (100:750:400). The charred TLC plates are scanned with a video densitometer. Comparing the density of each spot with the density of the corresponding standard curve is used to quantify the glucosylceramide and ganglioside GM3 bands [20-21].

3.5.16 Perfusion fixation of kidneys

Rats are anesthetized and perfused through the abdominal aorta as previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b)[20-21].

3.5.17 Periodic acid Schiff (PAS) staining

Paraffin sections are stained for PAS. The stained kidney sections are imaged with an Olympus microscope and semi quantitatively scored in a blinded manner by the renal pathologist (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b).

3.5.18 Immunofluorescence microscopy

Staining for type IV collagen and fibronectin are performed in frozen kidney sections as previously described (Sun et al. 2002; Jiang et al. 2005a; Jiang et al. 2005b)[20-21]. For adipophilin imaging, paraffin-embedded sections are used and adipophilin is detected by incubating with antibodies to adipophilin (1:500) (Roche Biochemicals, Indianapolis, IN, USA), and then with Alexa-488- (Molecular Probes Inc., Eugene, OR, USA) labeled secondary antibody. Lipid droplets and nuclei are stained with Nile red and 4', 6-diamidino-2-phenylindol [22-23].

3.5.19 Electron Microscopy

Tissue is fixed in 3% paraformaldehyde in a 6:4 mixture of cacodylate buffer (pH 7.4; adjusted to 300 mosmol with sucrose) and 10% hydroxyethyl starch. The tissue is then post fixed in 1% buffered osmium tetraoxide. The sample is dehydrated in a graded series of ethanol, and embedded in an epoxy resin. Tissue is surveyed with a series of one micrometer sections for a representative sample. The selected specimens are thin sectioned, viewed and photographed with a Phillips electron microscope 201 (Phillips Electron Optics, Inc., Mahwah, NJ, USA). The sections are read by the renal pathologist in a blinded fashion for determination of basement membrane thickness and podocyte morphology (Jiang et al. 2005b)[23].

3.5.20 Synapse

The therapeutic efficacy of HMG-CoA reductase inhibitors in various renal diseases has been shown extensively in both experimental settings and clinical renal patient. However, the precise molecular mechanism underlying statin-mediated renal protective effect remains unclear. Pharmacologically, statin is a class of drugs inhibiting HMG-CoA reductase, a direct target gene of transcription factor sterol response element binding protein-2 (SREBP-2), subsequently suppressing de novo cholesterol biosynthesis and lowering serum cholesterol level. It is proposed that statin- mediated renal protective effect in diabetic animal models is direct suppression of renal inflammation process and possibly independent of its systemic cholesterol lowering effect, although the hypothesis hasn't been experimentally validated. Further, renal in situ cholesterol/lipid lowering effect mediated by statins treatment hasn't been examined yet. Previously, we and others have shown that renal lipid metabolism may play important roles in renal inflammation, glomerulosclerosis and tubulointerstitial injury in diabetic nephropathy. Furthermore, the facts that lipids serve as primary inflammation mediators participating in multiple disease processes have been very well recognized. Thus, we surmise that the disturbance of renal lipid metabolism may be involved in STZ associated renal injury, and the normalization of lipid metabolism abnormality contributes to statins-

mediated renal protection. Based on the above mentioned rationales, we attempt to examine:

1. If renal SREBP-1, 2 expression/activation is modified in STZ rats. SREBP-2 is the principal transcription factor directly controlling the expression of HMG-CoA reductase gene expression. It is possible that SREBP-2 activity is altered and modified by statin treatment.
2. If HMG-CoA reductase expression is altered with or without the modulation of SREBP-2. Alternatively, HMG-CoA reductase could be modified post-transcriptionally.
3. If renal cholesterol or triglycerol content is altered in STZ rat kidney and normalized by statin treatment.
4. If certain other important lipid metabolism pathways altered, such as lipid transport, lipid oxidation pathways, in addition to *de novo* biosynthesis namely SREBP-2/HMG-CoA Reductase pathway in STZ rat and corrected by statin.
5. The statin may regulate certain key fibrosis associated factors such as VEGF, TGFb.

3.5.21 Parameters need to be analyzed

1. *Blood biochemistry*. Body/organ weight, serum lipids, BUN/Cr, e-GFR, cytokines (Interleukin 1b, TNFa, check one of them if possible, since these cytokines are elevated in patient with chronic kidney disease and suppressed by statin according to recent literature).
2. *Kidney lipid contents*. determine cholesterol/triglycerol content from kidney tissue. (n=4-5 from each group)
3. *Protein expression in kidney*. **Use 1/2 kidney.** Western blot for HMG-CoA reductase. Save the rest for future use, n=3-4 from each group. VEGF or TGF level can be re-blotted with same filter.
4. *Nuclear extract for SREBP-2 protein.* **Use ½ kidney.** Including both nuclear and cytosol lysate in western blot and compare the abundance of nuclear form and cytosol form SREBP-2. Antibody can be obtained from Santa Cruz. (n=3-4 from each group).
5. *Kidney RNA for RT-PCR analysis*. **Use ½ kidney.** Ideally extract and keep RNA in 80% ETOH. Determine gene expression for SREBP-2, SREBP-1, HMG-CoA reductase, ABCA-1, IL-1, TNFa, podocyte markers nephrin, and podocin. (n=4-5 from each group)
6. *Histology study*. **Use 1/3 kidney.** PAS staining for fibrosis. Microphage infiltration/MCP-1 expression (n=3-4 from each group). May include some other markers later, depends on the result.

3.6 Statistical and Data Analysis

The data could be expressed as the mean±SD. The statistical significance of the results between samples obtained from four groups is determined by one-way analysis of variance with Student-Newman-Keuls analysis for multiple comparisons. Significance is accepted at the $p<0.05$ level.

Discussion

There is crucial disagreement on the issue of functionality of these cells. Do the bone marrow-derived cells actively participate in extracellular matrix synthesis? Roufosse et al. in a mouse model of unilateral ureteric obstruction, detected bone marrow-derived α-SMA-positive cells. Using two reporter molecules under the control of the promoter and enhancer elements of the collagen I α2 chain gene, we did not however observe any functional bone marrow-derived fibroblasts or myofibroblasts producing collagen I. On the other hand, Iwano et al. in a mouse model of unilateral ureteric obstruction, and Broekema et al. in a rat model of unilateral I/R injury, demonstrated double immunostaining positivity of α-SMA-positive interstitial cells with pro-collagen I protein (Broekema et al. 2007).

In this project, with the mouse model, we aim to find the practical conditions to induce ES cells differentiating into renal stem cells and to find the ways using the renal stem cells to repair and regenerate obstructed kidney. For these, we will explore the techniques to induce ES cells to adopt a renal fate using co-culture with cell lines, metanephroi and novel growth factors. Meantime, we will characterize the expression profile of different renal subcompartments so as to identify the secreted proteins involved in renal differentiation and to isolate the specific cell surface markers identifying renal stem cells.

The theoretical background justifying the pursuit of the potential of bone marrow cells to participate in renal regeneration has been laid. Stem cells, both embryonic and from the adult bone marrow, in the right conditions, can express renal markers in vitro and give rise to renal cells in vivo.

In addition, injection of stem cells into the kidney or the bloodstream can lead to an improvement of renal function, although this does not always seem to be mediated by transdifferentiation into renal cells. Current views favour a predominant role for the delivery of a cocktail of angiogenic and

immunomodulatory mediators as the main means by which bone marrow cells enhance epithelial and endothelial cell survival. As far as engraftment of bone marrow cells as renal parenchymal cells is concerned, proving functionality of the engrafted bone marrow-derived cells is crucial in order to assign to them a role in improved renal function, rather than relying on morphological observations alone.

The kidney is a complex organ with over 30 different cell types, and present technology does not envisage constructing a whole kidney from stem cells. However, within existing kidneys where the basic scaffolding is intact, stem cells may contribute to a variety of specialised cell types, either promoting more efficient repair or correcting genetic defects. These would include: (1) acute tubular necrosis (ATN) caused by toxins or ischaemia/reperfusion (associated with kidney transplantation); (2) mesangial damage, often associated with immune complex deposition and diabetes; (3) defective podocyte function (Alport's disease); (4) vascular endothelial damage (e.g. in glomerulonephritis) (Alison et al. 2007; Alison 2009).

Whether stem cell injections will ever be used for the treatment of renal failure is at this stage still unknown. There is certainly some hope to be found in the numerous animal models that have been developed and analysed over the last few years.

