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Polyurethane sheet impregnated with Arabinogalactan can lead to increase of attachment of promastigotes and Amastigote of *Leishmania major* (MRHO/IR/75/ER) by *GP63* and *HSP70* genes

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ABSTRACT

The aim of this study was to investigate the effect of polyurethane sheet (PUS) and polyurethane sheet impregnated with Arabinogalactan (PUSIAG) on the cell attachment and viability of Promastigotes and Amastigotes of *Leishmania major* (MRHO/IR/75/ER), and mouse macrophages, and its whole skin cells (WSCs). In a sterile condition, 10 mL of Arabinogalactan 5% *w/v* was poured into a falcon. Then, a piece of PUS was placed inside it, and incubated at 37 °C for 24 h. Next, it was washed, and cut. Then, one piece of PUS and PUSIAG was separately added to 1 mL of cell suspension (Promastigotes, Amastigote, and WSCs), and then incubated for 1, 2, 3, and 4 days at 37 °C. After incubation times, the quantity of adhered cells was counted, and cell viability was measured by MTT assay. Also, for WSCs and macrophages, the expression of *integrin, fibronectin* and *GAPDH* was investigated, and for Promastigotes and Amastigotes, the expression of *GP63*, *Cpb*, and *18s rRNA* was measured. This study showed that with increase of exposure time, the percentage of attached cells was increased. There was a significant difference between attached cells to PUSIAG and PUS in case of Promastigotes and Amastigotes. It seems that Promastigotes and Amastigotes have higher interest to PUSIAG than WSCs and Macrophages. Also, this study showed with increase of exposure time, the percentage of viable cells was decreased. There were significant differences between cell viability of Promastigotes and Amastigotes when exposed to PUSIAG and PUS, especially in long time incubation. Also, when incubation time was increased the relative expression of *integrin* and *fibronectin* in WSCs and macrophages, and *GP63* and *HSP70* in Promastigotes and Amastigotes were increased.

1. Introduction

Leishmaniasis refers to a wide range of conditions caused by the intracellular flagellate protozoan transmitted by sand fly. It has a worldwide distribution observed in > 90 countries. Cutaneous Leishmaniasis (CL) is rendered as a dominant public health problem and induces a wide spectrum of disorders from self-healing infections to several chronic diseases. Currently, there is no effective vaccine and pharmaceutical treatment available for CL. However, no new strategies have been introduced for controlling CL. Of course, some new approaches for manufacturing vaccines which contribute to the prevention of CL and immunotherapy are under way due to increased knowledge concerning the cells involved in its etiology. [1]. A few weeks after the incidence of CL ulcer, the secondary bacterial and fungal infections are superimposed on it. In the course of time, bacteria and fungi replace the parasite Amastigote in the Leishmaniasis ulcer [2]. Cysteine Peptidases play a pivotal role in the regulation of GP63 gene expression and both GP63 and CP are key factors in the virulence of CL [3]. GP63 is a useful metalloprotease which can rapidly regulate the cellular signaling pathways and hosts predisposed to their challenge [4]. GP63 is one of the most prominent superficial proteases of which > 500,000 transcriptions (replication) (% 0.5-%1 of the total cellular protein content) are expressed. Since its significant role in the innate resistance of macrophages infected with the parasite has been demonstrated, it is rendered a suitable candidate for the development of anti-Leishmaniasis vaccine [5].

Polyurethane is composed of organic units joined by urethane links. Polyurethane are traditionally formed by reacting of di- or poly-isocyanate and polyol. Mostly, this polymer has thermosetting property that does not melt when heated. Because of high chemical and physical

