VARIABLE BACTERIAL RESPONSES TO OXIDATIVE STRESS IN DIFFERENT BACTERIAL SPECIES

By

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ABSTRACT

Background: Living organisms are exposed to oxidative stress due to internal or external stimuli. It results from the imbalance between the production and elimination of reactive oxygen species. This leads to loss of homeostasis. Objective: To test the effect of oxidative stress on the level of the production of reduced glutathione (GSH) as an antioxidant, malondialdehyde (MDA) as a measure of lipid peroxidation, and of the siderophore enterobactin as an oxidative stress response, in different bacterial species.

Materials and Methods: H2O2 minimum inhibitory concentration (MIC) was determined in Escherichia coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603, using broth-macrodisution method. The levels of GSH and MDA were measured in E. coli ATCC 25922 and K. pneumoniae ATCC 700603 and in clinical isolates of E. coli, K. pneumoniae and Staphylococcus aureus after exposure to lethal H2O2 concentration, using Glutathione Reduced Kit and Lipid Peroxide-Malondialdehyde Kit, respectively. The level of expression of entC gene, involved in enterobactin biosynthesis, in presence of 0.25 and 0.5 MIC of H2O2 was determined using quantitative reverse transcription-polymerase chain reaction.

Results: H2O2 MIC for both E. coli ATCC 25922 and K. pneumoniae ATCC 700603 was 1.5 mM. Exposure of E. coli to H2O2 resulted in a significant increase in GSH (p=0.0001) and MDA (p=0.0001) levels. However, in K. pneumoniae, a significant decrease in the GSH (p=0.0001) and MDA levels (p=0.0001) was recorded upon H2O2 exposure. No change in MDA and GSH levels was detected in S. aureus isolates exposed to H2O2. The expression of entC gene in both E. coli ATCC 25922 and K. pneumoniae ATCC 700603 was reduced in presence of 0.25 and 0.5 H2O2 MIC.

Conclusion: Bacteria responded differently to oxidative stress, with S. aureus bacteria as the least affected by oxidative stress. Enterobactin role in oxidative stress needs reevaluation.

Keywords: Oxidative stress, Malondialdehyde, Reduced glutathione, enterobactin, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus.

INTRODUCTION

Oxygen is essential for the growth of living organisms. Under normal physiological conditions, free radicals are generated due to the escape of electrons from the electron transport chain, leading to formation of reactive oxygen species (ROS) as superoxide anion (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (OH•; McBee et al., 2017). If the generated ROS exceeds the elimination capacity of the organism, this leads to loss of homeostasis, and the organism encounters a state of oxidative stress (Su et al., 2019).
Excess ROS, generated during the oxidative stress, results in adverse modifications of cell components as lipids, proteins and DNA. Polyunsaturated fatty acids of the cell membranes are highly susceptible to ROS damage; a process called lipid peroxidation. Lipid peroxidation is a chain reaction that results in the production of breakdown products such as malondialdehyde (MDA) and 4-hydroxynonenal; these products may cause disruption of the bacterial cell membrane (Ayala et al., 2014).

The attack of ROS may also affect the protein activity through nitrosylation, carbonylation, disulphide bond formation and glutathionylation. In addition, the breakdown products of lipid peroxidation may form conjugates with the protein (Repetto et al., 2012). Oxidative attack on DNA results in deoxyribose oxidation, removal of nucleotides, strand breakage, base modification, and DNA-protein crosslinks. This may lead to malfunctions or complete inactivation of the encoded protein and finally can lead to mutations (Sharma et al., 2012 and Su et al., 2019).

Microorganisms produce antioxidants to alleviate the oxidative stress. Antioxidants can be enzymes as superoxide dismutase (SOD) which catalyzes the conversion of superoxide anion into H$_2$O$_2$, catalase and peroxidase enzymes which decompose H$_2$O$_2$ into H$_2$O. Antioxidants can also be non-enzymatic molecules as reduced glutathione (GSH), ascorbic acid, and α-tocopherol (Staerck et al., 2017).