To reach the goal, we propose the following basic research objectives: (1) Use expression profiling to further dissect the processes of commitment to a metanephric fate during normal development. (2) Identify novel renal progenitor cell markers and growth factors to assist in the identification, isolation and/or reactivation of renal stem cells. (3) Examine the potential for ES cells to be differentiated into the lineages necessary for renal regeneration or endogenous repair.

The scientific objective of the application for the renal stem cell research could be to establish a model by using stem cells to repair and regenerate damaged kidney: Aim 1. To isolate stem cells from mouse embryo and/or adult tissue (such as adipose and renal tissue) via sorting for specific cell surface markers; Aim 2. To induce embryonic and/or adult stem cells into renal stem and/or progenitor cells; Aim 3. To damage mouse kidney by partial ureteral obstruction (PUO); Aim 4. To administrate the renal stem and/or progenitor cells to the damaged rat kidney; Aim 5. To observe the kidney repairing and regenerating effects such as physiological condition and biochemical status after stem cell treatment.

Correspondence to:
Hongbao Ma, PhD
Brookdale University Hospital and Medical Center
One Brookdale Plaza
Brooklyn, New York 11212, USA
ma8080@gmail.com
347-321-7172

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Haemocoelom excludes embryonic stem cells and asexual reproduction in invertebrates?*

P. Murugesan [a], T. Balasubramanian [a] and T. J. Pandian[b]

[a]C. A. S. in Marine Biology, Annamalai University, Parangipettai – 608 502
[b]C. A. S in Genomics, School of Biological Sciences, Madurai Kamaraj University, Madurai – 625 021

murugesaan74@rediffmail.com

Abstract: The terms embryonic and adult stem cells are explained. Previous studies on identification, description and isolation of the embryonic stem cells in different invertebrate groups are briefly summarized. Most invertebrates, which reproduce asexually, have retained the embryonic stem cells in their adult body. A hypothesis is proposed for the possible exclusion of embryonic stem cells and thereby asexual mode of reproduction by the coelom in arthropods and molluscs. [Stem Cell. 2010;1(1):52-57] (ISSN 1545-4570).

Key words: Types of stem cells, modes of reproduction and regeneration, theory of neoblasts, exceptional models

This communication attempts to establish a correlation between the presence of haemocoelom and the absence of embryonic stem cells and the consequent non-occurrence of asexual reproduction in arthropods and mollusks. Most invertebrates reproduce sexually but may switch over to asexual mode of reproduction, when need arises owing to biotic factors e.g. very high density (Skold, et al. 2002) or abiotic factors e. g. water quality parameters (O' Dea, 2006). The presence of embryonic stem cells is obligatorily required to facilitate the asexual reproduction, as in sponges, cnidarians, turbellarians, clitellates and echinodermates.

Based on their differentiation potential, the stem cells can be divided into two major types: i) *the embryonic stem cells* (see Neuringer & Randell, 2004), derived from the inner mass of early blastocytes, as in humans and echinoderms, have retained the capacity to generate all the two/ three germinal layers, from which fully developed progeny arises, and ii) *the adult stem cells,* hidden deep within the organs and surrounded by millions of ordinary cells in fully developed adult animals, have restricted potential to produce only certain types of cells. The processes of differentiation by embryonic and adult stem cells are known as *epimorphosis* and *morphallaxis*, respectively (Agata, et al. 2007). The first one involves the activation of embryonic stem cells to proliferate, form blastema and differentiate into the regenerated body parts, as in *Dugesia tigrina*. The second one, the morphallaxis involves the transformation of existing body parts or tissues into newly organized structures without cell proliferation e.g. *Crepidula plana*

Investigations since early 1900's on regeneration in triclad turbellarians showed that fully differentiated adult animals harbour unique "embryonic stem cells". These cells have retained the capacity for self-regeneration and ability to differentiate into a progeny. They are slow-cycling undifferentiated cells and divide asymmetrically into daughter cells, one of which is committed to differentiation and the other retains the capacity of the original stem cells, which can differentiate all the cell types required to generate a progeny. Because of their slow cycling, adult stem cells of human with limited potential (to produce certain cell types) can be identified by their prolonged retention of nucleotide analogues like bromodeoxyuridine (Borok, et al. 2006). This communication points out the need for identification and description of adult and embryonic stem cells in animals bestowed with the capacity for regeneration of a part of the body or an entire organism from 'bit and pieces' of the parental animal.

Animals vary widely in their ability to replace lost body parts through regeneration (Brusca & Brusca, 1990). The phylogenetic distribution of regenerating ability across animals implies that this capability has been gained and/or lost many times during the chequered history of evolution. Despite the recent surge of interest in adult stem cell research, comparative studies on identification and description of such stem cells in animal groups characterized by different abilities to regenerate the lost parts of body are wanted. To date, regeneration studies have focused almost on a few, very distantly related groups such as cnidarians, turbellarians, clitellates and echinodermates.

For reasons yet to be known, arthropods and mollusks, characterized by haemocoelom, have only a minimal capacity to regenerate a stump on the lost part of an appendage, as in arthropods or to regenerate the lost part of the inhalent and exhalent siphons, as in bivalves, but have no capacity to regenerate an entire animal. The deep evolutionary separation between

embryonic and adult stem cell model systems and the important anatomical differences between them make it nearly impossible to reconstruct, which evolutionary and developmental mechanisms are responsible for such wide differences in the ability of regeneration among these groups. On account of this fact, there is an urgent need for identification and description of tissue/animal regeneration in selected invertebrates, harbouring “stem cells”.

In sexually reproducing animals, the zygote, a product of fusion of two gametes, is developmentally totipotent, and has the capacity to generate all the two (as in sponges and cnidarians) or three (as in all other higher animal groups) germinal layers and a completely developed progeny. In parthenogenetic animals, the female produces diploid egg, from which a completely developed progeny arises. However, in asexually reproducing animals, the equivalent of ‘zygotes’ namely embryonic stem cells are retained in specialized “niches” and are capable of producing completely developed progenies.

It is known that adult bone marrow of human contains cells, which can make all of the blood cell types (Beeres, et al. 2005). But these stem cells could not be isolated as pure populations, as the techniques for recognizing adult stem cells were developed only after 1980’s. As indicated elsewhere, the inconspicuous nature of the stem cells in terms of numbers, size, shape and function make their identification and isolation a Herculean task.

These adult stem cells possess an array of protein on their surface; the surface proteins can be used as “markers”, which characterize individual cell types i.e. a type of “molecular marker”. For example, using molecules that recognize and attach the specific surface proteins, which can be blazed under certain wavelengths of light, a blood stem cell can be distinguished from a mature white blood cell. Unfortunately, not all stem cells can be identified in this way, as ‘molecular markers’ have not yet been identified for all the stem cell types, which occur in other animal groups, especially the invertebrates. Hence, there is a need for molecular biologists to develop suitable markers to identify the stem cells in adults of different invertebrate animal groups.

Modes of reproduction and regeneration

Asexual mode of reproduction among invertebrates is not homogeneous in its nature, as it proceeds by fragmentation and gemmulation in sponges, cladogenic, blastogenic buddings and strobilation in cnidarians, fission in turbellarians, architomic and paratomic fission in clitellata, and by fission and autotomy in echinodermates. Many scientists have endeavoured to trace the ultimate progenitor cells, from which a complete progeny arose and named those stem cells by different designations namely archeocytes and thesocytes in sponges, stem interstitial cells and amoebocytes in cnidarians, neoblasts in turbellarians, blastocytes and eleocytes in clitellata and coelomocytes in echinodermates and indicated that these cells are totipotent/omnipotent or pluripotent/polypotent/ multipotent. Of these the following must be mentioned:

Working on *Oscarella tuberculata,* a homoscleromorph sponge, which shares many morphological, cytological, biochemical and embryological features in common with eumetazoa, (Ereskovsky & Tokina, 2007) indicated that this sponge and bilaterians share highly conserved homologies in basic genetic machineries involved in cell differentiation and regulation of development. Thus their research work has provided the first bridge on polarity, axial formation and regulation mechanism of development between the two-layered sponges and the three-layered animals.