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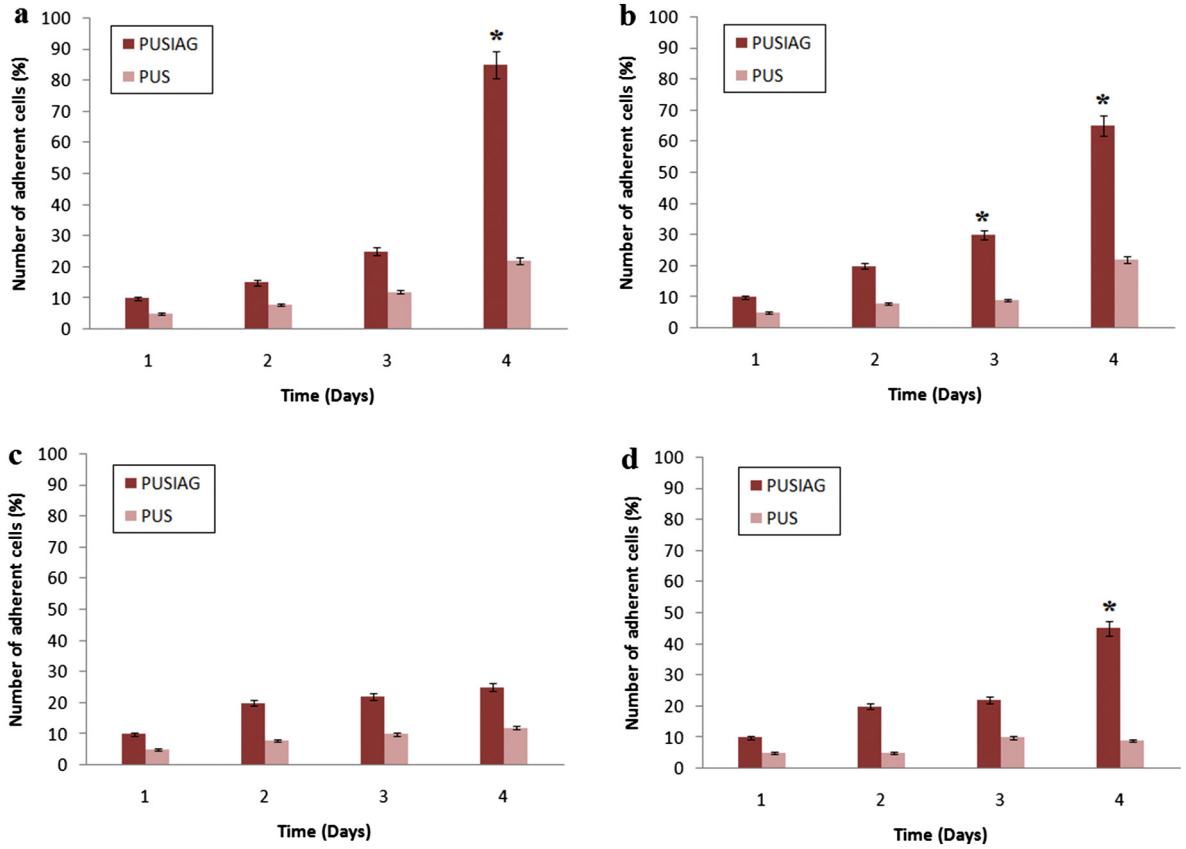

Fig. 1. The percentage of attached Amastigotes (a), Promastigotes (b), WSCs (c), and Macrophages (d) when exposed to PUSIAG and PUS. * $P < 0.05$ compared with PUS at same day, $n = 3$.

properties of polyurethane, it is used in the manufacture for different applications, e.g., high-resilience foam seating, microcellular foam seals, durable elastomeric wheels, etc. Moreover, polyurethane can be used in medical applications such as the artificial heart, intra-aortic balloons, pacemaker leads, heart valves, and hemodialysis membranes [6].

The aim of this study was to investigate the effect of polyurethane sheet (PUS) and polyurethane sheet impregnated with Arabinogalactan (PUSIAG) on the cell attachment and viability of Promastigotes, Amastigotes, Macrophages, and skin cells.

2. Methods

2.1. Materials

Arabinogalactan, polyurethane, NNN medium, RPMI1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropanol, chloroform were purchased from Sigma-Aldrich Chemical Co, (St Louis, MO, USA). RNA extraction buffer (RiboX) and cDNA mastermix were purchased from GeneAll Company, South Korea. The real-time Mastermix was provided by Applied Biosystems Company, USA. Also, all primers were sourced from Takapoo Zist Company, Iran.

