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Studies regarding the mechanisms involved in tissue regeneration, due to application of biomaterials obtained via biotechnology

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ABSTRACT

The processes of regeneration and repair tissues that occur under action of any substance are based on the effects determinated by this substance on some soluble mediators (cytokines, growth factors, pro-/anti-inflammatory agents) and on the macrophages involved in process. In this respect in this paper we will present an *in vitro* study of three kinds of biomaterials (P1, P2 and P3) obtained by submerged biosynthesis. These materials were synthesised using three strains of *Monascus sp*. The biocompatibility studies, performed with the three biomaterials on the adherent cells lines, type L929 murine fibroblast or RAW 264.7 murine macrophage, showed that only the biomaterial P1 is biocompatible. In the presence of biomaterial P2 or P3, the cell viability is under 70% and it is not depending on their concentration, meaning that these biomaterials are not biocompatible. In order to evaluate P1 biomaterial capacity to activate secretion of mediators involved in the process of tissue repair, for this biomaterial was determined the ability of inducing the secretion of VEGF (vascular endothelial growth factor) for the human monocyte THP-1 cells line. The results obtained *in vitro* have been confirmed that in the presence of biomaterial P1, the macrophages THP-1 adopt a profile associated with accelerating the process of tissue repair characterized by the absence of the secretion of pro-inflammatory cytokines (TNF-α, IL-6) respectively by the secretion of anti-inflammatory cytokines (IL-1RA).

KEYWORDS

regeneration tissues; human monocyte THP1

Introduction

The processes of regeneration and repair tissues that occur under action of any substance, are based on the effects on some soluble mediators (cytokines, growth factors, pro-/anti-inflammatory agents) and on the macrophages involved in process. The macrophage is an integral part of innate and adaptive immunity, and it is composed of two subtypes that are either pro-inflammatory or anti-inflammatory [1–2]. The classically activated or M1 macrophage phenotype, produces inflammatory cytokines, like tumor necrosis factor (named TNFα) [3–8]. The process of wound healing, involves the laying down of a cellular fibrous tissue to replace the region of lost cells. Initially, the formation of the new blood vessels is critical

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Table 1. Major steps in wound healing and their associated grown factors [6].

Factors	EGF	FGF	KGF	PDGF	TGF-α	TGF-β	TNF-α	VEGF
Fibroblast migration		X		X		X		
Fibroblast proliferation	X	X		X	X		X	
Monocyte migration		X		X		X	X	
Macrophage activation							X	
Epithelial migration	X	X	X		X			
Epithelial proliferation	X	X	X		X			
Angiogenesis		X		X	X		X	X
Collagen synthesis				X		X		
Collagenase synthesis	X	X		X			X	X
Wound contraction		X		X				

EGF epidermal growth factor;
FGF fibroblast growth factor;
KGF keratinocyte growth factor;
PDGF platelet-derived growth factor;
TGF transforming growth factor;
TNF tumor necrosis factor;
VEGF vascular endothelial growth factor.

in the transport of nutrients and cells to the new tissue, but after a certain time, they recede with the fibroblasts, leaving a collagenous scar that is remodeled and strengthened over time. Macrophages are essential directors of this process, secreting the growth factors, that entice and stimulate fibroblasts, endothelial precursor cells and (in skin wounds) the keratinocytes. These also oversee the deposition and remodeling of extracellular matrix [6–10].

Growth factors are specialized polypeptide molecules that bind to receptors on target cells and deliver messages regarding migration, proliferation, differentiation, survival and secretion. (Table 1).

Vascular Endothelial Growth Factors (VEGF) are a family of homodimeric proteins. VEGF is a potent inducer of blood vessel formation in early development (vasculogenesis) and has a central role in the growth of new blood vessels (angiogenesis); it promotes angiogenesis in chronic inflammation, healing of wounds, and of the tumors.

