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Effect of Helium/Neon Laser Radiation, Sodium Hypochlorite, and Other Selected Disinfectant Combinations on the Killing of Disinfectant-Resistant *Staphylococcus aureus* Isolated from Wounds

Mohemid Maddallah Al-Jebouri[1]*, Najim A. Yasin[2]

[1]Department of Medical Laboratory Technology, Al-Qalam University College, Kirkuk, Iraq
[2]Department of Microbiology, College of Medicine, University of Duhok, Duhok, Iraq
Email: *profaljebouri@yahoo.com

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Abstract

Laser as a new phototherapeutic approach was proposed with its basic theory since the beginning of the last century. It was used in wound disinfection, particularly with antibiotics, as well as disinfectant-resistant bacterial pathogens. This lethal photosensitization is thought to be due to membrane-induced damage arising from free radicals generated by photoactivated sensitizers and also membrane and wall damage induced by sodium hypochlorite disinfectant. The laser was a helium-neon (He/Ne) gas type with a measured output at 5 mW (Laser Beacon, I. N. C., Michigan, USA) used in the present study. Toluidine blue 0 and povidone-iodine as photosensitizers were used. Hypochlorite (Britain Drug Home) was utilized. Selected disinfectant-resistant strains of *S. aureus* were tested. Using the minimal inhibitory concentration of different antibiotics was assessed, and its effect on total viable counts (TVC's) of strains was concluded. The effect of laser, sodium hypochlorite, and laser-sodium hypochlorite on selected strains of *S. aureus* was observed. There were significant decreases in TVC's after exposure to the laser–sodium hypochlorite combination compared to laser, povidone-iodine, or sodium hypochlorite. Exposure of bacterial strains to chemical disinfectants at subminimal inhibitory concentrations caused a sharp decrease in total viable counts of disinfectant-resistant *S. aureus*, particularly after exposure to the laser-povidone-iodine-sodium hypochlorite combination, with significant statistical differences in viable counts of the pathogen tested ($P \leq 0.05$). The present study revealed that the effect of sodium hypochlorite reduced the TVC's moderately, whereas the combination of laser and sodium hypochlorite showed a dramatic and consistent reduction in TVC's with minimal variation, revealing strong and reliable antimicrobial synergy.

Keywords

Wounds, Infection, *S. aureus,* Laser Radiation, Sodium Hypochlorite, Killing

1. Introduction

The mode of action of disinfectants on bacterial cells is variable. Savlon, cetrimide, and chlorhexidine are bactericidal at high concentrations and bacteriostatic at low concentrations, and cause lesions in the cell membrane and leakage of components. Povidone-iodine causes disorder of protein structure, oxidation of (-SH) groups in amino acids, and membrane immobilization. Chloroxylenol (Dettol) causes poisoning of the protoplasm and disruption of the cell wall and its proteins [1]-[3]. Serial passage of bacteria through diluted disinfectants not only increases the minimal inhibitory concentration, but also brings about phenotypic changes in their antibiogram [4]-[6]. In a study conducted elsewhere [7] [8] on the effect of disinfectant exposure, it was found that *Staphylococcus aureus* strains become resistant to many antibiotics after growing in a series of diluted disinfectants. The mechanisms have not been fully studied, and it was suggested that the disinfectants probably altered sites in the bacterial ribosome, making it selectively less susceptible to certain antibiotics. Others suggested that enzymes involved in peptidoglycan synthesis might be destroyed, causing resistance to penicillins and cephalosporins. Destruction of periplasmic enzymes by groups of disinfectants is also another contributing factor [7] [9]. Unjustifiable use of disinfectants in improper and sublethal dilutions and wide usage for long periods should be avoided, since it gives a chance for survival and multiplication of bacteria due to the development of resistance to these agents as well as to many antibiotics [4]. These resistant variants, which multiply in the hospital environment, could lead to serious epidemic outbreaks. Also, the consequent changes in phage susceptibility patterns might affect the epidemiological reporting [10]. Low-power lasers deliver low doses of light over long periods and cause photochemical effects on irradiated cells [11] [12]. Helium-Neon lasers are the most common lasers used in the killing of bacteria. When its output is at 632.8 nm, the maximum power is still weak and not useful in surgery (50 - 60 nm), continuous waves penetrate up to 1.5 cm depth in tissue [13] [14]. *Helicobacter pylori* could be killed by low-power laser light in the presence of a photosensitizer [15]. Non-pigmented bacteria are not affected by low-power laser light [16]. Black-pigmented anaerobic bacteria such as *Porphyromonas gingivalis* and *Prevotella intermedia* would be susceptible to killing by low-power laser light as a result of endogenous photosensitization [17] [18]. Appropriate photosensitizers can render transparent organisms susceptible to killing by low-power laser light. Gram-positive *Sarcina lutea*, *Escherichia coli*, and *Pseudomonas aeruginosa* species could be killed by He-Ne laser light after treatment with toluidine blue O, TBO [19] [20]. Martinetto *et al.* [21] used haematoporphyrin as a photosensitizer and found that *S. aureus* and *E. coli* could be killed by He-Ne laser light. Povidone-Iodine (PI) was used by Al-Jebouri and Al-Obaidy as a photosensitizer, which was more effective than toluidine blue O [22].

2. Materials and Methods

Isolation and Identification

Wound specimens from patients residing in Tikrit Teaching Hospital were obtained with disposable sterile cotton on the third post-operative day. Each sample was sub-cultured on blood agar and mannitol salt agar and incubated at 37˚C for 24 hours. The purified colonies suspected of being *Staphylococcus aureus* were cultured on slants and kept at 4˚C for further investigations. The suspected colonies were conventionally identified following Cowan and Steel [23].