2.2. Preparation of PUSIAG

Five mL of Arabinogalactan 5% w/v was poured into a falcon containing 10 pieces of PUS (1 cm × 1 cm). Then, they were incubated at 37 °C for 24 h. Next, they were washed by distilled water to remove excess Arabinogalactan. After washing, all were kept in a sterile condition at 4 °C.

2.3. Preparation of Promastigotes of L. major

The standard isolates of *L. major* (MRHO/IR/75/ER) was sourced from Shahid Sadoughi University of Medical Sciences, Yazd, Iran. First, it was incubated in NNN medium enriched by RPMI1640 for 48 h at 37°C. After incubation, it was centrifuged at 3000 rpm, and washed by cold RPMI1640. Finally, the density of Promastigotes was adjusted to 2×10^5/mL.

2.4. Preparation of Amastigotes of L. major

Two mL of *L. major* Promastigotes (2×10^5/mL) were injected to peritoneum of male BALB/c mouse. After 5 days, 3 mL of RPMI1640 was injected to its peritoneum, and then aspirated. Preformed cell suspension was three times aspirated in sterile tube. Next, all were aseptically washed by RPMI1640, and then its density was adjusted to 2×10^5/mL.

2.5. Preparation of mouse peritoneal Macrophages

First, cold RPMI1640 was injected to peritoneum of male BALB/c mouse. Then, the peritoneal fluid was aspirated, and centrifuged at 3000 rpm for 10 min. Then, Macrophages were suspended in RPMI1640, and finally adjusted to 2×10^5/mL.

2.6. Preparation of whole skin cells (WSCs) of mouse

Ten male BALB/c mice with weight of 18–20 g were anesthetized, and then their dorsal skin was biopsied (a piece of 2 × 2 cm) and rinsed by normal saline. One mL of trypsin enzyme (0.025 mM) was added to each skin piece, and incubated at 37 °C for 5 min with mild agitation. After enzymatic digestion, all skin pieces were crushed with a mortar

