EFFECT OF CLINICAL *Klebsiella pneumoniae* EXTRACTED MELANIN ON SOME IMMUNE ASPECTS IN MICE

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ABSTRACT

Melanin is a pigmented material varies from yellow to black color dependent on the type of melanin. Not all the microorganisms can produce this material, most industrial researchers are looked after about melanin that produced from environmental microorganisms and little is known about from pathogenic bacteria and its role in pathogenesis. Therefore this study is aimed to focus on the role of melanin produces by one pathogenic bacteria(*Klebsiella pneumoniae*) isolated from an infected wound on host immune responses.The results of this study showed, that the increasing melanin concentration leads to decrease in the phagocytic index. and *in vivo* tests are done by using different concentration of extracted microbial melanin, These concentrations showed variable effects on tested cytokines(IL-1β , IL-12 , TNF-α , IL-10 , IFN-γ and IL-4) In conclusion, microbial melanin is act as virulence factor throughout suppress the most important pro-inflammatory cytokines and that squeal to counteract immune responses.

Key words: Melanin, microbial melanin, pathogenic Klebsiella pneumonia, pro-inflammatory cytokines

تأثير الميلانين المستخلص من بكتريا *Klebsiella pneumonia* السريريةعلى بعض الدلائل المناعيه في الفئران

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المستخلص

الميلانين هو من المواد الصبغية التي تتغاير بين اللون الاصفر الى اللون الاسود اعتمادا على نوع الميلانين .لاتمتلك جميع الكائنات المجهريه القدرة على انتاج هذه المادة ، يبحث معظم الباحثينفي انتاج هذه الماده من الكائنات المجهرية البيئية والتي يكون انتاجها قليل من الكائنات المجهريه المرضيه ودورها في الأمراضيه. هدفت هذه الدراسة في التركيز على دور هذه الصبغة المنتجة من قبل بكتريا *Klebsiella pneumoniae* المعزولة من اخماج الجروح على الاستجابة المناعية للمضيف. حيث بينت نتائج الدراسة الى ان زيادة تركيز الميلانين يؤدي الى تقليل دليل البلعمة ، بينما بينت نتائج الاختبار داخل الجسم الحي عند استعمال تراكيز مختلفة من الصبغة المستخلصة الى حدوث تاثير متغاير في مستويات الحركيات الخلوية (1 بيتا ،12،عامل النخر الورمي –الفا ،انترفيرون –كاما،و 4). ولهذا يمكن الاستنتاج الى ان الميلانين المايكروبي ممكن ان يعمل كعامل ضراوة من خلال تثبيط معظم الحركيات الخلوية لما بعد الالتهاب وبالتالي الى اعاقة الاستجاب المناعية.

الكلمات المفتاحيه: الميلانين، الميلانين الميكروبي، بكتريا الكلبسيلا نيموني، الحركيات الخلوية مابعد الالتهاب

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INTRODUCTION

Melanin's are one of the polymers of phenolic compounds. It's brown to black colored complex natural pigments. As well as widely distributed throughout all living forms in nature. This pigment has several biological functions which comprise thermoregulation, photo protection, acting as free radical sinks, cation chelators, and as antimicrobials. In plants, melanin acts as cell wall strengtheners (30), while in humans it regulates the skin color and also acting a vital role in protecting the skin against ultraviolet light (12).Whereas the microbial melanin acts as a protective agent against environmental stresses. For example, it's made the bacteria resistant to antimicrobials (24) and is involved in fungal pathogenesis (6). Melanin classification into 3 main types Eumelanins; are a black or brown pigment, Pheomelanins: are yellow to red pigment and Allomelanins: classified as black eumelanin and yellow-to-brown pheomelanin, whereas melanin's from plants, fungi, and bacteria are brown-to-black allomelanins (32).There are several key enzymes complicated in melanogenesis, such as tyrosinase which is involved in the formation of melanin, it helps in the conversion of tyrosine to DOPA (13), oxidoreductases, such as laccases, its belong to the blue copper-containing oxidases family, together with ascorbate oxidase and ceruloplasmin (35). Whereas polyketide synthases (PKS) belongs to multidomain protein related to fatty acid synthases involved in the biosynthesis of melanin. Several microorganisms utilize these enzymes to produce secondary metabolites like, antimicrobials, pigments, toxins and other products (22). P-hydroxy phenyl pyruvate hydroxylase (HPPH) is the key enzyme involved in pyomelanin biosynthesis. This enzyme pathway is belongs to the phenylalanine and tyrosine degradation, is ubiquitous among living organisms (25). And 4-hydroxy phenylacetic acid hydroxylase (HPA), belongs to a separate family of hydroxylases which catalyzes the development of allomelanin-like polymers. Tyrosine is also a substrate of HPA, but unlike tyrosinase (17). Bacterial melanin has several biological functions of like because melanin includes various groups which are able to donate and accept electrons, therefore it acts as an electron acceptor in bacteria (26) and has antioxidant activity(11). As well as has a role in nitrogen fixation by soil bacterium Azotobacter chroococcum (31). Also, melanin as anti-venom for that extracted from black tea has shown antivenin activity in mice (21). Researchers (36) reported that the crude melanin pigment from Streptomyces had shown antibacterial activity against *Escherichia coli*, and *Lactobacillus vulgaris* had the ability to inhibit Human Immunodeficiency Virus (HIV) replication in vitro (38). In addition, it acts as a virulence factor in pathogenesis of *Burkholderia cepacia* which serves as an example of how melanin production increases bacterial virulence (39). Because is little known about the pathogenic *K. pneumoniae* melanin and its role in bacterial pathogenicity. Therefore, this study was aimed to investigate the effect of pathogenic *K. pneumoniae* melanin on some immune parameters in mice.