Effect of disinfectant exposure on *Staphylococcus aureus*

Ten strains of the pathogen were exposed to five types of chemical disinfectants as shown in Table 1. The series of disinfectant exposures was as follows: Hibitane, Dettol, Cetrimide, Savlon, and Providine-iodine. All strains were inoculated into nutrient broth and incubated at 37˚C for three hours. The growth yielded 10^6 - 10^7 cells/ml. The plates of Muller-Hinton agar containing doubling dilutions of disinfectants were prepared. Strains grown in nutrient broth cultures were sub-cultured by streaking onto Muller-Hinton agar containing the first disinfectant and incubated at 37˚C for 8 hours. Colonies from the Subminimal Inhibitory Concentration (SMIC), which is the last visible bacterial growth, were picked up and inoculated into nutrient broth and incubated at 37˚C for three hours to expose to the second disinfectant and so on for the remaining disinfectants. The second exposure to the same disinfectant was repeated for the same strains as it was done in the first exposure. The strains after the second exposure were kept on nutrient agar slants at 4˚C for further studies.

Table 1. Disinfectants used in susceptibility testing of *Staphylococcus aureus* isolated from wounds.

Disinfectant	Scientific name	Commercial concentration	Concentration Used (μg/ml)	Manufacturer
Hibitane	Chlorohexidine gluconate	5%	0.5 - 128	I. C. I (Britain)
Cetrimide	Cetrimide	Pure powder	0.5 - 128	I. C. I. (Britain)
Dettol	Chloroxylenol	10%	0.5 - 128	S. D. I. (Sammara, Iraq)
Providine-iodine	PI	10%	0.5 - 1024	I. C. I. (Britain)
Savlon	Chlorohexidine: Cetrimide	0.3% Chlorohexidine: 3% Cetrimide	0.3 cetrimide:0.03% chlorohexidine/ −76.8 ctrimide:7.68 chlorohexidine	I. C. I. (Britain)

The effect of laser light exposure on *Staphylococcus aureus*

The laser was a Helium-Neon (He/Ne) gas type with a measured output of 5 mW (Laser Beacon, I. N. C, Michigan, USA) used in the present study. The emitted light had a wavelength of 632.8 nm [24].

Photosensitizers

Toluidine blue O (Sigma, USA) and providine-iodine (I. C. I., Britain) were used.

Sodium hypochlorite disinfectant

Sodium hypochlorite disinfectant (Britain Drugs Home, Britain) with a concentration of 5000 μg/ml was used alone or in combination with laser, other disinfectants, and photosensitizers.

The effect of laser and Toluidine Blue O (TBO) and povidone-iodine on *Staphylococcus aureus*

Four strains of non-disinfectant, firstly disinfectant, and secondly disinfectant-exposed strains of *S. aureus* were used in this test. The lethal photosensitization of *S. aureus* was performed using toluidine blue O and povidone-iodine sensitizers as described elsewhere [11] [25].

The effect of laser-sodium hypochlorite combination on *Staphylococcus aureus*

Four strains of *S. aureus* were selected and exposed to laser, sodium hypochlorite, and laser-sodium hypochlorite combinations. The series of exposure times to all these combinations for killing purposes was 0.5, 1, 2, 4, and 8 minutes. Sodium hypochlorite was used at a final concentration of 5000 μg/ml. The method used in this test was the same as that used for the laser-toluidine blue O combination as mentioned before. The exposed strains were kept on nutrient agar slants at 4˚C for further studies.

The effect of laser-toluidine blue O-sodium hypochlorite combination on *Staphylococcus aureus*

Four strains of disinfectant non-exposed, firstly disinfectant-exposed, and secondly disinfectant-exposed strains of *S. aureus* were used in this test. Each strain was grown in nutrient broth at 37˚C for 16 hours, then it was harvested by centrifugation and resuspended in an equal volume of 0.9% (W/V) saline [26]. Aliquots (100 ml) of saline suspension were transferred to the sterile test tube, and an equal volume from each filter-sterilized solution of toluidine blue O in 0.9% (W/V) saline and sodium hypochlorite in distilled water was added to each tube to give a final concentration equal to 12.5 μg/ml of toluidine blue O and 5000 μg/ml of sodium hypochlorite. The test tube was placed on the magnetic stirrer and exposed to laser light for 0.5, 1, 2, 4, and 8 minutes. Control tubes containing the bacterial suspension plus saline solution were treated in an identical manner to determine the effect of laser irradiation alone on bacterial viability [27]. Four more tubes, identical to those described above, were prepared. A further irradiation of the appropriate tubes, 10-fold serial dilution of the contents of each tube was prepared on the surface of blood agar plates.