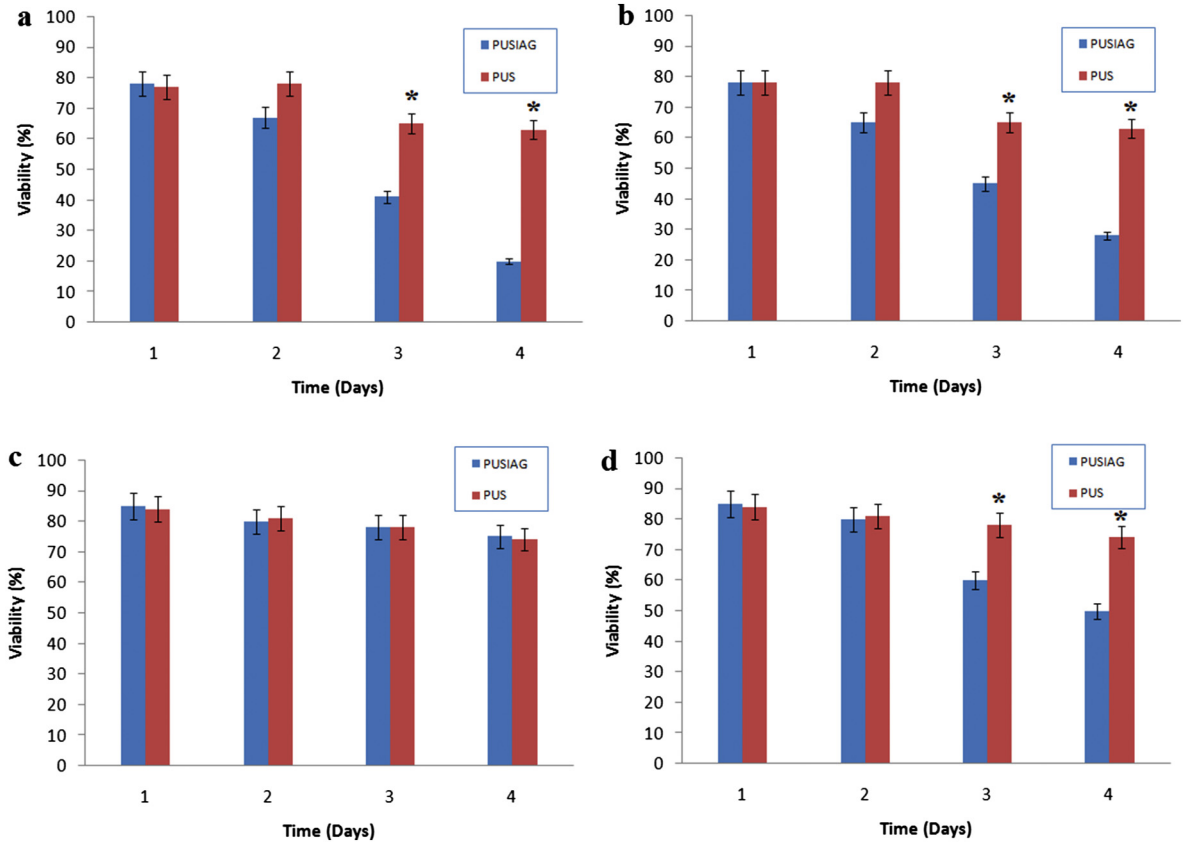

Fig. 2. The viability of Amastigotes (a), Promastigotes (b) WSCs (c), and Macrophages (d) when exposed to PUSIAG and PUS. * $P < 0.05$ compared with PUS at same day, n = 3.

Table 1
The expression of *integrin* and *fibronectin* when whole skin cells of mouse exposed to PUSIAG and PUS.

Gene name	PUSIAG	PUS
Day 1		
integrin	1.5	2
Fibronectin	2	2
Day 2		
integrin	2	2
Fibronectin	5	3
Day 3		
integrin	12	3*
Fibronectin	10	2r*
Day 4		
integrin	52	3*
Fibronectin	45	4*

* P < 0.05 compared with PUSIAG at the same day.

Table 2
The expression of *integrin* and *fibronectin* when mice macrophages exposed to PUSIAG and PUS.

Gene name	PUSIAG	PUS
Day 1		
integrin	2	2
Fibronectin	3	2.5
Day 2		
integrin	3	2.5
Fibronectin	3	2.5
Day 3		
integrin	12	3*
Fibronectin	9	3.1*
Day 4		
integrin	25	4*
Fibronectin	48	4*

* P < 0.05 compared with PUSIAG at the same day.

and pestle, and then centrifuged at 1500 rpm for 15 min. Finally, all cells were re-suspended in RPMI1640, and adjusted to 10^5 cells/mL. All mice were maintained at standard conditions in accordance with the guidelines of the National Institute of Health (Guide for the Care and Use of Laboratory Animals, 1996), and ethics committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

2.7. Exposure of PUS and PUSIAG with Promastigotes, Amastigote, Macrophages and WSC

In the next step, one piece of PUS and PUSIAG was separately added to 1 mL of cell suspension (Promastigotes, Amastigote, Macrophages, and WSCs), and then incubated for 1, 2, 3, and 4 days at 37 °C.

2.8. Attachment and toxicity

After incubation times, both PUBVSIAG and PUBVS were placed in formalin 10%, and then stained by hematoxylin and eosin. 30 slides were separately counted, and the number of adhered cells was recorded under a light microscope. For toxicity assay, 100 μL of 5 mg/mL MTT was added to each tube containing PUBVSIAG and PUBVS. After 5 h, their optical density was read at 450 nm, and then cell viability was determined for each group.

2.9. Q-PCR

Total RNA was first extracted by RiboX buffer. Next, 1 μg of total RNA and 1 μL of random hexamer were mixed. Next, 10 μL of cDNA

Table 3
The expression of *GP63* ad *Cpb* when promastigotes of *L.major* exposed to PUSIAG and PUS.

Gene name	PUSIAG	PUS
Day 1		
Gp63	10	1.1*
Cpb	9	1*
Day 2		
Gp63	9	1*
Cpb	12	1.2*
Day 3		
Gp63	21	2*
Cpb	25	2.3*
Day 4		
Gp63	75	4*
Cpb	65	4*

* $P < 0.05$ compared with PUSIAG at the same day.

