Reproducible measurements of human mesenchymal stem cells counting and proliferation in 3D scaffolds for Regenerative Medicine

C. Divieto, M.P. Sassi
Istituto Nazionale di Ricerca Metrologica INRIM
Torino, Italy
c.divieto@inrim.it

***Abstract*—Human mesenchymal stem cells are a good candidate to repair and to regenerate tissues for regenerative medicine applications. Their use in combination with 3D scaffolds has been largely studied *in vitro* to characterize their properties and differentiation potential prior to apply them *in vivo*. One of the most important clues *in vitro* is given by their proliferation trend, leading to information about their viability, their wellness, their interaction with scaffolds, etc. In order to measure the proliferation of hMSCs on scaffolds for regenerative medicine, it is important to adopt accurate counting methods in both research and diagnostic studies. This work aims to develop a reproducible method for hMSCs proliferation measurement in 3D cell cultures on coralline scaffolds (Biocoral®). Results demonstrated that: proliferation curves obtained in this work are reproducible at different initial cell densities on several scaffolds cultured with hMSC in long term experiments (3 weeks).**

Keywords—hMSCs; cell counting; cell proliferation; scaffolds; regenerative medicine; metabolic methods.

I. INTRODUCTION

In Regenerative Medicine treatments are based on the implant of human mesenchymal stem cells (hMSCs) seeded on three-dimensional (3D) scaffolds which mimic the 3D matrix hosting tissues and organs [1].

A certain number of cells, isolated from the patient, is seeded on 3D scaffolds, cultured in vitro and then cells and scaffolds are implanted in the receiver tissue or organ of the patient. The number of cells represents the cell dose required to treat the patient for the specific disease/disorder.

The correlation between the cell dose and the effect on the patient must be known to improve the treatment itself.

Cell proliferation (cell number over time) measurements in *in vitro* studies provide clues on the biocompatibility of the 3D culture system, its influence on the cells viability and metabolism. Importantly, by monitoring the *in vitro* proliferation several predictions about the *in vivo* behavior of cells-scaffold combination can be made.

In order to reduce the number of measurements and consequently to have only minimal manipulation and invasiveness, the possibility to have an accurate measurement method is fundamental.

The number of cells within a scaffold can be determined by several methods: by using the traditional trypsinization and cell counters; by image analysis of fluorescently labelled cells; by metabolic tests that assay metabolite activity of cultured cells, used when the number of cells is proportional to the number of cells performing that particular metabolic activity (e.g. MTT test) [2, 3].

These methods are in most cases destructive: traditional counting requires the detachment of the cells from the scaffold, MTT test or similar tests are cytotoxic or alter the cell structure, imaging is often invasive because of the need of making sections or slices of the sample and require cells staining. In any case, these methods prevent to perform a cell count over time on the same sample.

Therefore, in order to monitor over time the same cell population growing on 3D cell cultures (on scaffolds), it is necessary to employ non-destructive and non-invasive methods for cell counting.

The method chosen in this work for cell proliferation evaluation is a metabolic assay based on the reduction of resazurin into the end product resorufin. It is not cytotoxic and does not require any cell damaging steps [4], therefore it satisfies the requirement of non-destructiveness and non-invasiveness.

Biocoral® scaffolds have been chosen for this study to culture human mesenchymal stem cells over time. Biocoral® is a natural coral from exoskeleton of Madrepores. Its chemical composition is very similar to that of the human cancellous bone (98% of calcium carbonate), its high porosity ranges from 20% to 50% of the entire volume and its internal architecture is formed by a network of interconnected pores [5]. This biomaterial is fully and gradually resorbable, biocompatible and bio-functional, allowing osseointegration and growth of the new bone, followed by gradual resorption of the biomaterial itself. This makes the Biocoral a good biomaterial to support the formation of new bone [5, 6]. Biocoral osteointegration, osteoconductive and osteoinductive properties have been demonstrated by *in vitro* experimental studies and preclinical and clinical applications in neurosurgery, orthopedic surgery and dentistry in the last 20 years. [5, 6, 7]. Reproducibility and repeatability of this method on 3D cell cultures has never been tested. However, a first approach of evaluation of uncertainty of the method applied to 2D cell cultures has been published in 2012 [8].