The effect of laser-providine-iodine-sodium hypochlorite combination on

Staphylococcus aureus

Four strains of disinfectant non-exposed, firstly disinfectant-exposed, and secondly disinfectant-exposed strains of *S. aureus* were used in this test and exposed to laser, laser-providine-iodine-sodium hypochlorite combination. The series of exposure times was 0.5, 1, 2, 4, and 8 minutes. Providine-iodine and sodium hypochlorite were used at final concentrations of 64 and 5000 μg/ml, respectively. The method used in this test was the same as that used for the laser-toluidine O-sodium hypochlorite combination, which was described before.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics such as means, standard deviations, and frequency distributions were computed to summarize the data. Linear regression and multivariate linear regression models were applied to examine the relationships between variables. Where appropriate, interaction terms were included to evaluate the effect modification between predictors. An exponential decay model was used to describe the decreasing trends in the outcome variable over time, and the model was linearized using natural logarithm transformation for compatibility with linear regression frameworks. The significance of individual coefficients in the regression models was assessed using t-tests, with a p-value < 0.05 considered statistically significant. Assumptions of normality, linearity, homoscedasticity, and multicollinearity were checked prior to model interpretation. All graphical outputs and residual diagnostics were also generated using SPSS. In addition, for confirmation of model robustness, selected analyses were repeated using R version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Effect of laser, sodium hypochlorite, and their combination

The effect of laser, sodium hypochlorite, and laser-sodium hypochlorite combination on 4 strains of *S. aureus* was shown in Table 1. There were significant decreases in Total Viable Counts (TVCs) after exposure to the laser-sodium hypochlorite combination, e.g., TVCs of strain no. 4 before exposure were 590×10^8 and then reduced to 8×10^8 after exposure to laser-sodium hypochlorite for 4 minutes, while TVCs reached 570×10^8 after exposure to laser light alone for the same time of exposure, *i.e.*, 4 minutes. The differences in the TVCs after exposure to laser light alone were statistically not significant ($P \geq 0.05$) using the Microstat test. However, there were statistically significant differences in TVCs after exposure to the laser-sodium hypochlorite combination using the same test, and the P value was less than 0.05. The correlation coefficient is almost equal to 0.999 when different cells of values were compared and tested (**Table 2**). The forest plot revealed that the laser combined with sodium hypochlorite yielded the most substantial and statistically confident reduction in total viable counts of *S. aureus*. Laser alone did not show a significant reduction in bacterial total numbers, while laser with sodium hypochlorite revealed a significant decrease in viable bacteria.

The forest plot illustrates the mean reduction in total viable count (TVC × 10^8) for each treatment relative to the baseline, *i.e.*, before laser with 95% confidence intervals (**Figure 1**).

Table 2. The effect of Laser (L) and Laser-sodium hypochlorite combination on the Total Viable Counts (TVCs) for four selected strains of *Staphylococcus aureus* isolated from wounds before chemical disinfectant exposure.

Strain	TVC's x 10^8 before exposure to laser	TVC's x 10^8 after exposure to the laser	TVC's x 10^8 after Exposure to sodium hypochlorite	TVC's after exposure to Laser + sodium hypochlorite combination
1	500	490	290	14
2	505	505	310	30
3	460	470	260	20
4	590	570	305	8

Correlation coefficient equal to 0.999 for different cells.

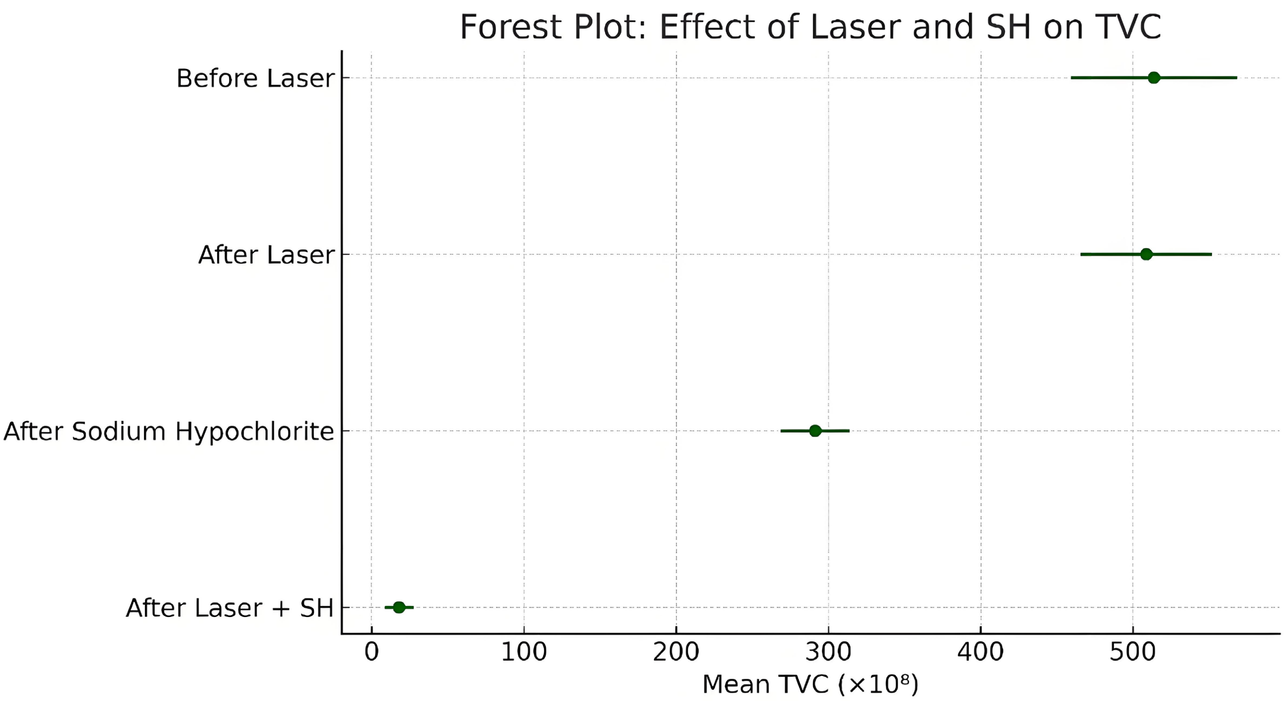

Figure 1. Forest plot of Total Viable Count (TVC) reduction by different combinations of treatment.