Table 4
The expression of *GP63* ad *Cpb* when amastigotes of *L. major* exposed to PUSIAG and PUS.

Gene name	PUSIAG	PUS
Day 1		
Gp63	9	1.1*
HSP70	9	1*
Day 2		
Gp63	10	1.2*
HSP70	12	1.2*
Day 3		
Gp63	23	2.3*
HSP70	24	2.3*
Day 4		
Gp63	77	5*
HSP70	63	4*

* $P < 0.05$ compared with PUSIAG at the same day.

Mastermix was added to 10 μL of total RNA, and incubated at 50 °C for 60 min. Then, 2 μL of cDNA, 2 μL of forward primer, and 2 μL of reverse primer of each gene were added to 10 μL of SYBR® Green Real-Time PCR Master Mix. Then, strip tubes were entered into Real-time PCR machine (ABI 1 plus, USA), and run. For WSCs and Macrophages, the expression of *integrin, fibronectin* and *GAPDH* (as a reference gene) was investigated. For both Promastigotes and Amastigotes, the expression of *GP63*, *HSP70*, and *18s rRNA* (as a reference gene) was calculated by ΔΔCT formula.

2.10. Statistical analysis

All results were reported as mean ± standard deviation (SD). To detect significance differences between groups, one-way ANOVA method was used. For this purpose, SPSS software (SPSS 20.0 Inc., Chicago, IL) was used, and *P*-values < 0.05 were considered as statistically significant.

3. Results

3.1. Attachment

Fig. 1 shows the percentage of attached Amastigotes (a), Promastigotes (b), WSCs (c), and Macrophages (d) when exposed to PUSIAG and PUS. As seen, with increase of exposure time, the percentage of attached cells was increased. Sharply, this pattern was seen for both PUSIAG and PUS. As an important finding, there was significant difference between attached cells to PUSIAG and PUS ($P < 0.05$). This was sharp for long incubation times (e.g. 3 and 4 days). It seems that Promastigotes and Amastigotes have higher interest to PUSIAG than WSCs and Macrophages.

3.2. Toxicity

Fig. 2 shows the viability of Amastigotes (a), Promastigotes (b) WSCs (c), and Macrophages (d) when exposed to PUSIAG and PUS. As demonstrated, with increase of exposure time, the percentage of viable cells was decreased. This pattern was sharply seen for both PUSIAG and PUS and for all types of cells. There were significant differences between cell viability of Promastigotes and Amastigotes when exposed to PUSIAG and PUS, especially in long time incubation ($P < 0.05$). Interestingly, this pattern was sharply seen for Macrophages ($P < 0.05$). It seems that PUSIAG had more toxic for Amastigotes than WSCs and Macrophages.

3.3. Gene expression

Table 1 shows the expression of *integrin* and *fibronectin* when whole skin cells of mouse exposed to PUSIAG and PUS. Table 2 shows the expression of *integrin* and *fibronectin* when mice macrophages exposed to PUSIAG and PUS. Table 3 shows the expression of *GP63* and *HSP70* when Promastigotes of *L major* exposed to PUSIAG and PUS. Table 4 shows the expression of *GP63* and *HSP70* when Amastigotes of *L major* exposed to PUSIAG and PUS. The first finding was that when incubation time was increased the relative expression of *integrin* and *fibronectin* in WSCs and macrophages, and *GP63* and *Cpb* in Promastigotes and Amastigotes were increased. In some incubation times (e.g. 3 and 4 days), there were significant differences between gene expression when exposed to PUSIAG and PUS ($P < 0.05$).

4. Discussion

Some of known polymers have not required characteristics for biomedical applications. An applied approach to solve this problem is modifying the surface of polymers. A grafted surface can be achieved either by graft polymerization or covalent coupling [7]. Recently, it has been shown that modification of polyurethane with natural molecules making them more bioactive [8]. Arabinogalactan is an example that leads to increase of bioactivity of polyurethane. Pakzad et al. showed that coating of polyurethane scaffold with Arabinogalactan could lead to increase of adhesion to fibroblast cells. This phenomenon is done by integrin pathway [9]. Arabinogalactan is a hydrocolloidal oligosaccharide, and increases the viscosity of different materials. Different plants can secrete Arabinogalactan. For example, *Amygdalas lycioides* which is an almond plant cans secret Arabinogalactan with some medical application [10].