978-1-4799-6477-2/15/$31.00 ©2015 IEEE

Aim of the presented work is to develop a repeatable and reproducible methodology to evaluate the cell proliferation in a 3D scaffolds overtime.

II. Materials and Methods

A. 3D Cell cultures

Bone marrow derived-hMSCs from donor were purchased from Lonza Group Ltd (Basel, Switzerland). hMSCs were expanded and maintained in non-differentiating cell growth aMEM (alpha modified Minimum Essential Medium - Lonza, Wokingham, UK) with FBS 10% v/v (Fetal Bovine Serum, Lonza, Wokingham, UK), L-glutammine 2mM, penicillin 0,06 mg/ml and 0.1 mg/ml streptomycin (Sigma-Aldrich) in traditional Petri dishes in incubator at 37°C with 5% CO2. Fresh medium was replaced every 3-4 days until cells reached about the 80% of confluence.

At the experimental day 0, cells at passage 7 were washed once with Phosphate Buffer Saline (PBS), detached with 0.25% Trypsin – 0.53mM EDTA solution, counted by means of a hemocytometer and suspended at several concentrations (cells/ml) in 100 µl non-differentiating aMEM growth medium and then seeded on Biocoral scaffolds previously placed in 24-well plates. Scaffolds with cells were incubated for two hours at 37°C in a 5% CO_2 atmosphere before adding the growth medium to a final volume of 1 ml and starting the static culture.

hMSCs were cultured on 3 scaffolds for 10 days and on 9 scaffolds for 21 days, every 2-3 days growth medium was refreshed and the cell proliferation assay was performed at several time points.

B. Biocoral® scaffolds

Biocoral® scaffolds (Inoteb, LeGuernol, Saint-Gonnery, France) used in this study, have truncated cone shaped with 9 mm in minor diameter, 11 mm in major diameter and 5 mm in height as shown in fig. 1A (digital camera acquisition). Optical microscope (Olympus BX51WI equipped with Objective: Olympus UIS2 Series UPLSAPO ×40; Digital camera: Olympus Digital B/W Camera XM10 Software: Olympus CellSens Dimension 1.4.1) was used to image the Biocoral® internal pores (fig. 1C) and scanning electron microscopies (fig. 1B and 1D) were used to image the Biocoral® surface and pore details.

C. Method for cell proliferation measurement

The resazurin assay is a fluorimetric and metabolic assay used for determining the number of living cells in a biological in vitro system. It is based on a redox reaction made only by living cells: cytoplasmic redox enzymes reduce the resazurin (dark blue in color, redox dye with a slight intrinsic fluorescence) into the end product resorufin (pink, highly fluorescent molecule, excitation wavelength of 579 nm and emission maximum at the wavelength of 584 nm). Resazurin solution is added to the 3D cell culture in the cell culture medium at 10% in volume. The resaruzin molecule can penetrate cells by passing the cell membrane and into the cytoplasm is reduced by cytosolic, microsomal and mitochondrial redox enzymes producing the fluorescent resorufin (reaction product). Resorufin diffused out of cells in the cell culture is considered proportional to the total metabolic activity of cells. The metabolic activity measurement is obtained by measuring the fluorescence intensity of the metabolic product (resorufin) over time. Fluorescence intensity were measured with the GloMax®-Multi Microplate Multimode Reader (Promega Corporation, USA). Excitation was performed at 525 nm wavelength while emission was measured in the range 580-640 nm. For each scaffold the volume on which the fluorescence measurement is performed is 0.1 ml with an uncertainty due to the pipetting error set as 1% obtained quadratically composing the experimental repeatability and the coefficient of variation according to the manufacturer.

To correlate fluorescence intensity to cell number, scaffold seeded at day 0 were analyzed. Calibration curves were made by seeding a serial number of cells on a series of Biocoral® scaffolds and measuring the emitted fluorescence intensity (see results in fig. 3).

D. Cell viability test

Cells seeded on scaffolds were fluorescently stained with Calcein AM (Invitrogen), a vital staining specific for living cells. Each scaffold was washes twice with PBS and incubated for 30 minutes at 37°C with 1 ml of Calcein AM 4 µM. Biocoral scaffolds were then imaged with a confocal microscope (Carl Zeiss Laser Scanning System LSM 510, Germany) to test cells adhesion and viability within the Biocoral® scaffolds.