Reduced glutathione (the tripeptide γ glutamylcysteinylglycine) is one of the most abundant non-protein thiols. GSH reacts with free radicals preventing the damage of cellular components; it is also involved in the formation and maintenance of proteins’ disulphide bonds (Ribas et al., 2014).

The role of the enterobactin siderophore in alleviation of oxidative stress produced by hydrogen peroxide and paraquat was reported in Escherichia coli (Adler et al., 2014 and Peralta et al., 2016). Also, Staphylococcus aureus siderophore transporter SirABC was found to be induced by oxidative stress (Nobre and Saraiva, 2014). In this study, the effect of the classical stressor; hydrogen peroxide on different bacterial species was determined. The level of the two antioxidant molecules; GSH, and enterobactin as well as MDA as a measure of lipid peroxidation, were determined.

**The present study aimed to** study the effect of H$_2$O$_2$ on different bacterial strains regarding GSH, MDA and enterobactin.

**MATERIALS AND METHODS**

**Bacterial strains:**

E. coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603 were used in the study. Clinical isolates of E. coli (n=59), K. pneumoniae (n=11) and S. aureus (n=9) were obtained from the Faculty of Medicine, Cairo University and Faculty of Medicine, Ain Shams University.

**Determination of H$_2$O$_2$ minimum inhibitory concentration (MIC):**

The MIC of H$_2$O$_2$ was measured using broth-microdilution method, according to the Clinical and Laboratory Standards Institute (2016). Briefly, the overnight culture of E. coli ATCC 25922 and K.
_pneumonia_ ATCC 700603 were diluted to have an optical density equivalent to that of 0.5 McFarland standard (contains approximately 2x10⁸ CFU/mL). This was diluted 1:150 to contain 5x10⁵ CFU/mL. Then, one mL of different H₂O₂ concentrations prepared by two-fold serial dilutions (6mM to 0.0117 mM) was inoculated with one ml of the prepared inoculum. The culture of the organism without addition of H₂O₂ was used as a positive control while un-inoculated broth was used as a negative control. Tubes were incubated overnight at 37 ºC for 20 hours, and the MIC was determined as the lowest concentration of H₂O₂ which completely inhibited the growth of the organism in the tubes.

**Determination of GSH and MDA levels:**

The effect of H₂O₂ on the levels of MDA and GSH was tested at lethal H₂O₂ (10xMIC level; Jenkins et al., 1988). Cell extracts were obtained by the method of Daily et al. (1978). Briefly, the tested strains were grown aerobically for 24 hours in brain heart infusion broth at 37 ºC in a rotary shaker at 250 rpm. The culture was divided into two portions; one portion was treated with 15 mM H₂O₂ (50% w/v) and the other was kept without treatment (control). The flasks were incubated in the shaking incubator for 90 minutes at 37 ºC. Cells were harvested by centrifugation for 10 minutes at 7000 xg and washed with 0.05 M potassium phosphate (pH 7.8) containing 1mM ethylenediaminetetraacetic acid (EDTA; potassium phosphate EDTA buffer). Washed cells were resuspended in 10 mL potassium phosphate EDTA buffer and centrifuged for 3 minutes at 7000xg. The supernatant was discarded and 5mL potassium phosphate EDTA buffer was added. Cells were disrupted for 3 minutes with a sonicator (Branson sonifier, USA). Cell debris was removed by centrifugation at 10000 xg for 5 minutes and the cell extracts were stored at -70 ºC until used. The level of reduced glutathione was assayed in the cell extracts using Glutathione Reduced kit (Biodiagnostic, Egypt) according to manufacturer’s protocol. The level of GSH was determined by measuring absorbance of the yellow color produced after the reaction with 5,5'-dithiobis (2-nitrobenzoic acid) at 405 nm. The concentration of GSH was calculated using the following equation:

\[
\text{GSH concentration (mmol/L) = Sample absorbance x 2.22}
\]