In cnidarians, the situation remains a little complicated. The structural cells i.e. ectodermal plus endodermal cell complexes are responsible for giving the polyp its form and the ‘stem cells’, i.e. amoebocytes maintained among the structural cells by controlled cell cycle give the polyp its behaviour and sex. The amoebocytes are known to migrate and proliferate at the site of budding. But, Gilchrist (1937) showed that the epidermis of polyp can regenerate a complete polyp. Hence it is not clear whether the true stem cells are maintained amidst the structural, i. e. subtentacular cells or interstitial cells. However heterogeneous asexual modes of reproduction in cnidarians are far more complicated to comprehend a single concept, as has been spectacularly achieved in triclad turbellarians.

The triclads display remarkable power of regeneration and have been the object of numerous researches, especially by the French school led by E. Wolff, who postulated polarity and axial gradient theory. However, the central question concerns the origin of the cells in ‘blastema’, from which any injured or removed part of the body is reconstructed. Amazingly, it was traced to the free basophilic cells buried in the parenchyma called ‘neoblasts’ and the theory of neoblasts was proposed as early as in 1889-1901 by Morgan. The neoblasts of endodermal origin are regarded as undifferentiated totipotent elements, which remain quiescent from the embryo stage up to the moment at which they participate in formative process. Capable of migrating by means of amoeboid movements, they reach the area in which mutilation has taken place.

Betchaku (1967) was the first to obtain selectively a culture of neoblasts. Subsequently, Franquinet (1976) and his collaborators (Franquinet, et

al. 1985) developed new culture media, which yielded a large number neoblasts but still mixed with other cell types. Using the selective adhesive property of the neoblasts to the substrate, they eliminated the other types of cells, which led to the culture of neoblasts with "high purity". Thus it was possible as early as in 1985 to have a highly pure culture of neoblasts, i.e. embryonic stem cells, something similar to what has been achieved with molecular markers for the adult stem cells in recent years. Some of these techniques may be handy to zoologists to isolate and culture the embryonic stem cells of other animal groups like the annelids.

It appears that regeneration research in anthozoans, clitellates and echinodermates proceeded in the direction of locating and quantifying the minimum required 'niche' of stem cells to induce successful epimorphosis. Annelids are excellent group to investigate regeneration abilities in a comparative context. As their bodies are composed of repeated segments, which largely possess the same structures (segmented nerve ganglia and fibers, musculature, gut, blood vessels, nephridia, chaetal bundles and so on), any mutilation made at different axial positions along the body results primarily in the removal of different quantities of a given organ system, rather than the removal of different organs/ systems or unique structures and thus facilitates comparisons among the annelid species. The ability to regenerate both anterior and posterior segment is widespread and probably ancestral for the phylum (Bely, 2006). Some sabellids and lumbriculids are capable of regenerating an entire individual from a single mid-body segment, which indicates that adequate number of embryonic stem cells is retained in every segment (Martinez, et al. 2005).

Small and medium sized sea star *Allostichaster insignis* divides throughout the year and the ramets of most individuals regenerates sufficiently to divide again after 6-9 months (Michael, et al. 2008). In the sea star *Ophiocoma echinata*, a piece of oral disc is necessary to complete regeneration and requires a long duration of 2 years to completely regenerate the 3 arms (Pomory & Lawrence, 2001) at the energy cost of 0.17 kJ/ day (Pomory & Lawrence, 1999). On the other hand, fragments of about 20 cm length are required to regenerate an individual with reproduction capacity in the branching coral *Acropora formosa* (Okubo, et al. 2007). According to the description of Reichensperger, regeneration in *Neocrinus decorus* is commenced promptly by two types of cells: 1. the phagocytic amoebocytes and 2. the coelomocytes, filled with rods and granules and abundant along the nerve cods; they become elongated in shape and assist the process of regeneration (Hyman, 1955).

Briefly the epimorphic regeneration occurring in sponges, cnidarians, clitellata and echinodermates originates from totipotent embryonic stem cells in the sense of Borok et al. (2006). Morphallaxic regeneration encountered among arthropods and mollusks originate from multipotent adult stem cells capable of generating the germinal layers/organ specific cell lineages. Hence the embryonic stem cells are not likely to occur in these two animal groups. Arthropods are capable of regenerating undifferentiated mass of tissues on autotomised fraction of appendages (Maginnis, 2006). Molluscs have retained multipotent adult stem cells capable of regenerating tissues/organs involving mesoderm and ectoderm alone. Many bivalves suffer the "siphon-nipping" i.e. the removal of the terminal fraction of the siphons by predators. Hodgson (1982) estimated the requirement of 92 hrs time and 0.6 kJ energy to regenerate 6 mm long siphon representing 20% of total length of the siphon. In *Octopus vulgaris,* O'Dor & Wells (1978) recorded the presence of arms with various stages of regeneration. A 'climax' is the case in which organ specific regeneration involving mesoderm and ectoderm has been reported in *Crepidula plana* by (Gould, 1952) heads removed from anaesthetized snails were replaced within 14 days but the snail failed to regenerate alimentary canal of endodermal origin.

The proposed hypothesis

From a careful visual survey through the Multi–Volume series on 'The Invertebrates' by Hyman, and that on 'Reproductive Biology of Invertebrates' by Adiyodi & Adiyodi, relevant available information on the presence of embryonic stem cells and occurrence of asexual reproduction in major groups of invertebrates was made. For a few minor invertebrate phyla, adequate and reliable information is not yet available.

Besides, a computer search was also made in Google.com using keywords: haemocoelom, asexual reproduction, embryonic stem cells, Invertebrates. From these sources, Table 1 was formulated and the following inferences were made:

1. The presence of the equivalents of embryonic stem cells has facilitated the occurrence of asexual reproduction in many major invertebrate groups,
2. However, in a couple of minor groups characterized by the presence of pseudocoelom and in the major groups of arthropods and mollusks possessing haemocoelom, asexual reproduction is not known to occur, and
3. Incidentally, the presence of embryonic stem cells or their equivalents has not so far been recorded in these animal groups.

These inferences lead us to propose a hypothesis, i.e. embryonic stem cells are obligatorily required to facilitate asexual reproduction; pseudocoelom of nematodes and rotifers, and haemocoelom of arthropods and mollusks appear not to have provided the required niche for retaining embryonic stem cells and thereby the non–occurrence of asexual reproduction in these animals. This hypothesis, however, is yet to be tested. Incidentally, a rare claim has been made by Vanderspoel (1979) on the occurrence of asexual reproduction in a haemocoelomate snail *Clio pyramidata*, which may prove an ideal model to test the hypothesis. Incidentally, it must also be mentioned that despite the presence of embryonic stem cells *Polycelis nigra–tenuis* has lost asexual mode of reproduction (Benazzi & Lentati, 1993). Likewise, a large number of polychaetes have secondarily lost the capacity for asexual mode of reproduction (Bely, 2006).

However sporadic occurrence of sex change from female to male or male to female in sequential hermaphrodites like annelids: e.g. *Sphaerosyllis hermaphrodita* (Westheide, 1990), arthropods: e.g. *Clibanarius* (Wenner, 1972), molluscs: e.g. *Xylophaga dorsalis* (Purchon, 1941) involve dedifferentiation and redifferentiation of organs related to reproductive system. Apparently, all of them appear to have retained multi-potent adult stem cells somewhere in the gonad. It is known that the components of reproductive system are of mesodermal origin; however, it is also known that vitellogenin is synthesized in the liver/hepatopancreas/fat bodies of females and transported and deposited in the maturing oocytes of ovary. Hence, these liver of endodermal origin and equivalent organs are 'feminine'. Therefore, all these animals, which change sex from male to female, may also serve as experimental models to test the proposed hypothesis.

Table 1. Correlation between coelomate type, asexual reproduction and embryonic stem cells in invertebrate groups

Invertebrate group	Coelomate type	Equivalents of embryonic stem cells	Occurrence of asexual reproduction
Sponges	-	Archaeocytes, thesocytes	Yes
Cnidaria	-	Stem interstitial cells, amoebocytes	Yes
Turbellaria	Acoelomate	Neoblasts	Yes
Clitellata	Eucoelomate	Blastocytes, eleocytes	Yes
Echinodermata	Eucoelomate	Coelomocytes	Yes
Arthropoda	Haemocoelomate	Absent?	No
Mollusca	Haemocoelomate	Absent?	No
Nematoda	Pseudocoelomate	Absent?	No
Rotifers	Pseudocoelomate	Absent?	No
Chaetognatha	Coelomate	Absent?	No

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E-mail: murugesaan74@rediffmail.com

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2/1/2010

STRONG HORSE TEA BY ALICE WALKER: *A REVIEW*

Samiya Taskeen[1] and Abida Taskeen[2]

[1] Hajvery University Lahore,Pakistan

Email: funnydoll5@yahoo.com

[2]Lahore College for Women University Lahore Pakistan

Email: Paristan12@yahoo.com

ABSTRACT: This review is about the history of those black skinned people who were treated like slaves in the past because it was believed that black skinned people were born to serve others in fact they are slaves who have no right to live like humans. Alice Walker in "STRONG HORSE TEA" focuses how Rannie Toomer lost her only child and how she has become the victim of white men's exploitation who suffered from superiority complex and they considered themselves torch-bearers and civilized but in reality they are in need to be civilized and they are savage, ignorant, cruel and prejudice. [Stem Cell. 2010;1(1):58-59] (ISSN 1545-4570).