Effect of laser and sodium hypochlorite combinations on total viable counts

Table 2 shows the effect of laser-sodium hypochlorite for 0.5, 1, 2, 4, and 8 minutes on the Total Viable Counts (TVCs) of the four strains of *Staphylococcus aureus* before and after exposure to disinfectants. There were significant decreases in TVCs after increasing time exposure ($P < 0.05$) using the Duncan test. The correlation coefficient ranged between 0.692 and 0.920 for different cells (**Table 3**). The interaction plot illustrates how total bacteria decreased over time for different exposure types. This explanatory diagram indicates a clear interaction effect for all groups, which decreased over time, but the reduction was more rapid in the secondly exposed group (**Figure 2**).

Table 3. The effect of Laser-Sodium Hypochlorite (LSH) on the Total Viable Counts (TVC's) for 4 strains of *Staphylococcus aureus* isolated from the wounds at various types of chemical disinfectant exposure times.

Time	Non exposed				Firstly exposed				Secondly exposed			
min	1	2	3	4	1	2	3	4	1	2	3	4
0	40	82	56	84	68	66	78	64	54	68	66	38
0.5	24	80	26	44	18	38	15	40	22	26	14	18
1	16	70	24	10	10	24	14	34	8	14	6	18
2	14	44	28	8	8	14	40	16	2	8	4	4
4	14	30	20	8	4	8	10	6	0	4	2	2
8	0	0	0	0	0	0	0	0	0	0	0	0

Correlation coefficients ranged between 0.692 and 0.920 for different cells.

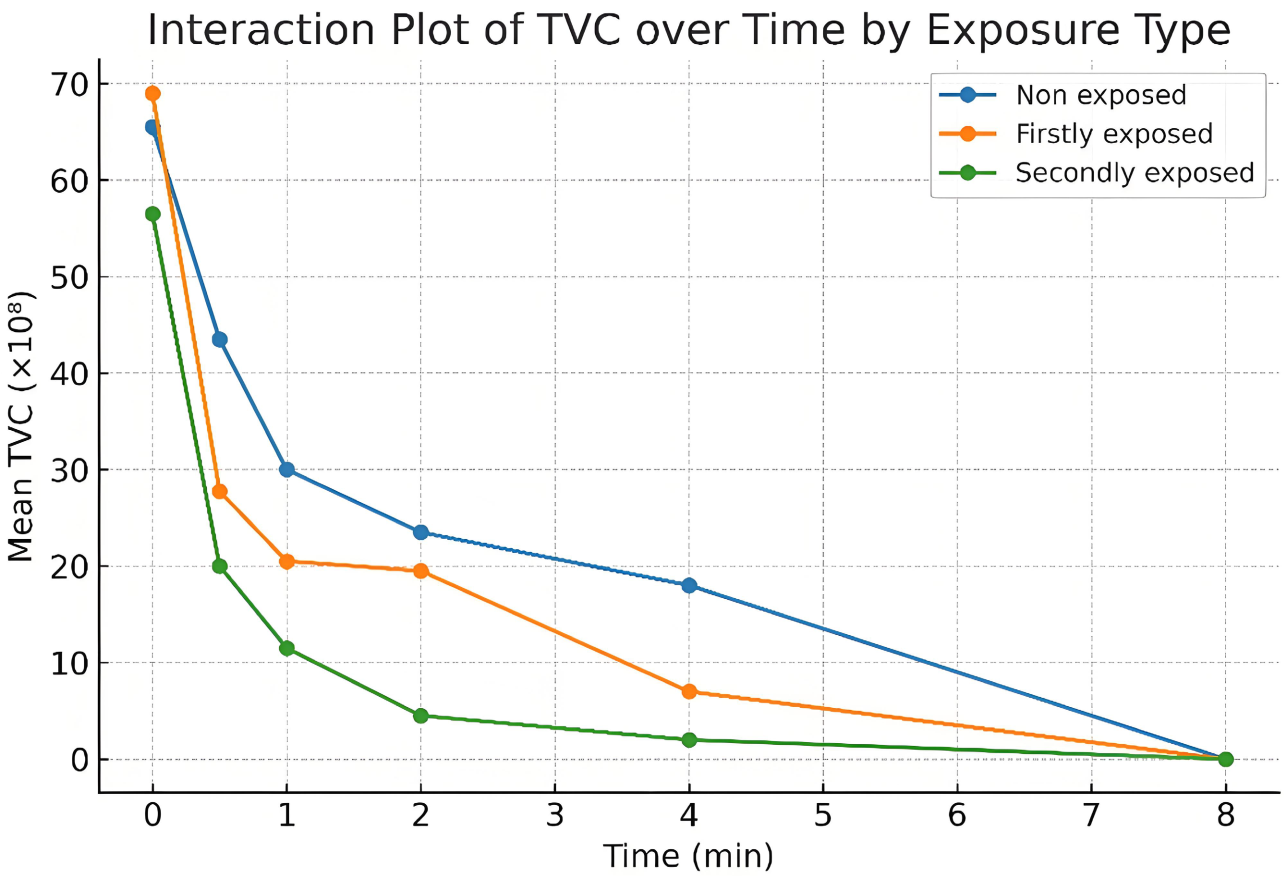

Figure 2. The interaction plot shows the relation between time of exposure and total viable counts after treatment with different combinations.