MDA level in the extracts was measured using Lipid Peroxide-Malondialdehyde kit (Biodiagnostic, Egypt) according to manufacturer’s instructions, where MDA reacts with thiobarbituric acid (TBA) in acidic medium forming thiobarbituric acid reactive product of pink color. The absorbance of the produced color was measured at 534 nm; the concentration of MDA was calculated using the following formula:

\[
\text{MDA concentration (nmol/mL) = (Sample absorbance/Standard absorbance) x 10}
\]

**Determination of entC gene expression:**

The effect of the oxidative stress exerted by H₂O₂ on the level of _entC_ gene expression in _E. coli_ and _K. pneumoniae_ was determined using quantitative reverse transcription-polymerase chain reaction (RT-PCR). _entC_ gene encodes the
isochorismate synthase enzyme responsible for the conversion of chorismate to isochorismate which is converted finally to enterobactin, through different enzyme-catalyzed steps. The sequence of the primers used for entC gene quantitation in *E. coli* was as follows:

F'ACCTCCTCTCCACAATTTGATTAC and R'AGCAGACAAGCCAAAGTCA, while the sequence of those used in *K. pneumoniae* was as follows:

F'TGGCTGAGGATGTACAGAAAC and R'GCAGCCTGAGGTGCTAAA.

*rpoS* and *rpoB* were used as housekeeping genes for *E. coli* and *K. pneumoniae*, respectively. The following primers were used for quantification of *rpoS* and *rpoB*, respectively:

F'ACGGCCGAAGAAGAAGTTTAT and R'TTACCACCAGACGCAAGTTTAC

and

F'CGAAATCGAAGGTTCCGGTAT and R'ATCGTCCACTTCGCCCTTTAC.

Tested species were incubated in luria bertani broth overnight at 37 °C. Overnight cultures were diluted to reach an optical density of 0.05 at 600 nm. H₂O₂ was added to 20 mL culture at a concentration of 0.25 of MIC (0.375 mM) and 0.5 of MIC (0.75 mM). Culture without H₂O₂ addition was used as a control. The cultures were incubated at 37 °C in a shaking incubator at 180 rpm until the exponential phase of growth (OD 600 =0.2). The synthesis and degradation of RNA were blocked by adding 1/5 volume of stop solution (90% ethanol /10% phenol). The RNA was purified using RNeasy mini kit (QIAGEN, Germany) according to manufacturer’s protocol.

RNA was quantified by measuring the absorbance at 260 nm. cDNA synthesis and the RT-PCR were carried out using KAPA SYBR® FAST One-Step qRT-PCR Master Mix (2X) Kit (Sigma-Aldrich, U.S.A) as per manufacturer’s recommendations.

**Statistical analysis:** SPSS version 18.0 was used for statistical analysis. Comparisons of the results of the H₂O₂ treated cultures with the untreated ones were accomplished using the Mann Whitney U rank test.
RESULTS

H₂O₂ MIC:
The H₂O₂ MIC of both *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 was found to be 1.5 mM.

Level of MDA and GSH under oxidative stress:
The levels of MDA and GSH were determined in response to oxidative stress exerted by lethal H₂O₂ concentration. *E. coli* strains (*E. coli* ATCC 25922 and the clinical isolates) recorded a significant increase in the level of GSH in presence of a lethal concentration of H₂O₂ (p=0.0001). However, *K. pneumoniae* strains (*K. pneumoniae* ATCC 700603 and the clinical isolates) treated with lethal concentration of H₂O₂ recorded a slight but a significant decrease in GSH level (p=0.0001). In *S. aureus* strains, no difference in the level of GSH by H₂O₂ treatment was detectable (p=0.9; Table 1). The level of MDA increased significantly in *E. coli* cells treated with lethal H₂O₂ concentration (p=0.0001), while in *K. pneumoniae*, a significant decrease in MDA level (p=0.0001) by H₂O₂ treatment was recorded. In *S. aureus*, MDA level was not affected by H₂O₂ treatment (p=0.97; Table 1).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Test</th>
<th>Number of tested clinical isolates</th>
<th>MDA (nmol/mL)</th>
<th>GSH (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>p Value</td>
</tr>
<tr>
<td>E. coli</td>
<td>Control</td>
<td>59</td>
<td>2.15±1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂O₂ treated</td>
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<td>3.97±1.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Control</td>
<td>11</td>
<td>2.9±0.048</td>
<td></td>
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<tr>
<td></td>
<td>H₂O₂ treated</td>
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<td>2.6±0.07</td>
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<tr>
<td>S. aureus</td>
<td>Control</td>
<td>9</td>
<td>7±0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂O₂ treated</td>
<td></td>
<td>7±0.07</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table (1): Level of malondialdehyde and reduced glutathione in untreated and H₂O₂-treated bacterial cells