Keywords: Alice Walker, Rannie Toomer, Strong horse tea

SUMMARY

Generally People believed that it is an age of machines where science has progressed a lot, man is touching the heights of sky and making discoveries in almost every walk of life, man has started to move in the air and the discovery of atomic bombs and missiles has proved that how much man has progressed and has known how to cure and control those diseases which were impossible to control in past. Modern man is thinking that he has learned how to live a healthy and wealthy life by following his savage or dark desires. On the one hand man is moving upwards where he is progressing a lot in different fields while on the other hand he is equally moving downwards were he shows the darker and criminal aspects of his personality by behaving rudely to their fellow human beings.

It is well known that the European countries started to explore and colonize so many areas of this world at the start of seventeenth century and the peak of this colonial power of Europe was shown by the second half of nineteen century. Germany Belgium, France and above all Great Britain obviously rolled over almost half of the world. A noticeable sense of confident and pride also came along with this marvelous achievement that was about European civilization that it is the best civilization on this earth. And at that time black-skinned persons were considered slaves and it was believed that they were born to serve others .Strange is that white skinned persons were considered civilized and torch bearers while on the other hand all black skinned persons were considered cheap, inferior and above all slaves which were in need to be civilized according to the white skinned people. But reality is that such white skinned people themselves were tyrant, cruel, cheap, savage, and inferior and prejudice and they themselves were in the need to be civilized because they were white at face but dark at heart.

Alice Walker is a well known female writer of USA who is famous for "The color purple". and it is obvious that she has written a lot on the rights of black people because she herself was a black woman even her heroines are black women struggling to emerge from a history of oppression in the society of white people and abused by Black males who have failed women and themselves.

Alice believes in the equality of men and women. "Strong horse Tea "reflects Walker's views about black people . The story based on a young female rannie toomer and her ill son. Rannie Toomer is shown

the victim of white men's savagery and exploitation as she is unmarried but having a child snooks. Her son is her world. When story opens Rannie Toomer is in the helpless situation because her only son snooks is too ill and he is dying of double Pneumonia and whooping cough and Rannie is in great difficulty. She tries her best to save her son, she wants to call a real Dr for his medical check up and treatment but Dr. would not come to check Black lady's child. Rannie Toomer does not realize her position and hopes that Dr will come. She is shown as a loving mother who is too attached to her son because she is having no other connections and that is why she does each and everything she could. The heroine Rannie Toomer in "The strong horse Tea" is shown an intelligent and mature lady who does not likes conservative things that is why she rejects Sara who is a neighboring lady and who is considered to be a witch and knows the treatment of pneumonia by herbs and magic, she advised Rannie Toomer to try some home remedies but Rannie scornfully rejects because she does not believe in that swap magic that Sara practices. She again and again calls for doctor but she did never think that she is a black lady for whom there is no pity and kindness in this world of white people. Rannie and her son Snooks suffered a lot mainly because of their skin color. Both son and mother are in bad condition and suffering from hunger, illness and are ignored especially because of the discriminative behavior of so-called civilized (white) people who treats every white like a slave and it would be an insult for a doctor to treat a slave child, no matter how innocent he (Snooks) is. Tired and hopeless Rannie Toomer goes again and again to the Doctor but white Dr does nothing infect he is constantly repulsed with her bad breath and unwashed appearance. She was hopeful that doctor will come and Snooks will be all right that is why she waits a lot but doctor gives no importance to Rannie rather it was just like the insult of white doctor to treat black lady's child, so doctor gives no time and importance to them.

Defeated and hopeless at last Rannie allows Sara to do something to save her child. After examining Snooks Sara announces that only one thing that can save his life is "STRONG HORSE TEA" (horse urine). Rannie goes outside into the rain, chases down the horse and uses her shoe as container to catch the tea and we see that while she is collecting a horse kicks her down but she continues. And the feelings of pity aroused as we see that Rannie is collecting tea on the one hand while on the other her son has just passed away in the hands of Sara.

It was the disaster and end of the world for a mother to lose her only child only because of the ignorance of the white Doctor. And it shows not only injustice, cruelty, savagery, exploitation of the white but the darker aspect of the society of white where success worship is its religion.

Rannie Toomer becomes the representative of all those black ladies who suffered a lot because of such skin discrimination. Alice Walker wants to convey that black people are not animals. It would be injustice to treat them like animals rather we must treat them like all human beings. It must be noticed that such discriminative sort of behavior leads humans to decay. The question we have to ponder over is "Are we the members of such evil practicing society?"

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Researcher would like to thank Mejor Nazar Abbas, Ms. Anjum Naseem and Mrs. Shaukat Jahan for their technical assistance in writing this review.

3/3/2010

Effect of *Bacillus thuringiensis var. israelensis* (H-14) on Culex, Aedes and Anopheles larvae (Cotonou; Benin)

Gbehou Nounagnon Achille [1,*], Houssou S. Christophe[2], Li Yilian [3]

1. School of Environmental Studies, China University of Geosciences, Hubei province, 388 lumo Road, 430074 Wuhan, P.R China, , 008615927014830
2. University of Benin, (Abomey-Calavi), Dean of the Faculty of Human sciences Box: 526- Abomey-Calavi- Benin
3. China University of Geosciences, Department of Environmental Sciences, 388 Lumo Road, Hongshan District, Wuhan City, Hubei province, 430074, P.R.China

*For correspondence: achillegbehou@yahoo.fr

Abstract: The use of insect-specific toxins from *Bacillus thuringiensis var. israelensis* is forming an increasingly important component of biological control strategies that are either being implemented or planned for use in mosquito control. In terms of morbidity and mortality caused by vector-borne diseases, mosquitoes are the most dangerous animals confronting mankind. They threaten more than 2 billion people and have substantially influenced the development of mankind, not only socio-economically but also politically. In this study, the use of *Bacillus thuringiensis var. israelensis* crystals for controlling insect's larvae was carried out at laboratory scale. Three species of insects were tested. The operational parameters for the most efficient use and monitoring of *Bacillus thuringiensis var. israelensis* toxins against insect's larvae in the laboratory were discussed. [Stem Cell. 2010;1(1):60-68] (ISSN 1545-4570).

Key words: Larvae, mosquitoes, *Bacillus thuringiensis var. israelensis,* operational parameters

1- Introduction

Amongst vector borne diseases, malaria occupies a predominant position since it is probably the leading cause of death in the world despite intense national and international efforts to control it (Pickett, 1990; Smyth, 1994). It is estimated that there are 300-500 million new cases every year, with 1.5 to 2.7 million deaths world wide particularly in Africa (WHO, 1992).

Malaria is a major health problem in Benin, where the entire population lives in areas with malaria transmission. Malaria is a leading cause of morbidity and mortality among children under five, accounting for 44 percent of outpatient visits and 40 percent of all hospitalizations.

This disease is the leading dominant disease and is one of the most important causes of mortality among infants and young children. Two main vectors are responsible of the disease transmission in Africa: Anopheles gambiae and Anopheles funestus.

To reduce the intensity of human contact - vector, WHO has recommended since 1996 the use of insecticide-treated nets and indoor spraying of insecticides at home residual where the context permits. Unfortunately today, the resistance of vectors to insecticides has been observed and constitutes a barrier to their use (VULULE et Al. 1994).

The first case of resistance to pyrethroids has been reported in Bouaké, Côte d'Ivoire because of the massive use of home Aerosol (Ellisa, N, Mouchet, 1993).

Later, the same remark was made in Kenya in the area where the nets impregnated with pyrethrum are used by the population.