III. Results and Discussion:

A. Characterization of Biocorals®

Scanning electron microscopy (Fig.1 B and D) and optical microscopy (C) imaging show the porous structure of biocorals. In B it is shown the seeding surface where cells are deposited. C internal pores are imaged. In D a single pore entrance is imaged showing the roughness of the internal surface where cells are supposed to adhere.

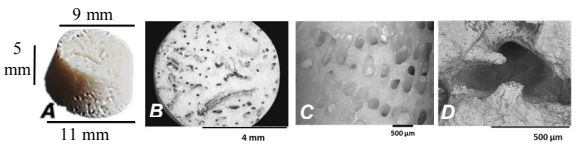

Fig. 1. Biocoral® scaffold aquired with digital camera (A) SEM (B and D) and optical microscopy (C).

A measurement of the pore size by optical microscopy revealed a pore size distribution ranging from 100 and 700 µm (fig.2). A calibrated XY linescale was used to obtain the pixel dimension. An image of the scale, in which a square of at least 300 µm × 300 µm was visualized, was taken. The analysis of the acquired image of the calibrated XY linescale allowed the definition of the pixel dimension. Mean diameter of pores (counts = 40) has been measured by comparing the length of the segment diameter of pores with the dimension of pixels.

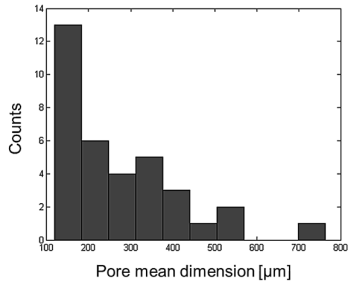

Fig. 2. Pore distribution of a representative portion of Biocoral® scaffolds.

B. Method linearity and resolution on 3D cell cultures

Calibration curves were made as described in section II C. The correlation between the fluorescence intensity and the cell number, was found linear in the range between 5×10^3 to 4.0×10^5 cells. The correlation coefficient was 0.97, as shown in fig. 3.

The uncertainty of cell seeding was 5% for each scaffold calculated quadratically composing the manual counts experimental repeatability and reproducibility. The uncertainty of fluorescence measurement was calculated quadratically composing the measurement uncertainty (3.2% according to a previous study [8]) and the experimental repeatability (5%) and was found to be 5.9%.

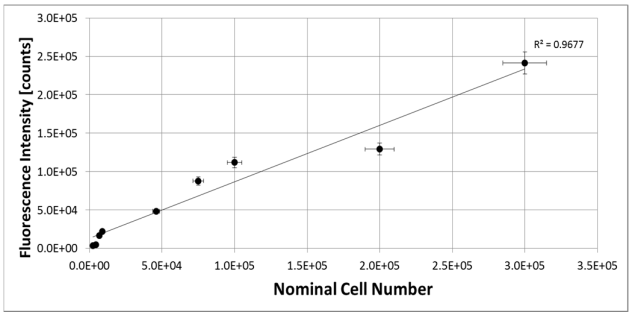

Fig. 3. Fluorescence intensity measurement of resazurin metabolized by nominal cells seeded on biocorals at day 0.

As shown by fig. 4, in the experimental conditions, the method for cell proliferation measurement was not able to distinguish between 2.5×10^3 (scaffold A) and 5.0×10^3 cells (scaffold B) seeded after manual counting and measured at day 0, after 24h of adhesion. The resolution of 5×10^3 cells for hMSCs on Biocorals® is enough for regenerative medicine purposes where cells are usually seeded at higher dose that 5×10^3 cells. Fig. 4 also shows that fluorescence intensity (I_f) increases over time. Error bars represent the uncertainty of the measurement calculated quadratically composing the fluorescence intensity measurement uncertainty (3.2% as above mentioned) and the experimental repeatability on 3 repeated fluorescence intensity measurements for each scaffold (mean repeatability was found 5.2% between scaffolds A, B, C). Repeatability of the method is better described in the next paragraph.