The characteristic feature of this proliferation is the formation of a substance called granulation tissue. The macrophages, multitasking cells derived from monocytes, replace the neutrophils, that are numerous, immediate after the wounding. A primary function of macrophages is the ingestion of unwanted materials: bacteria, cell remnants, debris, fibrin and foreign material. Macrophages also serve as directors for many other parts. These stimulate fibroblasts and keratinocytes (through release of PDGF, TGF-β, TNF, IL-1 and KGF), stimulate angiogenesis (by secretion of VEGF, FGF and PDGF), redirect and remodeling the extracellular matrix [6]. Growth factors, cytokines and chemokines are crucial for coordinating multiple cell types during the healing process, making cutaneous wound healing possible.

Collective findings from a wide range of the cytokine investigations, indicate that the net effect of the inflammatory response is determined by a delicate balance between pro- and anti-inflammatory cytokines [8]. Perturbations of this equilibrium, can drive the host defence immune response, either towards chronic inflammation or towards healing [8]. To date, various anti-inflammatory cytokines have been acknowledged in literature and these include IL-1RA (Interleukin-1 Receptor Antagonis), IL-4, IL-6, IL-10, IL-11, IL-13, TGF-β.

Extensive molecular research has established the central biological role of IL-1RA, as a highly competitive antagonist of its functional proinflammatory ligands, IL-1α and IL-1β.

For many years, these isoforms of the IL-1 cytokine family, have been recognised to participate in initiating and amplifying inflammation, upon induced tissue injury and infection.

Table 2. Major growth factors and cytokines that participate in wound healing with cell types and thei respective roles in both acute and chronic wounds are listed [7].

Growth Factors	Cells	Acute Wound	Function
EGF	Platelets Macrophages Fibroblasts	Increased levels	Reepithelialization
FGF-2	Keratinocytes Mast Cells Fibroblasts Endothelial cells Smooth muscle cells Chondrocytes	Increased levels	Granulation tissue formation Reepithelialization Matrix formation and remodeling
TGF-β	Platelets Keratinocytes Macrophages Lymphocytes Fibroblasts	Increased levels	Inflammation Granulation tissue formation Reepithelialization Matrix formation and remodeling
PDGF	Platelets Keratinocytes Macrophages Endothelial cells Fibroblasts	Increased levels	Inflammation Granulation tissue formation Reepithelialization Matrix formation and remodeling
VEGF	Platelets Neutrophils Macrophages Endothelial cells Smooth muscle cells Fibroblasts	Increased levels	Granulation tissue formation
IL-1	Neutrophils Monocytes Macrophages Keratinocytes	Increased levels	Inflammation Reepithelialization
IL-6	Neutrophils Macrophages[2]	Increased levels	Inflammation Reepithelialization
TNF-α	Neutrophils Macrophages	Increased levels	Inflammation Reepithelialization

Amongst its various localised and systemic effects, cytokins IL-1 are known as promoter of inflammatory cell infiltration at the site of tissue injury, induce fever and vascular dilation, promote the production of NO, COX-2 and prostaglandin, and induce the production of other mediators with cytokine, such as IL-6 [8].

Proinflammatory cytokines, particularly IL-1, IL6 (interleukin-6), and TNF-α (this subclass of cytokines is referred to as "proinflammatory cytokines", due to their ability to promote the inflammation, as a response at tissue injury and infection) are up regulated during the inflammatory phase of wound healing. IL-6 is produced by neutrophils and monocytes, and is important in initiating the healing response. Much like IL-1, TNF-α can induce the production of FGF-7, suggesting that it can indirectly promote reepithelialization.

Alone, the TNF-α has been shown to inhibit the wounds re-epithelization. TNF-α, at low levels, can promote wound healing by indirectly stimulating of inflammation and by increasing the produced growth factors of the macrophage. However, at higher levels, especially for prolonged periods of time, TNF-α has a detrimental effect on healing [7–8]. The success of the process of wound healing, depends on growth factors, cytokines, and chemokines involved in a complex integration of signals, that coordinate cellular processes. In the Table 2 is presented the major growth factors and cytokines involved in wound healing.