GSH: Reduced Glutathione; MDA: Malondialdehyde

Expression of entC gene under sublethal concentrations of H₂O₂:
The expression of entC gene in both *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 was reduced to approximately 20% and 1% of its original level in untreated cells in presence of 0.25 and 0.5 MIC of H₂O₂, respectively.

DISCUSSION
Oxidative stress is a universal phenomenon to which all organisms are exposed; however, the oxidative stress response differs from one organism to another and differs in the same organism according to the applied stressor. The mechanisms of homeostasis in bacteria are becoming a very attractive target for the development of new anti-infective agents and are a promising strategy to circumvent antimicrobial resistance (*Mourenza et al., 2020*).

H₂O₂ is considered one of the reactive oxygen species that results from normal cell metabolism and can cause damage to various cell components if exceeded a certain level. ROS, including hydrogen peroxide can play an important role in redox signaling (*Phaniendra et al., 2015*), where they are needed for the normal physiological functioning of cells. Redox signaling includes reversible modification...
either oxidation or covalent adduct formation with specific target proteins, allowing further translations of a signal. Cysteine residues of a target protein are the most susceptible to oxidation. Hydrogen peroxide is considered a critical signaling molecule involved in redox signaling (DiMarzo et al., 2018). It has a very good stability and is able to pass through transmembrane water channels (aquaporins), where specific isoforms (peroxiporins) are present for hydrogen peroxide transport (Bienert & Chaumont, 2014 and Prata et al., 2019). In this way, H$_2$O$_2$ acts as messenger to carry a redox signal from its generation site to the target site (Rani et al., 2015). In Gram negative bacteria, H$_2$O$_2$ modulates the activity of the transcription factor Oxy R; the main peroxide sensor that regulates the transcription of genes responsible for defense against cellular H$_2$O$_2$ (Jo et al., 2015). Similarly, PerR, a functional equivalent of OxyR, is used by many Gram positive bacteria for defense against oxidative stress (Ji et al., 2015).

In this study, the effect of H$_2$O$_2$ as a stressor was studied. H$_2$O$_2$ is a classical stressor and is one of the ROS produced normally in living cells under normal physiological conditions (Phaniendra et al., 2015). The response of the tested species to H$_2$O$_2$ was different. E. coli cells treated with H$_2$O$_2$ showed a significant increase in the MDA level compared to the untreated cells. MDA is a biomarker of oxidative stress; it’s one of the byproducts of lipid peroxidation (Hong et al., 2012). Elevation of the level of MDA under oxidative stress results from the attack of the ROS on the unsaturated fatty acids of the bacterial-cell membrane, where polyunsaturated fatty acids are the major substrates for lipid peroxidation in cell membrane (Ayala et al., 2014). Similar increase in MDA level by oxidative stress was reported previously in E. coli (Arenas et al., 2011; Joshi et al., 2011 and Hong et al., 2012).

In contrast to E. coli, K. pneumoniae strains subjected to H$_2$O$_2$ stress showed a slight but a significant decrease in the MDA level, although there was a reported significant increase in MDA level in K. pneumoniae exposed to the oxidative stress caused by lavender oil (Yang et al., 2020). This difference in the level of produced MDA by oxidative stress may be due to the difference in the used stressor. The lower level of MDA in H$_2$O$_2$ treated K. pneumoniae cultures compared to the control groups may also have resulted from a shift towards a higher level of saturation of membrane lipids to confer protection against oxidative stress and ROS attack, as suggested by Prione et al. (2016). However, similar to our results, a significant decrease in MDA level of Pantoea ananatis by oxidative stress was reported.