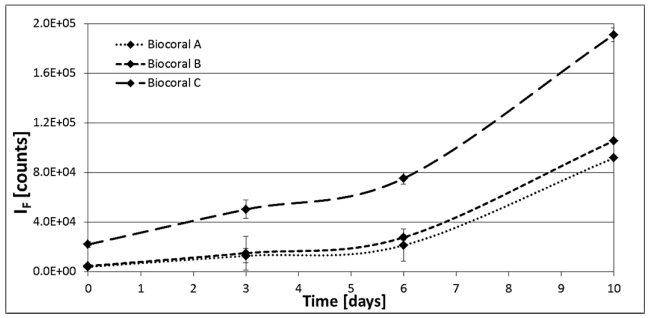

Fig. 4. Fluorescence measurement (I_f), at different time points, on 3 biocorals (A, B, C seeded with 2.5, 5.0 and 10.0×10^3 cells respectively, at day 0).

C. Measurement repeatability and reproducibility on 3D cell cultures

hMSCs were seeded on the 9 scaffolds in the same day and cultured for 21 days. Scaffolds D, E, F were seeded with 0.5×10^5 nominal cells at day 0, scaffolds G, H, I with 1.0×10^5 nominal cells at day 0 and scaffolds J, K, L with 3.0×10^5 nominal cells at day 0. The fluorescence intensity trend was followed over time as shown in figures 5, 6 and 7. Error bars represent the uncertainty of the measurement calculated quadratically composing the experimental repeatability and the experimental reproducibility.

The mean reproducibility was found to be: 10% between scaffolds D, E, F seeded with 0.5×10^5 nominal cells at day 0, 14.5% between scaffolds G, H, I seeded with 1.0×10^5 nominal cells at day 0 and 20.5% for scaffolds J, K, L seeded with 3.0×10^5 nominal cells at day 0. The mean reproducibility among the 9 scaffolds was found to be 15%.

The repeatability calculated as standard deviation of 3 repeated fluorescence intensity measurement for each day and for each scaffold, was found in the range from 0.0% to 5.2%.

By combining the data (see fig.8), it is noticeable that the fluorescence intensity of resazurin metabolized by hMSCs on Biocorals followed very similar trends over time.

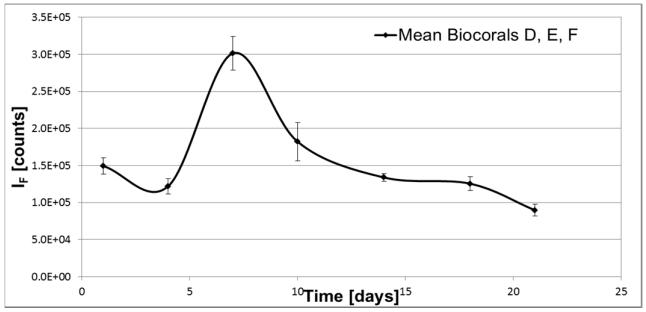

Fig. 5. Mean Fluorescence Intensity (I_f) from Biocorals D, E, F seeded at day 0 with 0.5×10^5 hMSCs at passage 7.

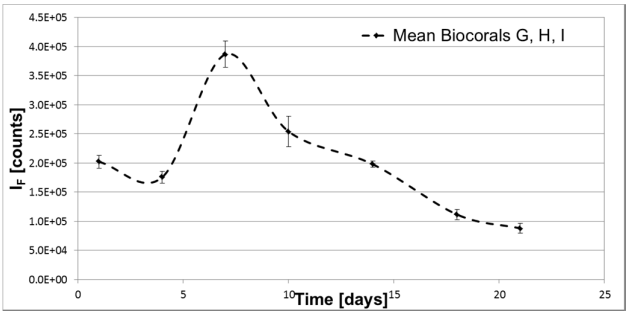

Fig. 6. Mean Fluorescence Intensity (I_f) from Biocorals G, H, I seeded at day 0 with 1.0×10^5 hMSCs at passage 7.

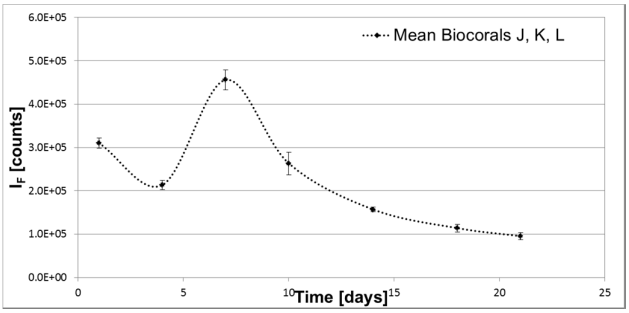

Fig. 7. Mean Fluorescence Intensity (I_f) from Biocorals J, K, L seeded at day 0 with 3.0×10^5 hMSCs at passage 7.