Materials and methods

The aim of this work was to study influence of the aqueous *Monascus extract*, on the major growth factors and cytokines, which are involved in wound healing, in particular: VEGF stimulating factor, IL family, and tumor necrosis factor (TNF-α or TNF alpha). *Monascus* extract was obtained by submerged biosynthesis, using Lin's modified medium [11], and three types of *Monascus* strains: *Monascus sp.1* (P1); *Monascus sp.2* (P2); *Monascus sp.3* (P3). The three extracts obtained, was named: Biomaterial P1, biomaterial P2, Biomaterial P3. All extracts were sterilised by passing on the Millex GP filter unit of 0,22 μm. Biocompatibility of these extracts was studied *in vitro*, using two types of cells lines, respectively L929 murine

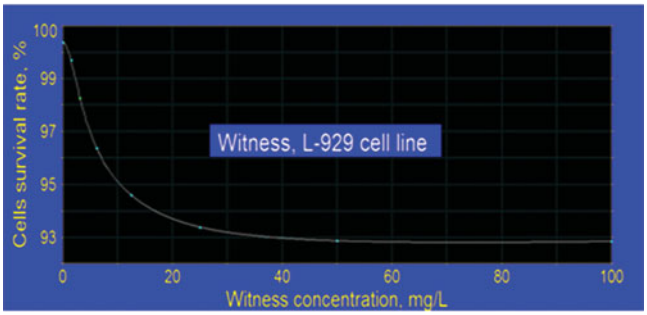

Figure 1a. Viability of L929 cells line (witness, no treated).

fibroblast cell line and RAW 264.7 murine macrophage cell line. The aqueous *Monascus* extract was added in the cells culture media, in concentrations range (0–100) $\cdot mg \cdot L^{-1}$. As the witness, was considered the cells culture media, which was supplemented with sterile water. Human macrophage cell line, type THP-1, was used to study *in vitro*, the influence of the *Monascus* extract on the process involved in wound healing. The cells of THP-1 stimulated with TNF-α (20 ng /mL), was used as positive control. In order to induce the polarization of THP-1 cells in phenotype M1, lipopolysaccharide (LPS) and interferon γ (IFN-γ) are used. THP-1 cells were treated with IFN-γ in the presence of PMA (5 ng/ml) /LPS (10 ng /ml) for 72 hours [8]. To generate phenotype M2 macrophages, THP-1 cells were treated with the PMA (Phorbol 12-Myristate 13-Acetate) in the presence of IL-4 (25 ng /mL) /IL-13 (25 ng /ml) for 72 hours [9]. At the end of incubation, the culture supernatants were collected and the cytokine profile asociated with phenotype M1 (TNF-α, IL-6) or M2 (IL-1RA) were determined using ELISA technique [10].

Results and discussion

Biocompatibility studies performed on L 929 cells lines (mouse fibroblast cell line), reveal a viability of 85%, if the cells culture media is supplemented with Bio-product P1. This percent is maintained to all P1 concentrations, and the rate of survival cells is constant (figure 1a–1b). In the case of Bio-products P2 and P3, the cells viability is less than 72%, and decrease quickly. In these cases, the survival rate is not constant (figure 1c–1d).

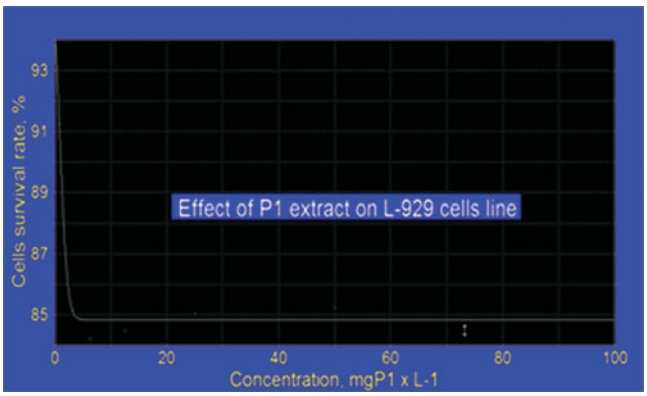

Figure 1b. Effect of Monascus P1 bioproduct regarding viability of L929 cells line.