In Benin in 1996, according to the work of Laleye and Akinotcho, a resistance of Anopheles gambiae ss to pyrethrum has been reported in Cotonou. This resistance was expressed by 20% mortality of Anopheline adults exposed to papers impregnated with pyrethrum.

Most of chemical larvicides create environmental problems because they are lethal to non-target species. To meet the challenges of vector resistance to chemical larvicides and environmental safety, we considered the use of biological insecticides Bacillus thuringiensis var. israelensis (Bti)

The biological agent has proved to be useful for control of mosquito species in a variety of breeding habitats (Mulla et al. 1984; Karch et al. 1992), and shown very high environmental safety. However, experimental evaluation of the agent specifically as control agent against malaria vectors is still limited.

The performance of the microbial agent may be affected by water quality parameters such as organic content, salinity, pH, and water temperature, all which vary by ecology and type of breeding habitat. These variables provide the basis for

evaluating the efficacy of this compound in a variety of ecological and epidemiological settings.

The larvicidal activity of the granular formulation of *Bacillus thuringiensis var. israelensis* was evaluated.

The main objective was to determine the optimal application rates and duration of activity for the biological larvicide.

2- Materials and methods

2-1 Literature review

2-1-1 Characterization of Bacillus thuringiensis israelensis

In 1975-76, a World Health Organization sponsored project in Israel examined mosquitoes for the presence of pathogens or parasites. During this survey, a new *Bt* strain was discovered with high toxicity to mosquito larvae (Goldberg and Margalit 1977) which was later identified and designated *Bt* var. *israelensis,* serotype H14 since raised to subspecies status as *B. thuringiensis israelensis*. This strain was significantly more toxic to mosquitoes than other known bacterial strains at that time. It was collected from mosquitoes in the Negev desert of Israel. While dipteran active *Bt*s were known, *Bti was* found to be relatively specific to Diptera and was quickly shown to be toxic to a range of mosquito and black fly species. Therefore, it was considered to have commercial potential as a control agent of nuisance Diptera around the world. Rapid development of *Bti* strains occurred in the early 1980 and several products were developed. The need for a more environmentally benign mosquito control agent and rising incidence of resistance to chemical pesticides provided a platform for rapid *Bti* development.

The species in the complex are only differentiated from one another by a few characters, most of which are located on plasmids. Therefore, characterization of *B. thuringiensis has* been problematic and several systems have been used. Phenotypic methods used include flagellar serotyping, description of crystal morphology, biochemical reactions and bioassays. Classification of subspecies or varieties based on serotyping using H-serovars resulted in identification of almost 60 varieties (Hansen *et al.* 1996). Serotype does not necessarily relate to the presence of -endotoxins, which determine host specificity, as flagellar genes are carried on the chromosome, while toxin genes are usually encoded on plasmids.

Characterization methods based on phenotypic characters are insufficient when used alone in studies on the environmental ecology and fate of *B. thuringiensis*, as these methods do not provide unambiguous identification. A number of DNA-based methods have been developed for characterization: specific primed polymerase chain reaction (PCR); Random amplified polymorphic DNA (RAPD), DNA: DNA colony hybridisation (Hansen *et al.* 1996) and rRNA-based probe (Akhurst *et al.* 1997). These methods can distinguish individual strains and isolates, allowing the tracking of the environmental fate of strains used for pest control.

Such methods can also be used to identify the presence/absence of specific endotoxin genes, which mean it is possible to establish whether a particular strain has lost or acquired specific - endotoxin genes in the environment.

2-1-2 Action

The products contain the spores and parasporal crystals of *Bti* H-14 serotype which must be ingested by the larval stage of the mosquito to cause mortality. Following ingestion, the parasporal crystals are solubilised in the alkaline larval midgut, followed by proteolytic activation of the soluble insecticidal crystal proteins. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell, which leads to death of the larva.

Bacillus thuringiensis var. israelensis treated mosquito larvae generally cease feeding within 1 hour, show reduced activity by two hours, extreme sluggishness by four hours and general paralysis by six hours after ingestion (Chilcott *et al.* 1990).

2-1-3 Occurrence

An understanding of the ecology of *Bti* in the environment is essential in assessment of its environmental risk. While originally recovered mainly from insects, improved isolation and identification techniques have indicated that *Bacillus thuringiensis may* be ubiquitous in soil. The lowest percentage recovery of *Bacillus thuringiensis from* soil reported was in the USA (60% of soils sampled) (Meadows 1993). In New Zealand, Chilcott and Wigley (1993) found that between 60-100% of soils sampled contained *Bt*, depending on source (urban, horticulture etc.). *Bacillus thuringiensis* is also indigenous in many other environments, being found in stored products, dust, on deciduous and coniferous plants and in aquatic environments. *Bacillus thuringiensis has* also been isolated from insect habitats such as rotting wood, wasp nests and stored products in many countries.

2-2 Area of study

This study was conducted in Cotonou, Benin's economic capital due to the high rate of malaria which is filled each year. The city was built on a

sandy beach to create a harbour close to the only waterway between the Gulf of Guinea and Lake Nokoué. The total area is 74 square kilometer. It is the largest city in Benin and situated between latitude 6.2°-6.3° N and longitude 2.2°-2.3° E. The relief is relatively flat and the elevation ranges between 0.3 and 6 meters.

The climate is equatorial, alternating with two rainy seasons (April - July and September - October, 800 to 1,200 mm of rain per year) and two dry seasons. From December to January, the city is affected by winds. The annual temperature varies between 18 and 35 °C. The population has multiplied rapidly from 3,300 inhabitants in 1921 to 383,000 in 1981 and 780,657 in 2006.

Cotonou always faces the most devastating floods during the rainy season in West Africa. These floods preceded by heavy rains in the catchment areas of Lake Nokoue, and consequent massive releases of water from the lake, inundated the city, and slums were the worst hit. Over half of Cotonou (Benin) suffers every year from several months of flooding, allowing mosquito larvae breeding and leading to an increase in malaria transmission.

2-3 Mosquitoes

Mosquitoes are vectors of many diseases around the world like malaria, dengue fever, yellow fever and many types of encephalitis.

In Cotonou and many provinces of Benin, insecticides are used to control mosquito populations, but larvicides like *Bacillus thuringiensis var. israelensis* are not used. Almost 100 % of the products used in the last 15 years to control mosquitoes and black flies are insecticides. Depending of the species, the adult female mosquito will lay eggs either on the water surface (egg rafts or single eggs) or on damp soil (single eggs). In favourable conditions, eggs will hatch and give birth to larvae. There are four different larval instars. The last aquatic stage is the pupa from which will emerge the adult. *Bacillus thuringiensis var. israelensis* is active only against the larvae and there is no toxic activity neither against the eggs or the pupae.

Mosquitoes can breed in many types of habitats. One can find them in roadside and irrigation ditches, pastures, woodland pools, tidal waters, salt marshes, polluted waters (with organic and/or inorganic matter), small containers, tires and tree holes. They are also present in your own backyard in abandoned pools, bird baths, roofs, clogged gutters, etc. Almost everywhere where stagnant waters are present, you get high probabilities to find mosquito larvae. Different formulations of *Bacillus thuringiensis var. israelensis* can be used to control larvae in these various breeding sites. The type of formulations and dosages must be adjusted to the types of sites encountered.

2-4 Larvae sampling

This research was a laboratory experiment which investigated on the control of malaria vectors with the use of a bacterial agent (*Bacillus thuringiensis var. israelensis*). The types of mosquito larvae which had shown a resistant to the pyrethroids were tested with the Bti in the process of evaluating its use in the control of malaria vector in Cotonou.

The mosquito larvae were collected from three areas (**Fifadji, Ladji** and **Placodji**). A high rate of resistant to the insecticides was found in those three areas (Akogbéto & al. 1999).

The larvae collected were brought to the laboratory and then sorted and classified according to their evolutionary stage (1 to 4). For the results accuracy, two larvae stage were chosen (L2 and L4).

Estimates of larval density were then made by calculating the number of larvae dipped in each container. Larval samples were preserved in 70% alcohol and identified later by standard larval taxonomy.

After that a test of sensitivity of larvae to the Bti was made. Every 30 minutes, the mortality rate of the larvae was recorded. A control experiment also was set up.