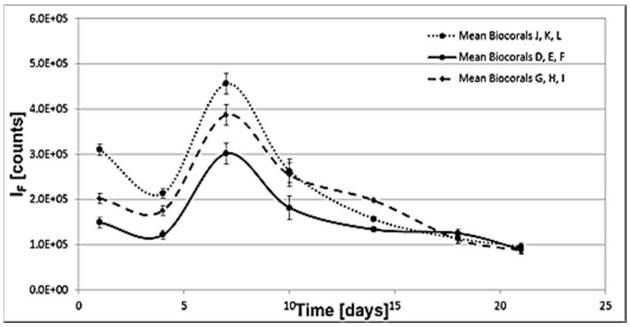

Fig. 8. Measurements of the mean fluorescence intensity (I_f), at different time points, on 3 series of biocorals. Biocorals D, E, F were seeded with 0.5×10^5 nominal hMSCs at day 0; biocorals G, H, I were seeded with 1.0×10^5 at day 0; biocorals J, K, L were seeded with 3.0×10^5 at day 0. Error bars represent the uncertainty of the measurement calculated quadratically composing the experimental repeatability and the experimental reproducibility.

D. Cell proliferation curves on Biocorals®

The calibration curve, shown in fig. 2, was used to compute the cell proliferation curves on biocorals over time obtained by plotting the calculated cell number versus time (days). The cells were seeded at day 0 in the range of 2.5×10^3 and 3.0×10^5 cells/biocoral and their proliferation was followed over time. As an example of proliferation curves, fig 9 shows what happened when the difference in the initial cell number (cell dose) is 5 times: proliferation in biocoral C, seeded at 10×10^3, is gradual and continuous, whereas the proliferation in biocorals D, E, F (mean proliferation is shown), is fluctuating over time. In addition, after day 7, in scaffold from D, E and F cells seem to gradually decrease their number until day 21. At day 10 they reach the same number that was found in scaffold C, seeded with a 5 times lower number of cells.

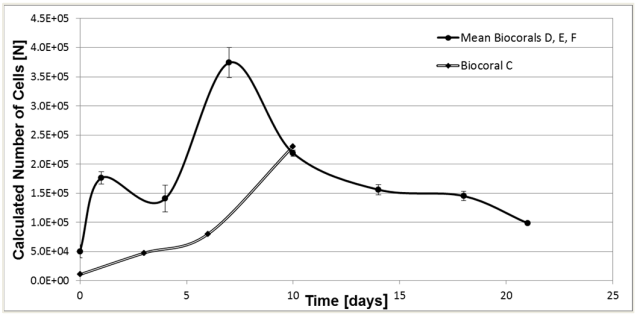

Fig. 9. Cell proliferation curves on Biocorals. C is seeded with 10.0×10^3 nominal cells (hMSCs) at day 0 and D, E, F (their mean curve is showed) with 50.0×10^3 nominal cells (hMSCs) at day 0.

E. hMSCs viability assessment

After cell proliferation assessment by resazurin, cell viability was evaluated by staining with Calcein AM: only living cells are stained in green (fig 10). In fig. 10 are shown cells on the external surface of the scaffold (A), delineating the scaffold confine (×5 magnification); in fig.10B cells within the scaffold (×20) and in fig.10C it is visible one pore containing three cells (×60).

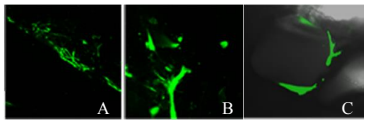

Fig. 10. Confocal microscopy images of hMSCs on Biocorals®. In green are shown hMSCs after 21 days of 3D cell culture at ×5, ×20 and ×60 magnification.