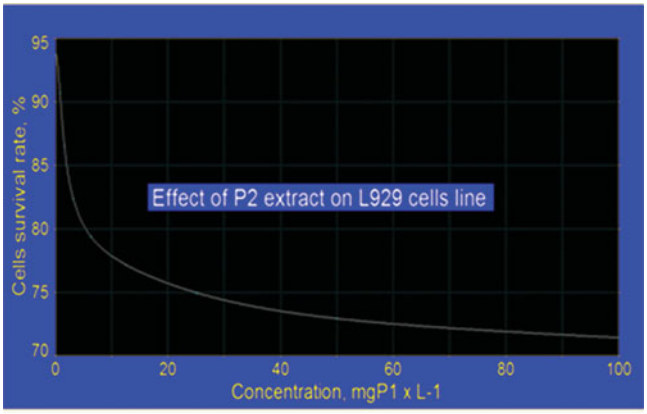

Figure 1c. Effect of Monascus P2 bioproduct regarding viability of L929 cells line.

The studies performed on RAW 264.7 cell lines (mouse monocyte macrophage) revealed the same type of viability as for witness sample (figure 2a, 2b). In this case the cell viability decreases slowly from 100% until 77%, in the range of P1 concentration of (0–100) mg L^{-1}. If the cells culture media is supplemented with bio product P2 or P3, the cells viability is 62% and respectively 70%, for concentrations higher than 10 mg $\cdot L^{-1}$ of bioproduct P2 or P3. The values are similar for concentrations of P2 and P3 of 100 mg $\cdot L^{-1}$(figure 2c, 2d).

Tacking into consideration the results obtained from biocompatibility study, it can be concluded that only bioproduct P1 is biocompatible. Thus the study was continued only with P1 Bio-product.

In order to evaluate the capacity of P1 Bio-product to activate secretion of mediators involved in the process of tissue repair, we proceeded to study the influence of different concentration of this biomaterial to growth factors of human macropahge (THP-1 cells line), like VEGF, TNF alpha, IL 6 and IL-1RA. Results obtained revealed the following:

- for concentrations of P1 between 0–20 $\mu g \cdot L^{-1}$, the VEGF secretion increase until 200 $pg \cdot mL^{-1}$ (figure 3) while the values of IL-1RA, increased from 10000 $pg \cdot mL^{-1}$ until 22000 $pg \cdot mL^{-1}$ (figure 4).
- a different behaviour was observed in case of IL-6. Thus, in the begining, the IL-6 concentration is increasing up to 70 pg mL^{-1}, with P1 concentration (up to 2.5 μg mL^{-1}).

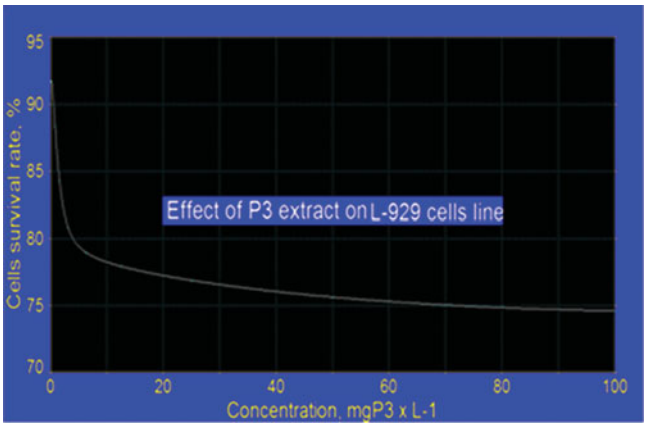

Figure 1d. Effect of Monascus P3 bioproduct regarding viability of L929 cells line.