Effect of laser, povidone-iodine, and sodium hypochlorite combinations on the total viable counts

The effect of laser, laser-providine-iodine, and Laser-Providine-Sodium Hypochlorite (LPISH) was shown in Table 4. There were significant decreases in TVCs after exposure to different combinations of LPISH, e.g., TVCs of strain no. 4 before exposure were 400×10^8, and the number reduced to zero after exposure to

LPISH. The differences in TVCs among all types of irradiation were statistically significant ($P < 0.05$) using the Duncan test. The correlation coefficient exceeded 0.9 (Table 4). Figure 3 shows a general decrease in the total number of viable bacteria, and three minutes' time was almost an inflection point of divergence-like action, particularly with no agent compared to the laser effect for 30 minutes (Figure 3).

Table 4. The effect of laser, Laser + povidone-iodine, and Laser + povidone-iodine + sodium hypochlorite on Total Viable Counts (TVC's) for 4 strains of *Staphylococcus aureus* isolated from wounds after the second exposure to chemical disinfectant

Time min	No L* + PI + SH	L/32 min	L + PI/1 min	L + PI + SH/1 min
1	540	500	6	0
2	400	190	2	6
3	310	300	0	4
4	400	190	7	0

The correlation coefficient ranged between 0.901 - 0.999 for different cells. *, L = Laser; PI = Povidone-Iodine; SH = Sodium Hypochlorite.

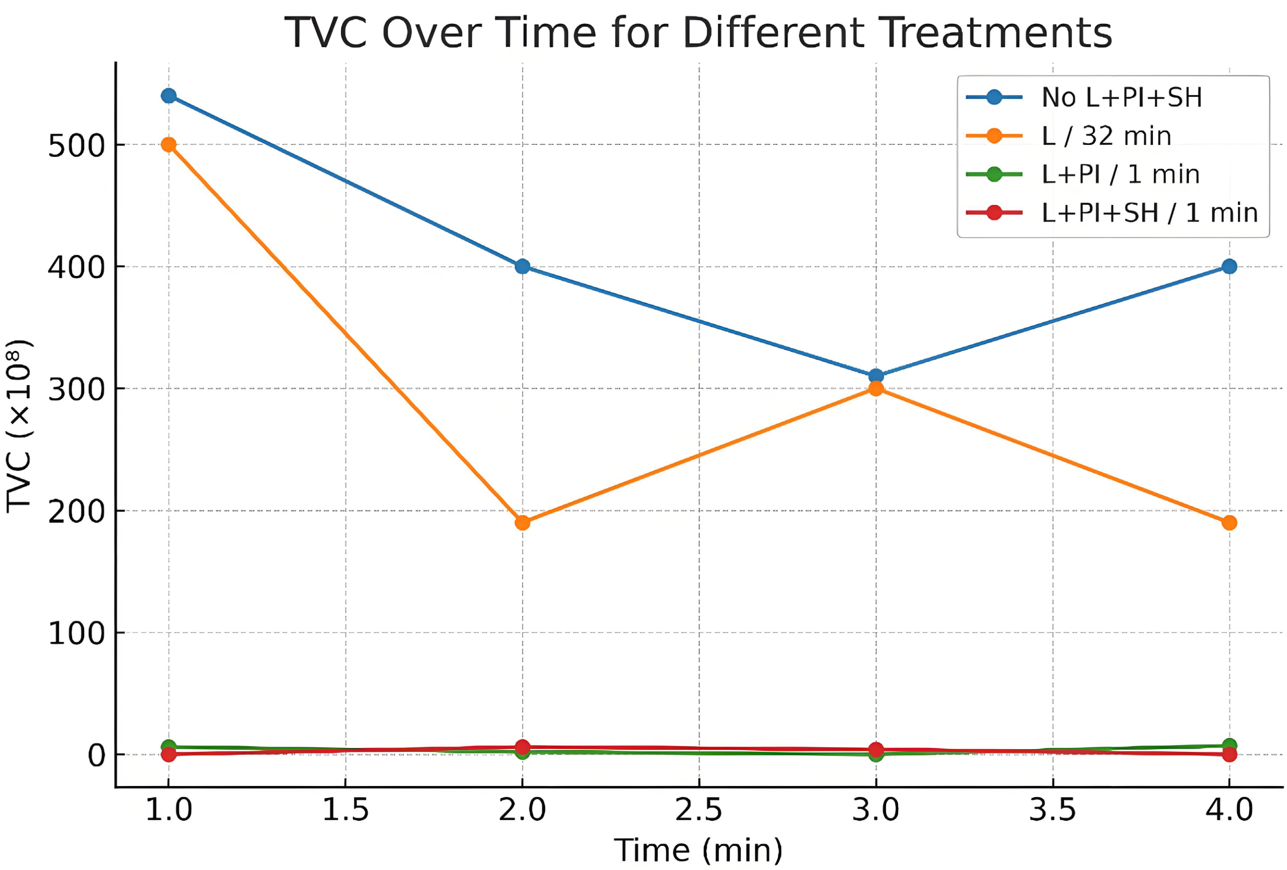

Figure 3. Total viable counts of *S. aureus* exposed for different times to different combinations of antimicrobial agents.

Effect of different types of exposure with reference to time

Table 5 shows the effect of the Laser-Providine-Sodium Hypochlorite combination (LPISH) for 0.5, 1, 2, 4, and 8 minutes on the TVCs for the 4 strains of *S. aureus* before and after exposure time to disinfectants. There were significant decreases in TVCs after an increase in the exposure time, e.g., TVCs of the non-disinfectant-exposed strain no.1 were 50 × 10^8 and then reduced to zero after 2 minutes' exposure time to LPISH (Table 5). The difference in TVCs among various times, types of disinfectant exposure, and types of strain was statistically

Table 5. The effect of Laser + Providine-Iodine + Sodium Hypochlorite (LPISH)* combinations on the Total Viable Counts (TVC's) for 4 strains of *Staphylococcus aureus* isolated from the wounds at various times and exposures to chemical disinfectants.