Biocoral® allowed hMSCs seeding, colonization and proliferation and did not interfere with cellular viability

IV. DISCUSSION

By merging the proliferation curves on biocorals A, B, C ending at day 10 and (as mean of 3 scaffolds) on biocorals D-E-F, G-H-I, J-K-L ending at day 21 (in fig.9 it is shown an example with scaffolds C and mean of D-E-F), several consideration can be made. Results show that when cells are seeded at higher densities (number of cells/adhesion surface), their proliferation fluctuate over time. Whereas cells seeded at lower densities grow gradually but continuously. Our hypothesis is that cells at high density within the biocoral pores are strongly influenced from the internal porous structure of the scaffold. When the cell density is high (presumably more than 1.0×10^3/adhesion surface), cells are able to fill the pores space rapidly. Consequently, they decrease the proliferation rate due

to cell contact growth inhibition. After some days, presumably the time necessary to colonize a new pore, cells are again able to proliferate and so on. To support this hypothesis is a consideration about the cells and pore dimensions: hMSCs cells dimensions are typically of 50-100 μm in diameter when are adhering to a substrate, pores size is in the range of 100-700 μm. Few cells are needed to fill a pore (see also fig.10C).

After day 7, cells seeded at high density reach the highest number in culture and then decrease their proliferation. To test that cell are still alive within the scaffolds after 21 day, we performed the viability test with calcein AM, demonstrating that decrease in cell proliferation is not due to cell death.

Our hypothesis is that cells are filling all the pores within the biocoral and reducing their proliferation rate.

V. CONCLUSION

The method here proposed for 3D cell proliferation monitoring in a non-invasive way, presents a good repeatability and reproducibility. This is strongly reinforced if considering the intrinsic variability of this complex system of 3D cell cultures: the Biocoral® has a not reproducible internal structure which is formed by "naturally made" interconnected pores and canals.

This method can be used to evaluate the cell proliferation on the same 3D cell culture system over time allowing to deduce several considerations about the cell-scaffold interactions without perturbing the system. As an example, it is possible to set the cell number to be seeded in order to modulate the proliferation rate and reach the desired cell density within the scaffold over time. In this context, the method could be a good support for the cell dose determination in regenerative medicine applications. However, this study represent a first approach toward the development of a reproducible method for 3D cell proliferation measurement and need to be improved and supported by more data. Further works are in progress to evaluate the reproducibility of this method on a larger number of scaffolds.

REFERENCES

[1] F. Pampaloni, E.G.Reynaud and E.H.K.Stelzer, "The third dimension bridges the gap between cell culture and live tissue" Nature Reviews Molecular Cell Biology, vol.8, pp.839-845, October 2007.

[2] R.J Gillies, N.Didier and M.Denton "Determination of cell number in monolayer cultures", Analytical Biochemistry, vol.159, pp.109-113, November 1986.

[3] N.J.Marshall, C.J.Goodwin and S.J.Holt "A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function", Growth Regulation, vol.5, pp.69-84, June 1995.

[4] J.O'Brien, I.Wilson, T.Orton, F.Pognan "Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity", European Journal of Biochemistry, vol.267, pp.5421-5426, September 2000.

[5] C.Mangano, F.Paino, R.d'Aquino, A.De Rosa, G.Iezzi, A. Piattelli, L.Laino, T.Mitsiadis, V.Desiderio, F.Mangano, G.Papaccio, V.Tirino "Human Dental Pulp Stem Cells Hook into Biocoral Scaffold Forming an Engineered Biocomplex", PLoSONE 6, 4, April 2011.

[6] T. Gao, T.K. Tuominen, T.S. Lindholm, B. Kommonen, T.C. Lindholm "Morphological and biomechanical difference in healing in segmental tibial defects implanted with Biocoral® or tricalcium phosphate cylinders", Biomaterials,vol.18, pp.219-223, February 1997.

[7] A.Giuliani, A. Manescu, E.Larsson, G.Tromba, G.Luongo, A.Piattelli, F.Mangano, G.Iezzi, C.Mangano. "In Vivo Regenerative Properties of Coralline-Derived (Biocoral) Scaffold Grafts in Human Maxillary Defects: Demonstrative and Comparative Study with Beta-Tricalcium Phosphate and Biphasic Calcium Phosphate by Synchrotron Radiation X-Ray Microtomography", Clinical Implant Dentistry and Related Research, vol.16, pp. 736-750, October 2014.

[8] C. Divieto, G. Sassi and M.P.Sassi "Uncertainty analysis of cell counting by metabolic assays" 20th IMEKO World Congress 2012, 2, pp. 1190-1193, 2012.