In S. aureus, no variation in the level of MDA by H$_2$O$_2$ treatment was detectable. This may be due to the presence of phosphatidylethanolamine (PE) as a minor component of the phospholipids of S. aureus cell membrane (Onyango and Alreshidi, 2018), while PE is a major component of the phospholipids of the E. coli outer membrane (Bogdanov et al., 2020). Poyton et al. (2016) revealed that the rate of oxidation increases linearly with the increase in PE content in the membrane. The difference in response to oxidative stress between S. aureus and E. coli can also be attributed to the presence
of a thicker peptidoglycan wall in S. aureus as suggested by Dakal et al. (2016) that may hinder the penetration of H₂O₂ inside S. aureus cells.

H₂O₂ has similar chemical properties to that of water and can be transported by the aquaporins (Bienert and Chaumont, 2014). Therefore, S. aureus can have a limited transport of H₂O₂, which can also explain their lack of response towards H₂O₂. However, some reports recorded a significant increase in the MDA level in S. aureus exposed to oxidative stress by blue light, which is an oxidative stress inducer (Wu et al., 2018).

The level of GSH was also measured as a defense mechanism used by many species against oxidative stress. There was a significant increase in GSH level in H₂O₂-treated E. coli cells. Similar increase in the GSH level was reported when different E. coli strains were exposed to different oxidative stressors (Arenas et al., 2011 and Smirnova et al., 2012). On the other hand, Korshed et al. (2016) reported a significant decrease in the GSH level in E. coli JM109 strain by silver nanoparticles-induced oxidative stress. It is unclear why H₂O₂-treated K. pneumoniae strains recorded a significant decrease in the level of GSH compared to untreated cells. K. pneumoniae are able to overcome the oxidative stress by increasing the level of various biomarkers as glutathione –S-transferase (Kulkarni et al., 2014).

In the present study, H₂O₂-treated S. aureus strains showed no significant difference in GSH levels. This may also be accounted for by the lack of canonical aquaporins in Gram-positive bacteria and consequently the limited H₂O₂ transport. On the contrary, the ROS produced due to treatment of S. aureus with silver nanoparticles which crossed the cell wall and the cell membrane reported a reduced GSH level (Dakal et al., 2016; Yuan et al., 2017 and Hamida et al., 2020).

Although, it was reported previously that enterobactin production increases with oxidative stress (Peralta et al., 2016), we reported a reduction in the level of enterobactin in both E. coli and K. pneumoniae strains tested under the effect of different peroxide concentrations. According to Achard et al. (2013), catechols not only function in iron uptake by the cells but they have the ability to scavenge the reactive oxygen species that enhance oxidative stress through Fenton reaction. This variation in enterobactin level on exposure to oxidative stress was suggested to arise from the effect of different regulators that predominate in low cell densities situation (Adler et al., 2014). According to Faulkner and Helmann (2011), peroxide stress may increase the expression of the E. coli Fur protein in a dose dependent manner; Fur protein is a negative regulator of the entC gene and this may be involved in modulating the effect of oxidative stress on enterobactin production. The role of catechol siderophores in alleviating the oxidative stress was reported previously in different organisms as Bacillus anthracis (Lee et al., 2011), Acinetobacter oleivorans (Kim et al., 2015), Salmonella enterica ser Typhimurium (Achard et al., 2013), and E. coli (Adler et al., 2014). In addition, catechol siderophores were reported to protect the bacteria from the oxidative stress caused by antimicrobial agents (Zhang et al., 2017).
CONCLUSION

Different bacterial species respond differently to oxidative stress. The used stressor may also affect the response of different organisms. The role of enterobactin in oxidative stress needs further evaluation.

REFERENCES


VARIABLE BACTERIAL RESPONSES TO OXIDATIVE STRESS IN...

الإستجابات المتنوعة لأنواع مختلفة من البكتيريا تجاه ضغط الأكسدة

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خلفية البحث: تتعبر الكائنات الحية لضغط أكسدة ناتج من كل من مؤثرات داخلية أو خارجية وذلك نتيجة عدم التوازن بين إنتاج والتخلص من جزيئات الأكسجين النشطة مما ينتج عنه فقدان الإنزيمات الداخلية للخلية.