2-5 Characterization of larval habitats

- pH
- Average depth
- Volume
- Water temperature
- Light intensity

2-6 Materials

- Granular formulation of Bti, serotype H-14 (VectoBac G – 200 International toxic units per mg [ITU/mg])
- Anopheles gambiae larvae (L2 and L4)
- Culex larvae (L2 and L4)
- Aedes larvae (L2 and L4)
- A graduated ladle
- Graduated test-glass
- Glass containers
- Sucking pipettes
- Deionized water
- Pasteur pipette
- Glass beads

2-7 Experiment Procedures

The *Bacillus thuringiensis var. israelensis* solution was prepared following the procedure of de Barjac and Larget (1984), cited by Dulmage *et al.* (1990): 50 mg of primary powder was suspended in

10 ml of deionized water and homogenized on a mixing machine for 10 min at 500 strokes/min using a bead mill (20-ml penicillin flask with several 6-mm glass beads). This homogenate was further diluted with deionized water to form a stock solution of 10 mg/L. A mixing machine was used for resuspension. Subsequent dilutions were made directly in the 500-ml test dishes.

The larvae were tested against the above mentioned *Bti* preparations at concentration of 10 mg/L. Using a Pasteur pipette with the narrow tip removed, 25 individuals were transferred to each test dish containing 500 ml. A control experiment was always run at each step of the procedure. The tests were run at room temperature. Larval mortality was assessed 30 minutes to 6 hours after *Bacillus thuringiensis var. israelensis* application. Each test was carried out at least twice and the standard deviation was less than 3%.

3- Results and discussions

Table 1: Larvicidal activity of Bti on larvae (L2) of Anopheles gambiae

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	24	22	19	14	12	11	9	8	6	4	1	0
X	0	1	3	6	11	13	14	16	17	19	21	24	25
Mortality (%)	0	4	12	24	44	52	56	64	68	76	84	96	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2: Larvicidal activity of Bti on larvae (L4) of Anopheles gambiae

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	24	24	20	17	10	7	5	3	2	1	1	0
X	0	1	2	5	8	15	18	20	22	23	24	24	25
Mortality (%)	0	4	8	20	32	60	72	80	88	92	96	96	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3: Larvicidal activity of Bti on larvae (L2) of Culex

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	20	6	3	2	1	0	0	0	0	0	0	0
X	0	5	19	22	23	24	25	25	25	25	25	25	25
Mortality (%)	0	20	76	88	92	96	100	100	100	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4: Larvicidal activity of Bti on larvae (L4) of Culex

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	25	19	11	7	4	3	2	1	0	0	0	0
X	0	0	6	14	18	21	22	23	24	25	25	25	25
Mortality (%)	0	0	24	56	72	84	88	92	96	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: Larvicidal activity of Bti on larvae (L2) of Aedes

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	22	16	4	3	2	1	0	0	0	0	0	0
X	0	3	9	21	22	23	24	25	25	25	25	25	25
Mortality (%)	0	12	36	84	88	92	96	100	100	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6: Larvicidal activity of Bti on larvae (L4) of Aedes

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	25	24	23	15	12	7	5	5	3	2	0	0
X	0	0	1	2	10	13	18	20	20	22	23	25	25
Mortality (%)	0	0	4	8	40	52	72	80	80	88	92	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

The larvicide had caused significant mortality of larvae during the experiment. Reduction of larval population was pronounced at each experiment, except the control experiment. The mortality rate of 100% was observed at 360 minutes (graph 1 and 2) for both type of larvae (L2 and L4) for Anopheles species. The L2 larvae population for Anopheles species have lower mortality speed than L4. Both Anopheles larvae had virtually the same sensitivity to Bti.

The impact of *Bacillus thuringiensis var. israelensis* was also followed in the laboratory on two larval stages of Culex spicies (L2 and L4). The results showed the death rate of 100% after three hours and half of exposure time and five hours for L2 and L4 respectively. A death rate of 50% of the larvae was observed after fifteen and one hour and twenty minutes for L2 and L4 larvae respectively. All these variations of population death rate of tested larvae are perceptible on graph 3 and 4. We can then say that the L2 larvae have a high sensitivity compared to the L4 larvae. The same experimental protocol was used again on Aedes larvae. After three hours and a half, we observe a 100% mortality rate for larvae L2 and five hours for L4 larvae (graph 5 and 6). However, after a one hour period of time, the death rate of 50% was observed for the larvae L2 and after 2 hours, the same percentage was reached for L4 larvae. Contrary to what we observed on the level of Aedes and Culex larvae, *Bacillus thuringiensis var. israelensis* has a less intense activity on the larvae of *Anopheles gambiae*. The death rate of 100% was observed after six hours and a half for both of L2 and L4 larvae. The two types of *Anopheles gambiae* larvae have practically the same sensitivity to *Bacillus thuringiensis var. israelensis*. The effectiveness of *Bacillus thuringiensis var. israelensis* on the larvae L2 of Aedes and Culex is higher compared to that of Anopheles of the same stage. The results obtained show that the larvae of Culex and Aedes are more sensitive to the crystals of *Bacillus thuringiensis var. israelensis*. This remark would be caused by behavioral and physiological variations for species studied. The position of the larvae in water would be one of the causes for these variations. Indeed, whereas the larvae of Anopheles are parallel to the water surface, those of Culex and Aedes are immersed because of their respiratory siphon. These results are connected with those provided by Lacoursière et al., (2004). This degree of susceptibility is also related to the dispersion of the crystals of *Bacillus thuringiensis var. israelensis*. The results obtained show that the larvae of anopheles are less affected than the larvae of Culex and Aedes when they are exposed to the same quantity of crystals of *Bacillus thuringiensis var. israelensis*. For Aly et al., (1987), the larvae of Anopheles would show a higher death rate if the crystals of *Bacillus thuringiensis var. israelensis* are delivered under a floating formulation. Moreover, the food behavior would be the essential component for this observation. The larvae of Culex and Aedes feed on

themselves actively through the entire water column, and since the crystals forms a deposit slowly towards the bottom of the lodging; they have a facility to introduce these crystals more than the larvae of Anopheles. The larvae of Anopheles which practically nourish themselves at water surface will not have time to introduce a sufficient quantity of crystals before their sedimentation. The low level of mortality recorded on the level of the L4 larvae could be explained by the fact why they are nourished little at the beginning of the pupal stage (stage where the metamorphosis at the adult stage occurs). The nymphs are completely insensitive to the crystals of *Bacillus thuringiensis var. israelensis* because they do not need to be fed on.

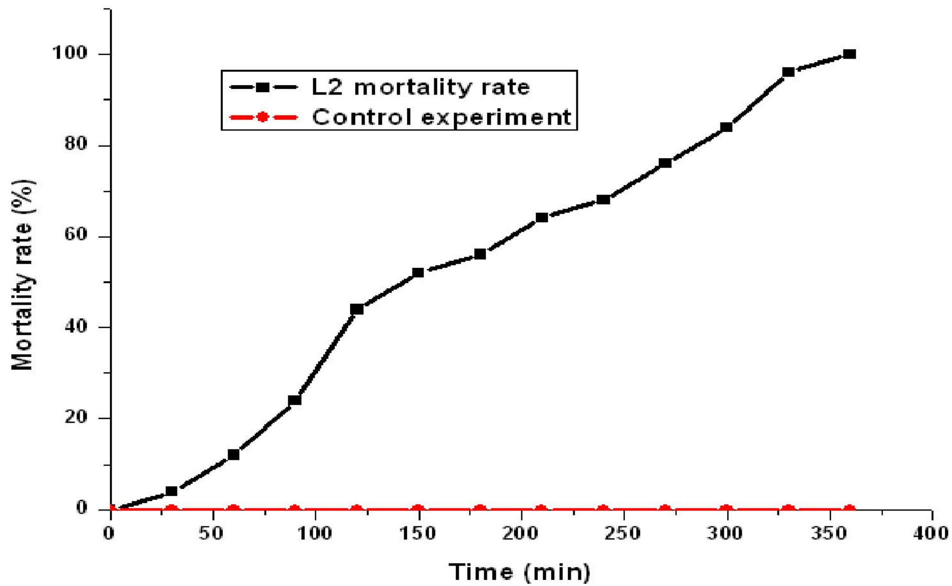

Figure1: Effect of *Bacillus thuringiensis var. israelensis* on Anopheles L2 larvae