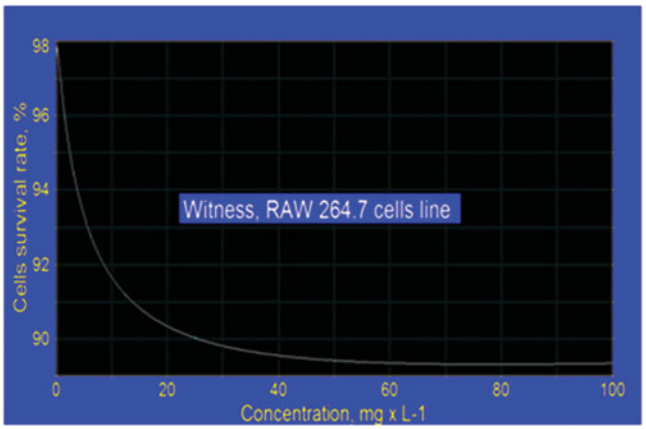

Figure 2a. Viability of RAW 246–7 cell line without treatment (witness).

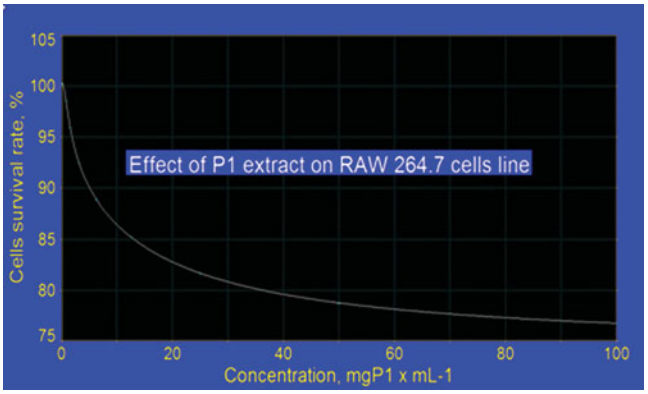

Figure 2b. Effect of P1 bioproduct regarding viability of RAW 246–7 cell line.

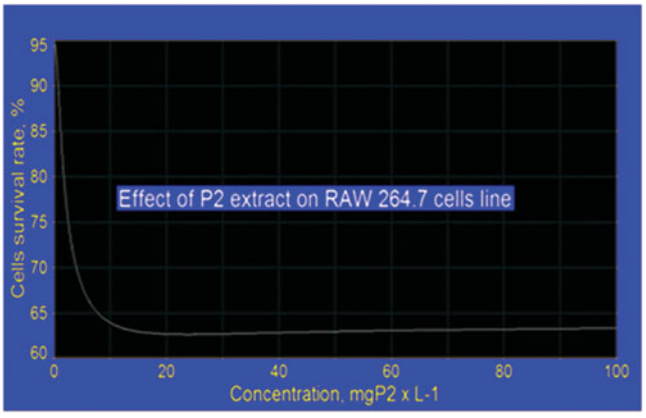

Figure 2c. Effect of P2 bioproduct regarding viability of RAW 246–7 cell line.

Increasing more the P1 concentration determine a decrease of Il – 6 concentration up to zero (figure 5).

– concerning the TNF – α secretion, from figure 6 it can be seen a fast decrease of its concentration from 400pg/mL to 125 pg/mL when P1 concentration is increasing from

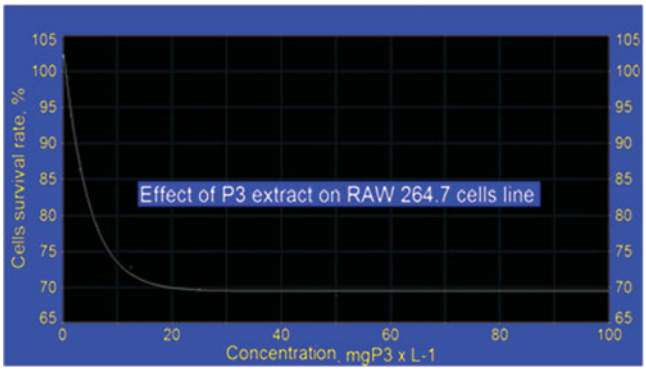

Figure 2d. Effect of P3 bioproduct regarding viability of RAW 246–7 cell line.