الهدف من البحث: يهدف هذا البحث إلى دراسة تأثير مستوى ضغط الأكسدة على مستويات إنتاج الجلوتاتيون المختزل (GSH) كمضاد للأكسدة و ثنائي المالون الديهيد (MDA) كناتج من نواتج أكسدة الليبيدات بالإضافة إلى استجابة الاتروباكتين وذلك في أنواع مختلفة من البكتيريا.

مواد وطرائق البحث: تم قياس التركيز المشبكي الأدنى (MIC) لفوق أكسيد الهيدروجين (H2O2) لكل من بكتيريا الإشريكية الفولونية (Escherichia coli) و بكتيريا الكبستيلية الرنانة (Klebsiella pneumoniae) ATCC 25922 و بكتيريا الكبستيلية الرنانة ATCC 700603 باستخدام طريقة التخفيف المكروبية السائلة كما تم قياس مستوى كل من الجلوتاتيون المختزل و ثنائي المالون الديهيد في مجتمعات من البكتيريا مجموعة لم تتعرض لفوق أكسيد الهيدروجين والمجموعة الأخرى تعرضت لتأثير فوق أكسيد الهيدروجين المشبكي وذلك في كل من بكتيريا الإشريكية الفولونية و بكتيريا الكبستيلية الرنانة و بكتيريا المكروية العنقودية و المأخوذة من حالات مرضية. كما تم قياس مستوى التعبير الجيني لجين الإتريوباكتين entC المسؤول عن تخليق الإنزيم المحفز لإنتاج الاتروباكتة الذي يشارك في عمليات التحكم في ضغط الأكسدة الخلوي وذلك باستخدام طريقة تفاعل البلمرة المتساعد الكمي وقد تم ذلك القياس تحت تأثير 0.25 و 0.5 وحدة MIC.
نتائج البحث: وقد وجد أن التركيز المثبط الأدنى (MIC) لفوق أكسيد الهيدروجين
(ESHERICHIA COLI) (MIC) يساوي 1.5 مل مول لكل مرن بكتيريا الإشريكية الفولونية
(KLEBSIELLA PNEUMONIAE) و بكتيريا الكبسيل الرئوية ATCC 25922. وقد أدى تعرض بakteيريا الإشريكية الفولونية لفوق أكسيد الهيدروجين إلى زيادة معنوية في كل من تركيز البلاكيناتين المختزل وثنائي المالمون الديهايد. أما في بكتيريا الكبسيل الرئوية فتعود فرضها لفوق أكسيد الهيدروجين أدى إلى انخفاض معنوي في كل من تركيز البلاكيناتين المختزل وثنائي المالمون الديهايد كما لم تبد بكتيريا المكروية العنقودية أي تغيير في تركيزات كل من البلاكيناتين المختزل وثنائي المالمون الديهايد أما مستوى التعبير فقد أدى إنخفاضا في كل من بكتيريا الإشريكية الفولونية entC الجيني للجنين و بكتيريا الكبسيل الرئوية (ESHERICHIA COLI) ATCC 25922. وأدى التعبير في كانت في كل من بكتيريا الإشريكية الفولونية (KLEBSIELLA PNEUMONIAE) ATCC 700603. المعروضة لتأثير 0.25 و0.5 وحدة (MIC) من فوق أكسيد الهيدروجين.

الاستنتاج: نستنتج من هذه الدراسة أن البكتيريا تستجيب بطريقة مختلفة بضغط الأكسدة وأن البكتيريا المكروية العنقودية هي أقل الأنواع تتأثر بضغط الأكسدة في هذه الدراسة كما أن دور الإنتروباكتين في الاستجابة لضغط الأكسدة يحتاج لمزيد من التقييم.

الكلمات الدالة: ضغط الأكسدة، وثنائي المالمون الديهايد، البلاكيناتين المختزل، إنتروباكتين، الإشريكية الفولونية، الكبسيل الرئوية، المكروية العنقودية.