Time	TVC's × 10^8 for non-disinfectant exposed strains				TVC's x 10^8 Firstly, disinfectant-exposed strains				TVC's x 10^8 Secondly, disinfectant-exposed strains			
	1	2	3	4	1	2	3	4	1	2	3	4
0	50	40	36	120	40	50	52	86	50	40	48	90
0.5	14	28	28	72	12		16	44	8	10	8	30
1	4	28	18	42	10	16	0	14	0	6	4	0
2	0	12	2	2	6	10	0	10	0	0	0	0
4	0	4	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0

*, L = Laser; PI = Povidone-Iodine, SH = Sodium Hypochlorite. The correlation coefficient ranged between 0.224 and 0.963 for different cells.

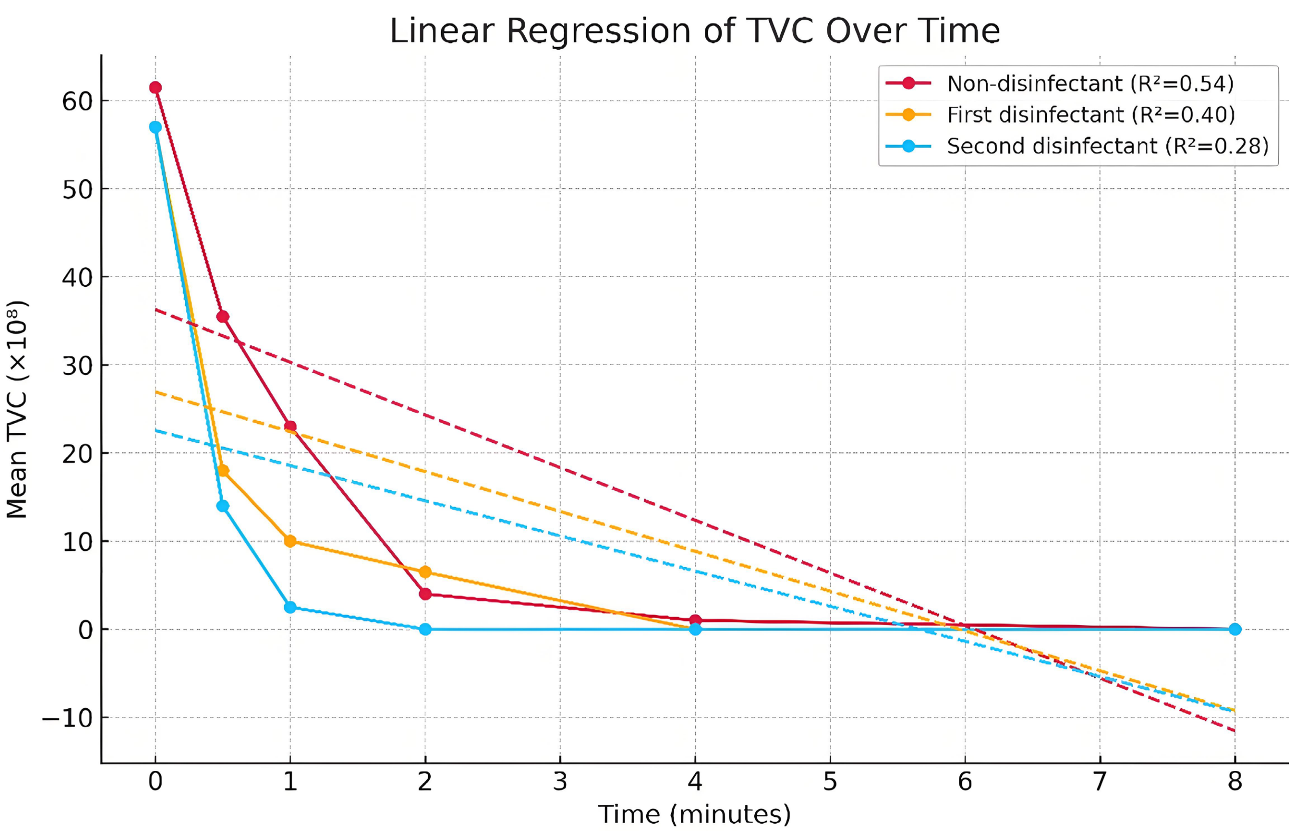

Figure 4. Linear regression of Total Viable Count decay over time of exposure.

significant ($P < 0.05$) using the Duncan test. The correlation coefficient ranged between 0.224 and 0.963 for different cells. Figure 4 shows that all groups had negative slopes. Total viable bacteria decreased over time, and the second disinfectant-exposed group revealed the steepest slope, demonstrating the fastest decline. R^2 values were moderate, indicating that a linear model partially explained the decay mode of behavior (Figure 4).