This study showed that with increase of exposure time, the percentage of attached cells was increased. There was a significant difference between attached cells to PUSIAG and PUS in case of Promastigotes and Amastigotes. It seems that Promastigotes and Amastigotes have higher interest to PUSIAG than WSCs and Macrophages. Also, with increase of exposure time, the percentage of viable cells was decreased. Here, there was significant difference between cell viability of Promastigotes and Amastigotes when exposed to PUSIAG and PUS, especially in long time incubation. Moreover, when incubation time was increased the relative expression of *integrin* and *fibronectin* in WSCs and macrophages, and *GP63* and *HSP70* in Promastigotes and Amastigotes were increased.

Although coating of polyurethane lead to increase of its bioactivity, polyurethane is attractive for adhesion and migration of cells. Huey et al. showed both adhesion and migration of endothelial and fibroblast cells on nano-polyurethane composites. They saw high expression of *P-FAK, P-RhoA* and *MMP2*, as well as α and β-integrin [11]. Attia et al. showed that $\alpha_5\beta_1$ integrin in fibroblastic cells was greatly increased when exposed to fibronectin-coated polyurethane [12]. Juan et al. showed that endothelial cells could grow on biphasic polyurethane

matrix [13]. Cheung et al. incubated polyurethane scaffolds with human fibroblastic cells. They showed the expression of *TGFβ*, *TGF2*, and integrin β, was increased, after 7 days [14].

The *HSP70* gene encodes the heat shock protein which is a chaperone and plays the important roles during transmission from vector to mammalian host and therefore differentiation from the promastigotes to Amastigote because of exposing various stress, including, pH, temperature, and oxidants from macrophages [15]. Based on the literature, some known factors that could increase the gene expression of *HSP70* from *Leishmania donovani* and *Leishmania chagasi*, include toxic oxidants [16], tumor necrosis factor [17] and pentavalent antimony treatment [17]. *HSP70* is one of the important keys for failing the pentavalent antimony treatment [18]. *GP63* gene encodes a zinc-dependent metalloprotease which presents on the surface of Promastigotes *Leishmania* parasites [19]. This important molecule modulates the immune subversion and evasion of *Leishmania* parasite. It is well known that this protein can experience cleavage of the prominent protein tyrosine phosphates (PTPs) in cytoplasm and result in infection in mammalian [20]. Based on our knowledge, PTPs make negative regulators of the signaling pathways and results in inhibition of inflammatory and Leishmanicidal functions. Therefore, more expression of *GP63* could be considered for the persistence of *Leishmania* infection. In our study, we showed high expression of *GP63*. It could exacerbate the outcome of CL, it is necessary for co-treatment of Antimonial and antibiotics against SBH group A be prescribed. Ardehali et al. (2011) tried to assess supernatant of culture media fluid containing SBH group A and showed that this upper layer can halt the growth of Leishmaniasis parasite Promastigotes [15].

5. Conclusion

Taken together, it seems that Promastigotes and Amastigotes have higher interest to PUSIAG than WSCs and Macrophages. Also, this study showed PUSIAG had higher toxicity for both WSCs and Macrophages than WSCs and macrophages. All data indicated that PUSIAG can be used as dermal patch for CL. Regarding the application, PUSIAG must be more investigated in vitro and in vivo.

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Conflict of interest

There is no conflict of interest to declare.

Authors' contribution

Ali Fattahi Bafghi and Mahmoud Dehghani Ashkezari– Conceived, designed the experiments and wrote the manuscript; Soheila Pournasir - performed the experiments- Mahmoud Vakili analyzed the data and all authors – edit and revised the manuscript.

Ethics approval and consent to participate

Ethical Committee of Shahid Sadoughi University of Medical Sciences has approved the protocol of the study.

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