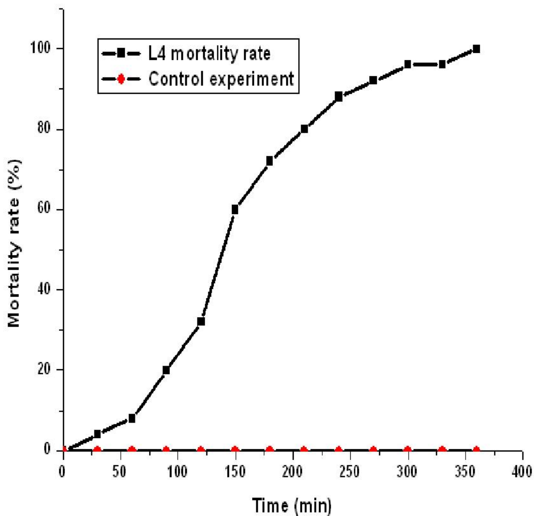

Figure2: Effect of *Bacillus thuringiensis var. israelensis* on Anopheles L4 larvae

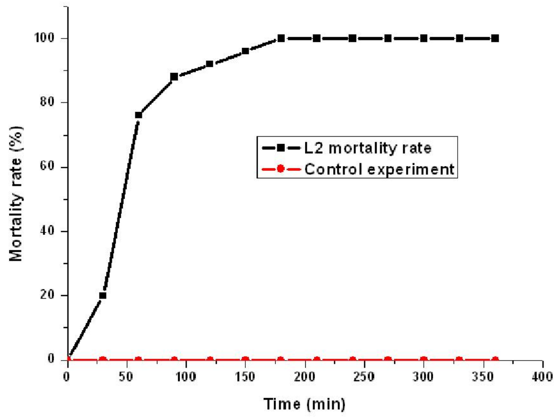

Figure3: Effect of *Bacillus thuringiensis var. israelensis* on culex L2 larvae

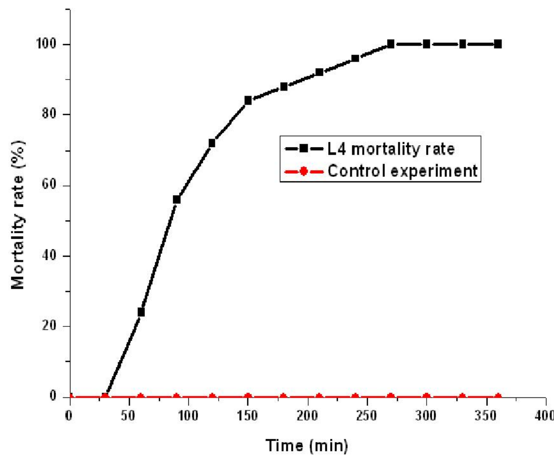

Figure4: Effect of *Bacillus thuringiensis var. israelensis* on culex L4 larvae

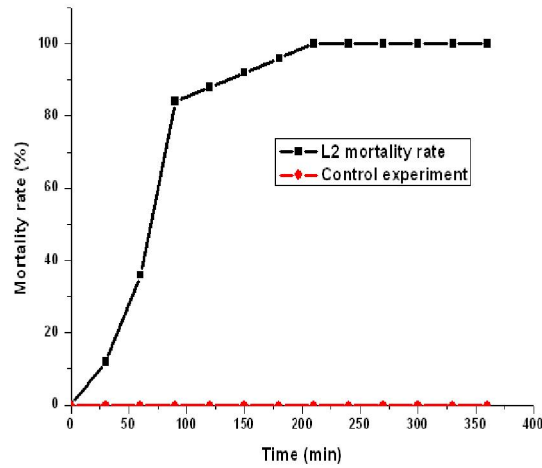

Figure5: Effect of *Bacillus thuringiensis var. israelensis* on Aedes L2 larvae

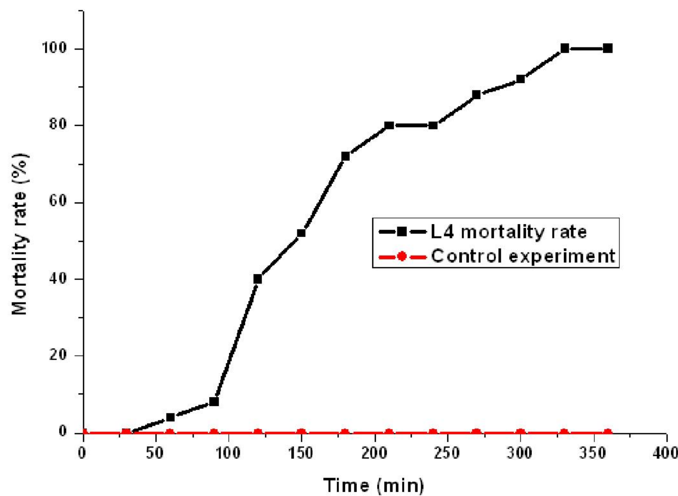

Figure6: Effect of *Bacillus thuringiensis var. israelensis* on Aedes L4 larvae

4- Conclusion

Since its commercial arrival in the early 1980's, *Bacillus thuringiensis var. israelensis* has been considered as an environmentally safe biopesticide for the control of mosquitoes and black flies. Compared to chemicals, the high degree of specificity, the low impact on non-target organisms and the short persistence have meant that *Bacillus thuringiensis var. israelensis* formulations are now useful in malaria vector control.

A promising future of this microbial control agent in mosquito control programs is ensured by its high efficacy, its specificity, its feasibility to be fermented on an industrial scale, its long shelf-life, its transportability, and finally and maybe the most important, there is actually no known field resistance documented until today.

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Determination Of Thermal Stability Of Oral Polio Vaccine (Opv) At Different Temperature Under Laboratory Conditions

Muhammad T[1], SS Baba[2], LT Zaria[2], AD El-Yuguda[2] And IB Thilza[3],

[1]who National Polio Laboratory University Of Maiduguri Teaching Hospital.
[2]department Of Veterinary Microbiology And Parasitology University Of Maiduguri.
[3]department Of Veterinary Medicine, University Of Maiduguri.

muhammadt6@gmail.com

ABSTRACT: Expanded program on immunization is one of the strategic universally accepted methods for the control of childhood diseases which include poliomyelitis. In Nigeria both monovalent and trivalent oral polio vaccines are routinely used. Thermal stability was determined using 16 vials obtained from different storage facilities, had titres which ranged from $\log_{10}$ 6.5 to 8.4. These values still fell within the normal limits recommended by WHO as minimum accepted values (P1= $\log_{10}$6.0, P2=5.0 and P3= 5.8). It was observed that the storage facilities in all the three tier of vaccination centres had adequate power supply ranging from solar refrigerators, standby generators and the National Electricity supply. Also, polio vaccine vials have vaccine vial monitor (VVM) device which usually indicate change in color when cold-chain is not maintained. This necessitated the change of vaccine carrier when the need arose during the house to house immunization exercise. Adequate potency obtained in this study confirmed ideal storage condition of vaccines in Maiduguri. [Stem Cell. 2010;1(1):69-73] (ISSN 1545-4570).

Keywords: Oral Polio Vaccine; Thermal Stability; Storage Facilities

INTRODUCTION

In May 1988, the 41st World Health Assembly committed the Member States of the World Health Organization (WHO) to the global eradication of poliomyelitis by the year 2000 (4). The resolution specified that the polio eradication initiative should be pursued in ways that would strengthen the Expanded Programme on Immunization (EPI). National Immunization Days (NIDS) were initiated in a number of developing countries including Nigeria to accelerate polio eradication strategies. In recent years, Nigeria has contributed over 90% of the polio cases reported globally despite immunization campaign. In developing countries, immunity induced following oral polio vaccine (OPV) is very low, about 30%. Failure of OPV has risen from 5% in 1960s to an alarming 30% currently. The factors for vaccine failure may be due to interference by antibodies in breast milk, presence of nonpolio enteroviruses preventing colonization by the vaccine virus strains, helminthic infestation or presence of non-specific inhibitors in saliva of infants. Decreased potency of the vaccine, break in reverse cold chain and thermal stability could be another probable reason which needs to be investigated (5).

MATERIAL AND METHODS

The potency of live oral poliomyelitis vaccine (OPV), both total virus content and individual serotypes separately, is determined in an *in vitro* assay using L20B cell line. The preparation to be assayed and the reference preparation are diluted in 2% MM.Tenfold dilution steps of the virus suspensions was initially made,but the dilution range selected should encompass at least three dilutions that will infect between 0% and 100% of the cultures inoculated.

The cells are examined for the presence of a specific viral cytopathic effect on days 3–5, with a final reading on days 5–7. The observations are recorded and the titer in $CCID_{50}$ per human dose calculated on the basis of the final observation.