Effect of laser, toluidine blue O, sodium hypochlorite, povidone-iodine, and their combinations on total viable counts

Table 6 shows the effect of agents on the TVCs of *S. aureus* strains before and after exposure to the disinfectants and/or laser irradiations at different times of exposure. It was found that the laser + providine-iodine + sodium hypochlorite combination was the most effective factor compared to other agents of exposure, especially against secondarily disinfectant-exposed strains. The differences in the TVCs among the types of disinfectant exposure after exposure to laser + sodium hypochlorite were statistically significant ($P < 0.05$) using the Duncan test. The differences in TVCs after laser + sodium hypochlorite exposure for 0.5, 1, 2, 4, and 8 minutes were statistically significant ($P < 0.05$) using the same test. The differences in TVCs after 0.5, 1, 2, 4, and 8 minutes were statistically significant ($P < 0.05$). There were significant differences in TVCs between types of various disinfectant exposures and various exposure times after laser + toluidine blue O + sodium hypochlorite exposure ($P < 0.05$). The differences in TVCs after laser + providine-iodine + sodium hypochlorite combination at various exposure times of 0.5, 1, 2, 4, and 8 minutes were statistically significant ($P < 0.05$) using the Duncan test. There was a highly significant decrease in TVCs between laser + providine-iodine + sodium hypochlorite exposure and nonexposed total viable counts ($P < 0.05$) using the Duncan test. This study revealed that the *S. aureus* was killed after exposure to Laser-Providine-Iodine-Sodium Hypochlorite for 1 minute among secondly disinfectant-exposed strains, e.g., TVCs of strain no. 3. The second disinfectant-exposed strain before exposure to LPISH was 500×10^8 and then became zero after exposure to the same combination for one minute of treatment (Table 6). The forest plot reveals that the effect of sodium hypochlorite reduced the TVCs moderately, whereas the combination of laser and sodium hypochlorite showed a dramatic and consistent reduction in TVCs with minimal variation, revealing strong and reliable antimicrobial synergy (Figure 5).

Effect of exposure time on total viable counts of *Staphylococcus aureus*

It was found that different numbers of viable counts of the pathogen under study were significantly affected by three modes of exposure to different combinations of laser, povidone-iodine, toluidine blue O, and sodium hypochlorite for 32, 8, and 4 minutes of exposure. The statistical analyses showed significant differences in numbers of viable counts exposed to different times, and the P value was less than 0.05 using Duncan test (Table 7). The correlation coefficient did not exceed 0.240 with respect to time of exposure but showed a high association between values according to various combinations of exposure agents, with an almost 0.900 value (Table 7). Figure 6 shows that the lowest TVC levels appeared in multi-treatment combinations (e.g., L + PI + SH, L + TBO + SH), particularly in the second exposure; non-exposed groups have the highest TVC, as expected, whereas the Laser (L) treatments consistently reduced TVC more than single-agent treatments.

Table 6. Total Viable Counts (TVCs) of 4 strains of non-disinfectant exposed, first and second disinfectant exposed *Staphylococcus aureus* strains isolated from the wounds before and after exposure to Toluidine Blue O (TBO), PI (Povidone-Iodine (PI)), and Laser (L), Sodium Hypochlorite (SH) combinations (×10^8).

Exposure type*	Time (min)	Non-exposed				Firstly exposed				Secondly exposed			
		1	2	3	4	1	2	3	4	1	2	3	4
Non	32	500	505	460	500	460	380	430	380	560	600	500	500
TBO	32	400	350	400	350	120	280	300	200	75	80	60	85
PI	32	60	43	140	120	100	300	290	200	75	80	60	85
SH	32	50	50	68	120	60	64	90	86	80	82	88	90
L	32	410	370	410	460	300	260	300	220	500	190	300	190
L + SH	8	0	0	0	0	0	0	0	0	0	0	0	0
L + TBO	32	14	18	12	60	25	12	8	2	6	8	1	1
L + TBO + SH	8	0	0	0	0	0	0	0	0	0	0	0	0
L	16	460	440	450	410	350	270	305	250	520	215	300	240
L + SH	4	14	30	20	8	4	8	10	6	0	4	4	2
L + TBO	16	28	18	15	58	16	10	19	39	30	7	21	35
L + TBO + SH	4	4	20	0	10	2	4	0	4	0	2	0	0
L + PI	16	20	0	8	0	0	0	0	0	0	0	0	0
L + PI + SH	8	0	0	0	0	0	0	0	0	0	0	0	0
L	8	500	455	460	520	345	255	300	245	510	300	320	235
L + SH	2	14	44	28	8	8	14	40	16	2	8	4	4
L + TBO	8	48	17	29	70	42	13	18	52	29	15	15	36
L + TBO + SH	2	10	22	40	18	8	14	0	10	4	14	8	4
L + PI	8	9	0	0	0	2	0	0	4	0	0	0	0
L + PI + SH	4	0	4	0	0	0	0	0	0	0	0	0	0
L	4	490	505	470	270	360	310	370	295	565	360	390	360
L + SH	1	16	70	24	10	10	24	14	34	8	14	6	18
L + TBO	4	68	28	40	86	76	29	35	61	48	25	20	52
L + TBO + SH	1	20	34	64	22	14	20	10	16	18	16	8	12
L + PI	4	27	0	32	8	29	0	0	0	0	0	0	0
L + PI + SH	1	4	28	18	42	10	16	0	14	0	6	4	0
L + PI	2	30	1	0	20	5	0	0	10	29	0	0	6
L + PI + SH	0.5	14	28	28	72	12	18	16	44	8	10	8	30
L + PI	1	14	52	0	13	5	15	0	8	6	2	0	7
L + PI	0.5	17	40	1	8	5	14	3	12	35	5	3	12

Correlation coefficient ranged between 0.892 and 0.934. *, TBO = Toluidine Blue O; L = Laser; PI = Povidone-Iodine; SH = Sodium Hypochlorite.