MATERIALS AND METHODS

According to Saud and Alaubydi method (19), melanin pigment was extracted from pathogenic *K. pneumoniae* isolated from the clinical sample.

Phagocytosis Assay

Dependent on (7), six heparin-containing tubes supplemented with 1ml of the healthy human blood sample in a sterile condition. *Candida albicans* was obtained and supplemented by (College of Science/ Biotechnology dept.) . By using sabouraud agar throughout overnight incubation at 37ºC, the yeast was activated. The collected yeast cells were diluted with sterile normal saline in a percentage 1:4 (10^3/ml). On the other hand, serial concentrations of pigment (500, 750, 1000, 1250, 1500 µg\ml) were prepared by using phosphate buffer pH 8 as diluents. Aliquot of 0.1ml from each pigment concentrations was added to blood tubes except the control (included the blood sample and 0.1ml of diluted activated *Candida albicans*). All tubes were incubated at 37ºC for 90 minutes. A duplicate of blood smear were prepared on clean slides of microscopic for each sample, after drying, the blood smear stained with Giemsa stain for 10 min. then washed with tap water, drying, and microscopic reading. The

phagocytic index is calculated according to the following equation (19):

Phagocytic index =

No. (Phagocytic cell)/ No. (Phagocytic + non phagocytic cell) x 100

Preparation of antigen –adjuvant emulsion

According to (14), melanin pigment treated as antigen, it was prepared at concentration (500, 750, 1000, 1250, 1500) µg\ml by dissolving pigment powder with 5 ml of phosphate buffer pH 8, then each concentration mixed well with olive oil until the emulsion become homogenized and milky like.

***In vivo* tests:** This experiment was done in Biotechnology Center/ University of Al-Nahrain. Thirty albino mice weight (25-30)g were used and divided into six groups, each group included five mice., one of them represented as control. These groups were treated as following:

Control group: intraperitoneal (i.p) injected with 1 ml of (Olive oil and phosphate buffer) emulsion. As well as each group (1,2,3,4,5) were i.p injected with (500, 750, 1000, 1250 and 1500 µg\ml) with partial purified prepared melanin emulsion for 3 days respectively. After one week from the first dose, another booster dose was given. All groups are monitored for 14 days from the first dose. And then the laboratory animals are anesthetized by inhalation using chloroform, blood samples were pooled from the heart using 3ml syringe then separate the serum by centrifugation at 5000 xg, after that collected and preserved in sterile tubes and stored at -20ºC. Further immunological tests were done such as IgG antibody titer (Abcam's IgG Mouse ELISA kit), and several cytokines (e.g IL1 beta, IL4, IL10, IL12, TNF-α, and INF-Gamma) by ELISA technique.