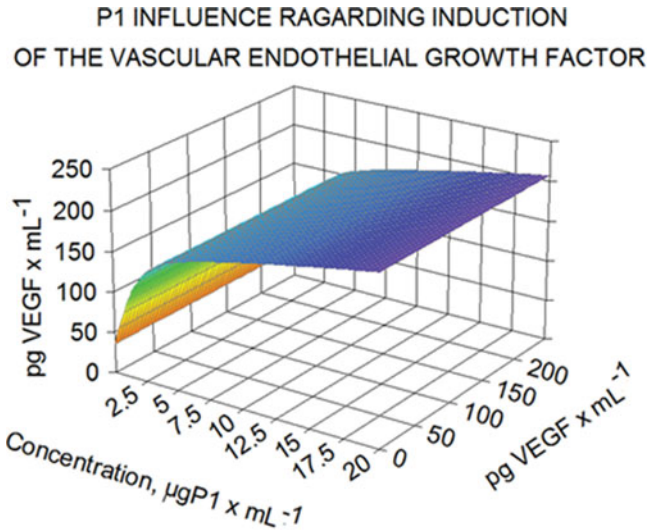

Figure 3. Ability of P1 bioproduct to induce the secretion of vascular endothelial growth factor (VEGF) for the human macrophage THP-1 cells line.

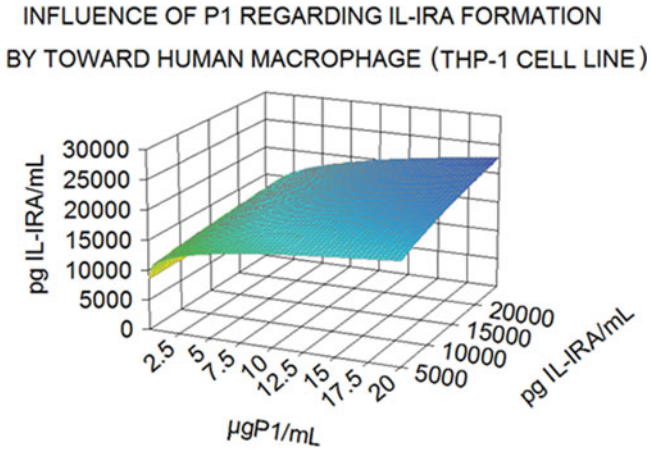

Figure 4. Effect of P1 bioproduct regarding IL-1RA formation.

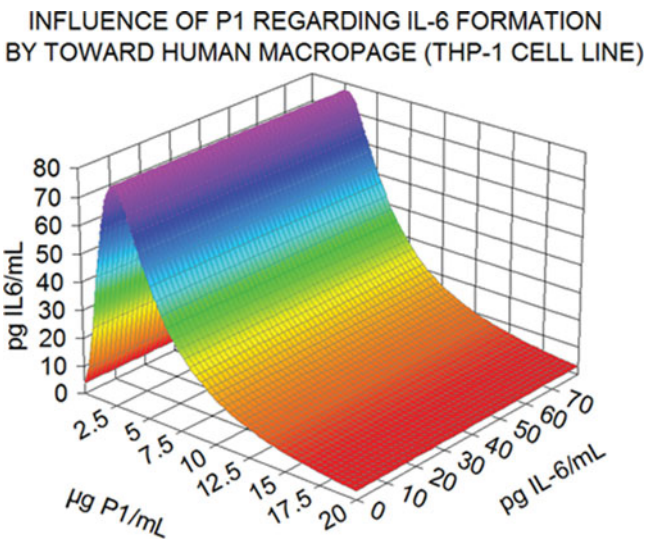

Figure 5. Influence P1 regarding IL-6 formation by toward human macrophage (THP-1 cells line).

0 to 2 μg mL^{-1}. An increase of P1 concentration had as result a slower decrease of TNF – α secretion up to zero.