Reference preparation

For each assay of trivalent OPV vaccine include a vial of live attenuated poliomyelitis vaccine, the titer of which has been well established, as a working reference preparation to control the accuracy and reproducibility of the testing system (validity).

Medium and dilutions

Diluent: Eagle's MEM supplemented with 2% fetal bovine serum. Using the refrigerated diluent,

prepare tenfold dilution. The subsequent dilutions for inoculation into the microtitre plates are prepared in 0.5 $\log_{10}$ steps. The range of dilutions used will depend on the type of virus and the formulation of the vaccine under test. The range chosen should include the expected titre of the vaccine type being tested.

Cells

Use L20B cells line .The passage level of these cells, which should be within 15 passages of the tested stock. Watch for any change in growth characteristics such as excess acidity of medium or slowing in the time taken to achieve a complete monolayer.

The number of cells used in the assay is usually about 1–2 x 10^5 cells per ml of test medium. This concentration should provide a confluent monolayer in microtitre plate wells within two to three days.

RESULTS

Table 1. Thermal comparism of Trivalent Oral Polio Vaccine (TOPV) titre at different storage facilities

Temperatures (^oC)	Storage facilities		
	FG	St	L.G
25	8.26±0.01	8.20±0.28	8.15±0.00
30	8.24±0.01	8.20±0.14	8.15±0.00
35	8.17±0.03^a	8.00±0.14^a	8.00±0.14^a
40	8.06±0.01^a	7.80±0.00^a	7.09±0.01^a
45	7.85±0.07^a	7.80±0.00	7.65±0.00^a
50	7.65±0.07	7.55±0.07	7.55±0.07
55	7.25±0.07^a	7.20±0.00	7.03±0.05^a
60	7.20±0.00^a	7.05±0.00	6.90±0.00^a

Values with the same superscript differ significantly at P 0.05

OPV- Oral Polio Vaccine

FG- Federal Government

St- State Government

LG- Local Government

SD- Standard Deviations

Table 2. Thermal comparism of Monovalent 1 Oral Polio Vaccine (MOPV-1) titre at different storage facilities.

Temperatures (oC)	Storage facilities		
	FG	St	LG
25	8.30±0.42	8.20±0.00	7.75±0.00
30	8.30±0.28 ^{a}	7.75±0.00	7.09±0.01 ^{a}
35	8.10±0.00^{a}	8.00±0.00^{a}	7.73±0.01^{a}
40	7.90±0.00 $^{a, b}$	7.09±0.01 ^{a}	7.00±0.14 ^{b}
45	7.80±0.14 $^{a, b}$	7.08±0.03 ^{a}	6.80±0.14 ^{b}
50	7.83±0.50	7.08±0.04	6.50±0.14
55	7.28±0.37 ^{a}	7.08±0.03 ^{b}	6.40±0.16 $^{a, b}$
60	7.13±0.39 ^{a}	7.04±0.05 ^{b}	6.25±0.30 $^{a, b}$

Values with the same superscript differ significantly at P 0.05

Table 3. Thermal comparism of Monovalent 3 Oral Polio Vaccine (MOPV-3) titre at different storage facilities.

Temperatures (^{o}C)	Storage facilities		
	FG	St	L.G
25	8.40±0.00	8.25±0.35	7.90±0.00
30	8.40±0.00 ^{b}	8.25±0.07^{a}	7.90±0.00$^{a, b}$
35	8.20±0.00 ^{b}	8.10±0.00 ^{a}	7.75±0.07 $^{a, b}$
40	8.05±0.07^{b}	8.04±0.07 ^{a}	7.51±0.00 $^{a, b}$
45	7.90±0.00 ^{a}	7.86±0.00 ^{b}	7.30±0.14 $^{a, b}$
50	7.70±0.14 ^{a}	7.66±0.00 ^{b}	7.10±0.14 ^{b}
55	7.30±0.14	7.25±0.35	6.90±0.00
60	7.00±0.14	6.80±0.14	6.50±0.00

Values with the same superscript differ significantly at P 0.05 across the different storage facilities.

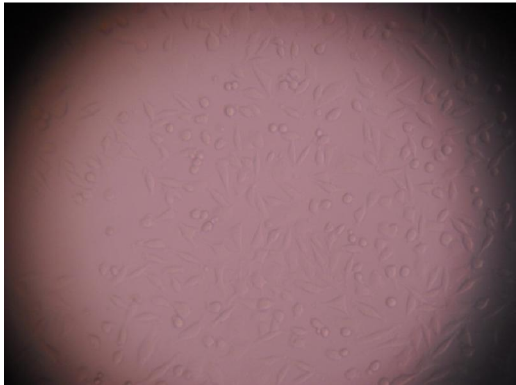

Fig. 1: Negative control L20B cell line

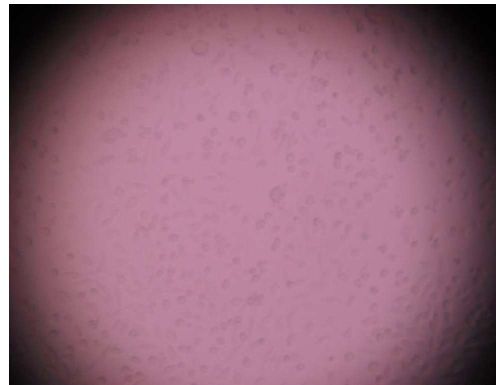

Fig. 2**:** Showing 50% CPE of polio virus on L20B cell line

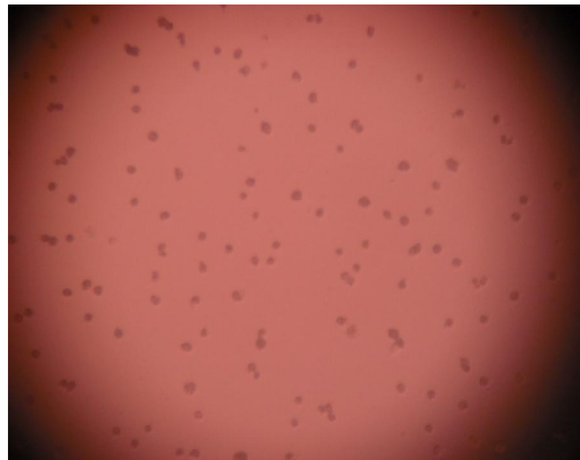

Fig. 3**:** Showing 100% CPE of polio virus on L20B cell line

DISCUSSION

An additional factor in the adequate stability of vaccine samples in this study was the thermo-stabilization with 1M magnesium chloride [3]. In the early 1960s, it was found that the infectivity of enterovirus could be preserved even when they were heated at 50°C if molar MgCl2 was added, a property that is used in the field where stabilised vaccines are used effectively to halt outbreaks of polio. In laboratory, the vaccines show so little loss in virus titre after long term storage at -20°C that the predicted half-life was calculated to be 92 years [3]. It was also noted that vaccine stabilized with MgCl2 suffered no significant loss of potency, after as many as nine cycles of alternate warm and cold conditions [2]. Thermostability requirements were defined by WHO as OPV that loses less than 0.5 log 10 of titre of each of the vaccine strain after exposure to 37°C for 2 days [6]. But current regulations require that for maintenance of potency, the vaccine must be stored and shipped frozen and that after thawing, it must be stored in the refrigerator at not more than 10°C for a period not exceeding 30 days after which time it must be discarded [6].

In a study of stabilizing OPV at high temperature at WHO, there was a general consensus that a vaccine capable of withstanding 45°C for seven days with less than 0.5 $TCID_{50}$ per dose. Reduction of the potency of each of the three serotypes, will offer substantial benefit to global eradication effort (1). In this study, though a significant drop in titer was observed when subjected to varying

temperatures; the minimum titer obtained is still above the minimum stipulated cut-off titer recommended by WHO $\log_{10}(6.0)$. Thus, all the vaccine has been stabilized with 1M magnesium chloride which helps to stabilize the vaccine at high temperature 50°C.

CONCLUSION

In this study, we did not titrate individual serotype in trivalent vaccine. This would have given information on thermal stability of individual vaccine strain i.e. type-1, type-2 and type-3. But as the total titre is fixed in OPV, composite titer estimation is sufficient to assess the thermal stability. Continuous monitoring of efficiency of cold chain maintenance and vaccine potency testing would contribute towards good vaccine strategy.

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