Statistical analysis

Statistical analyses were performed by use of IBM SPSS computer program version 21. Differences between the groups were statistically analyzed by ANOVA table. Data are expressed as mean ± standard error (SE). A P value of ≤ 0.05 was regarded as statistically significant

RESULTS AND DISCUSSION

Effect of melanin on phagocytic index: Many pathogenic microbes are produce melanins which has an important role in virulence and pathogenicity throughout protecting the microbe from immune responses of the host. In mammalians, a major aspect of the innate immune defense system against invading pathogens involves melanin. The overall measurement of phagocytosis index results is summarized in Table 1, which showed the obvious effect of increasing melanin concentration leads to decreasing in a phagocytic index, therefore melanin pigment is considered as a virulent factor for pathogenic bacteria. This result was agreed with Bustamante, *et al*. (5), who they explained the inhibition effect of melanin on phagocytic ability of neutrophil throughout negative charge of melanin that neutralized the activity of neutrophil defensin. As well as it enhances virulence by protecting microbe against free-radical fluxes generated by immune effector cells, suggesting that even a small amount of melanin may provide significant protection against reactive nitrogen species .

Table 1. The phagocytic index by using melanin *in vitro*

Group no.	Total no. (phagocytic and non-phagocytic cell)	Phagocytic cells	Phagocytic Index
500 µg\ml	41	28	68%
750µg\ml	26	15	57.6%
1000 µg\ml	26	13	50%
1250 µg\ml	37	12	32.4%
1500 µg\ml	34	10	29.4%
Control	42	36	85.7%

***In vivo* experiment**

The results of the quantitative detection of different interleukins (IL-1β, TNF-α, INF-γ, IL-10, IL-4, and IL-12) were revealed the following:

Interleukin 1 β (IL-1β): It is one of the strong pro-inflammatory cytokines, secreted from

various cell types including peripheral blood monocytes, macrophages, B cells, helper T lymphocytes and NK cells (10). the present results of IL-1β showed the different conc. of melanin do not recorded significant alteration in all treated groups except the group 3 that treated with 1000 µg/ml Table 2. These results disagree with that found by other researchers (27) they mentioned that, the IL-1β level did not altered due to treated lab. animals with different conc. of synthetic melanin. On the other hand, these results reflected the low toxicity of melanin, as a reason of an immune response without activation of caspase-1 or secretion of IL-1 beta at these concentrations of pigment. Whereas increasing of IL-1 beta promotes cell growth, tissue repair, and immune response regulation and has a significant role in many chronic and acute inflammatory diseases (24). Therefore this microbial pigment does not consider as inflammatory response inducer .

Tumor necrosis factor – α (TNF-α):

It is a cytokine associated with acute and chronic inflammation. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as $CD4^+$ lymphocytes, NK cells, neutrophils, mast cells, and eosinophil's (34). As showed in Table 2, all groups revealed significant increasing except group one, These observations raise the predictions of using melanin for treatment of diseases associated with some cancerous cases, for imbalanced cytokine production, and for other immunotherapies. This result is confirmed with that of El-Obeid, *et al*., (12) who documanted TNF-α can enhance immunogenicity and promote tumor regression

Interleukin 10 (IL-10) and INF-γ:IL-10 is recognized as cytokine synthesis inhibitory factor which is a product of Th_2 cells following antigenic stimulation that block cytokine production from Th_1 cells. IL-10 achieved this effect by inhibiting the ability of macrophages and dendritic cells to activate Th_1 cells (26), however, IL-10 can also be produced by most if not all $CD4^+$ T cell subset, including Th_1 and Th_{17} cells, B cells, neutrophils, and macrophages. In this regard, it has been found that IL-10 inhibits the protective immune response against pathogens by blocking the production of pro-inflammatory cytokines, such as TNF- alpha. It's also capable to inhibit phagocytosis and microbial killing through limiting the production of reactive oxygen and nitrogen intermediates in response to INF-gamma (9,28). Table 2 showed the significant ($P\leq 0.05$) elevation of IL-10 in the groups 2,3 and 4 comparable with the control one. The present result is not agreed with that recorded by Mohagheghpour *et al*.,(27), who they mentioned the in *vivo* test of synthetic melanin led to suppress IL-10 production, this differences between both studies may be due to the source and the conc. of usable melanin in the experiments. Whereas, Interferon–γ is mostly synthesized by Th_1 lymphocytes, after their activation with immune and inflammatory stimuli, rather than viral infection (15). Interestingly, natural killer cells can furthermore function as adaptive effectors once activated by T cell-induced IFN-γ or when IgG ultimately elicits antibody-dependent cell cytotoxicity (16). IFN-γ secreted to activate macrophages and to induce their microbicidal functions (29). Although it's essential for the development of an immune response, that extends the lifespan of an infected animal. IFN-γ/IL-10 ratio reflecting the Th_1/Th_2 balance in serum (32).The result in Table 2 is elucidated no significance ($P\leq 0.05$) elevation occurs among all treated groups comparison with control one, that reflects the role of IL-10 in suppression IFN-γ secretion from Th_1, and then counteracting cell-mediated immunity and the response is the bias toward stimulation humoral immune response and production of immunoglobulin. From previous results, microbial melanin mostly suppresses this type of cytokines with some exception of IL-10 and TNF-α. Because pro-inflammatory cytokines production mediates the damaging squeals of inflammation in case of infections, therefore this point is to improve the bacterial melanin consider as one of the bacterial virulence factors. This result is confirmed to that reported by Areej *et al*., (1) they showed plant melanin had an inhibitory effect on some pro-inflammatory cytokines, but it increased TNF-α and secretions in BJAB cells