The Bio-product P1, at all tested concentrations, induce the secretion of large amounts of VEGF, statistically significantly, in comparison with the basal secretion of unstimulated cells. This behavior is due to a dose-response relationship. In the presence of bio product P1, cells did not produced detectable amounts of TNF-α and IL-6 as compared to the cells differentiated in the presence of LPS /IFN; this fact suggesting that this Bio-product does not induce the macrophage polarization to an phenotype type M1. Regarding the secretion of IL-1RA, cells cultured in the presence of bio product P1, secrete higher amounts of IL-1RA, at all concentrations tested, compared with unstimulated cells, which suggests that the bio product P1, induce the macrophage polarization towards an M2 phenotype, phenotype associated with an accelerated process of tissues healing.

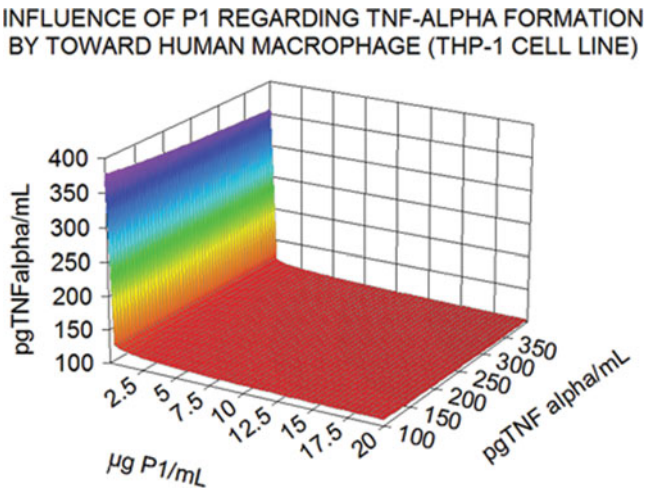

Figure 6. Influence P1 regarding TNF alpha formation by toward human macropahge (THP-1 cells line).

Conclusions

The biocompatibility studies, performed with the three biomaterials on the adherent cells lines, type L929 murine fibroblast or RAW 264.7 murine macrophage, showed that only the biomaterial P1 is biocompatible. In order to evaluate P1 biomaterial capacity to activate secretion of mediators involved in the process of tissue repair, for this biomaterial was determined the ability of inducing the secretion of VEGF for the human monocyte THP-1 cells line. The results obtained *in vitro* have been confirmed that in the presence of biomaterial P1, the macrophages THP-1 adopt a profile associated with accelerating the process of tissue repair, characterized by the absence of the secretion of pro-inflammatory cytokines (TNF-α, IL-6) and the increasing of the secretion of anti-inflammatory.cytokines (IL-1RA).

References

[1] Littlefield, M. J., Teboul, I., & Voloshyna, I. (2014). *Int. J. Immunol. Immunother.*, *1*, 1–8.
[2] Galván-Peña, S., & O'Neill L. A. (2014). *Front Immunol.*, *5*, 420.
[3] Liu, Y. C., Zou, X. B., Chai, Y. F., & Yao, Y. M. (2014). *Int. J. Biol. Sci.*, *10*, 520–529.
[4] Xu, W., Zhao, X., Daha, M. R., & van Kooten, C. (2013). *Mol. Immunol.*, *53*, 179–186.
[5] McNelis, J. C., & Olefsky, J. M. (2014). *Immunity*, *41*, 36–48.
[6] Kraft, K. P. (2010). *Organogenesis*, *6* (4), 225–233.
[7] Barrientos, S., Stojadinovic, O., & Golinko, M. S. (2008). *Wound Repair and Regeneration*, *16*(5), 585–601.
[8] Sultani, A., Stringer, A. M., & Bowen, J. M. (2012). *Chemotherapy Research and Practice*, *2012*, 11.
[9] Engström, A., Erlandsson, A., Delbro, D., & Wijkander, J. (2014). *Int. J. Oncol.*, *44* (2), 385–392.
[10] Stewart, D. A., Yinmeng, Y., & Makowski, L. (2012). *Mol. Cancer Res.*, *10* (6), 727–738.
[11] Lee B. K., Park N. H., & Piao, Y. H. (2001). *Biotechnol. Bioprocess Eng.*, *6*, 341–346.