Interleukin 4 (IL-4): Is a cytokine that induces differentiation of naive helper T cells (Th_0 cells) to Th_2 cells (33). It has many biological roles, including the stimulation of activated B and T- cells proliferation, and the differentiation of B cells into plasma cells. It is a key regulator of humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE and up-regulates MHC class II production. IL-4 decreases the production of Th_1 cells, macrophages, IFN-γ, and dendritic cell IL-12.Overproduction of IL-4 is associated with allergies (18). The results of IL-4 in this study showed that, there is no significant increase had been occurred at ($P\leq 0.05$), except group 3 which showed light significance. Because IL-4 is related mostly to an allergic condition as in report of Gour and Wills-Karp (18), therefore this result reflects the positive effect of different conc. of bacterial melanin on the host. This agreed with that of Choi *et al*. (8), who they mentioned about IL-4 has the ability to suppress melanogenesis and vice versa. Melanin is thought to contribute to microbial virulence by protecting microbial cells from oxidative attack during infection. However, there is also evidence from various systems that melanin's have immunomodulatory properties, which possibly could contribute to virulence by altering immune responses as Aron *et al.* (3) reported. Therefore the microbial melanin production led to counteract of an immune system to eradicate the microbes, this finding is acted as another improvement about microbial melanin is one of the bacterial virulence factors.

Interleukin -12 (IL-12): Is an interleukin that is naturally produced by dendritic cells (23), macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation. It is involved in the differentiation of naive T cells into Th_1 cells. It is known as a T cell-stimulating factor. It stimulates the production of tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK)cells and reduces IL-4 mediated suppression of IFN-γ. Also plays an important role in the activities of natural killer cells and T lymphocytes, and mediates enhancement of the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T lymphocytes (18). the results of IL-12 in table 3 revealed, all treated groups do not record significant increasing at ($P\leq 0.05$) except for group 3 comparison with the control group , that reflects the positive effect of IL-12 on the elevation of TNF-α level, and negative effect on IL-4 and INF-γ levels, moreover elucidated the relationship between IL-10 and IL-12 ,that means increasing IL-10 restricted the effect of IL-12, and directed the immune response to humoral immunity ,all these results are agreed with that reported by Gour and Wills-Karp (18).

Table 2. Levels of different cytokines (TNF- α, INF-γ, IL-1β, IL-4, IL-10 and IL-12)

Group	Cytokines in pg\ml ± SE					
	TNF-α	INF-γ	IL-1β	IL-4	IL-10	IL-12
Control	20±3.2	4.5±4.6	12± 6.2	11±3.4	22±2.3	10±1.2
Group 1 (500µg\ml)	22±2.5	4.2±3.5	11± 3.8	12±1.1	27±1.6	11±5.1
Group 2 (750µg\ml)	27±5.3*	4.6±7.1	13.8±1.3	16±3.1 *	28.3± 3.2*	13±2.6
Group 3 (1000µg\ml)	45.3±*	4.6± 4.8	20±4.2*	17±4.7 *	30.6± 4.4*	16±2.1*
Group 4 (1250µg\ml)	43±3.7*	5.4± 5.2	12± 3.5	16±4.1 *	36±3.4 *	14±3.2*
Group 5 (1500µg\ml)	27±4.1*	5.5± 2.1	12±1.4	16±5.3*	25±2.3	12±1.2

***= significant results($P\leq 0.05$) compared with the control group**

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