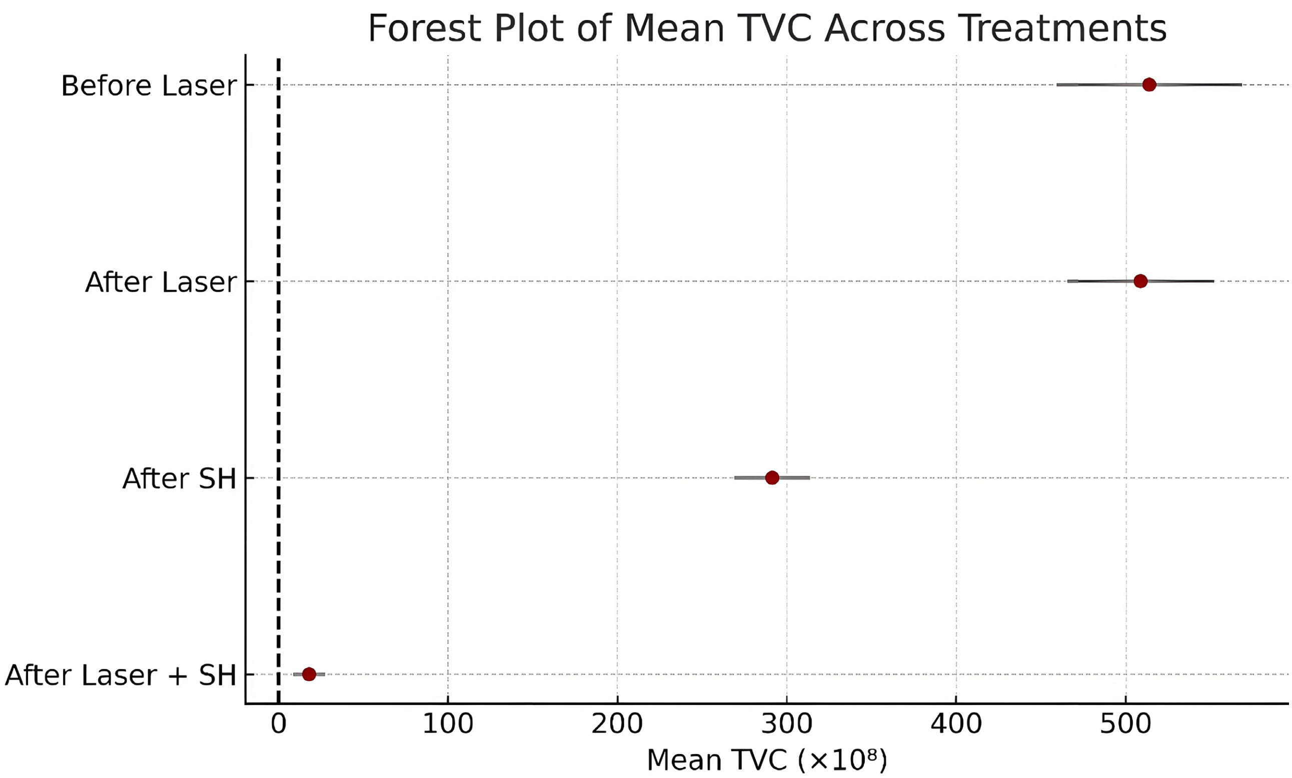

Figure 5. The forest plot illustrates the mean Total Viable Count (TVC × 10^8) for each treatment condition along with standard deviation error bars.

Table 7. Total Viable Counts (TVC's) of 4 strains of non-disinfectant-exposed, first and second disinfectant-exposed *Staphylococcus aureus* strains isolated from the wounds before and after exposure to Toluidine Blue O (TBO), Povidone-Iodine (PI), Laser (L), Sodium Hypochlorite (SH) combinations for three times of exposure.

Type of exposure*	Time/Strain	Nonexposed				Firstly exposed				Secondly exposed			
		1	2	3	4	1	2	3	4	1	2	3	4
Non	32	500	505	460	500	460	380	430	380	560	600	500	500
TBO	32	400	350	400	350	120	280	300	200	75	80	60	85
PI	32	60	43	140	120	100	300	290	200	75	80	60	85
SH	32	50	50	68	120	60	64	90	86	80	82	88	90
L	32	410	370	410	460	300	260	300	220	500	190	300	190
L + TBO	32	14	18	12	60	25	12	8	2	6	8	1	1
L + PI + SH	8	0	0	0	0	0	0	0	0	0	0	0	0
L	8	500	455	460	520	345	255	300	245	510	300	320	235
L + TBO	8	48	17	29	70	42	13	18	52	29	15	15	36
L + PI	8	9	0	0	0	2	0	0	4	0	0	0	0
L + TBO	8	48	17	29	70	42	13	18	52	29	15	15	36
L + PI	8	9	0	0	0	2	0	0	4	0	0	0	0
L + SH	4	14	30	20	8	4	8	10	6	0	4	4	2
L + TBO + SH	4	4	20	0	10	2	4	0	4	0	2	0	0

Continued

L + PI + SH	4	0	4	0	0	0	0	0	0	0	0	0	0
L	4	490	505	470	270	360	310	370	295	565	360	390	360
L + TBO	4	68	28	40	86	76	29	35	61	48	25	20	52
L + PI	4	27	0	32	8	29	0	0	0	0	0	0	0

Correlation coefficients ranged from 0.121 to 0.240 due to time of exposure and from 0.876 to 0.916 depending on types of exposure.
*, TBO = Toluidine Blue O; PI = Povidone-Iodine; SH = Sodium Hypochlorite; L = Laser.

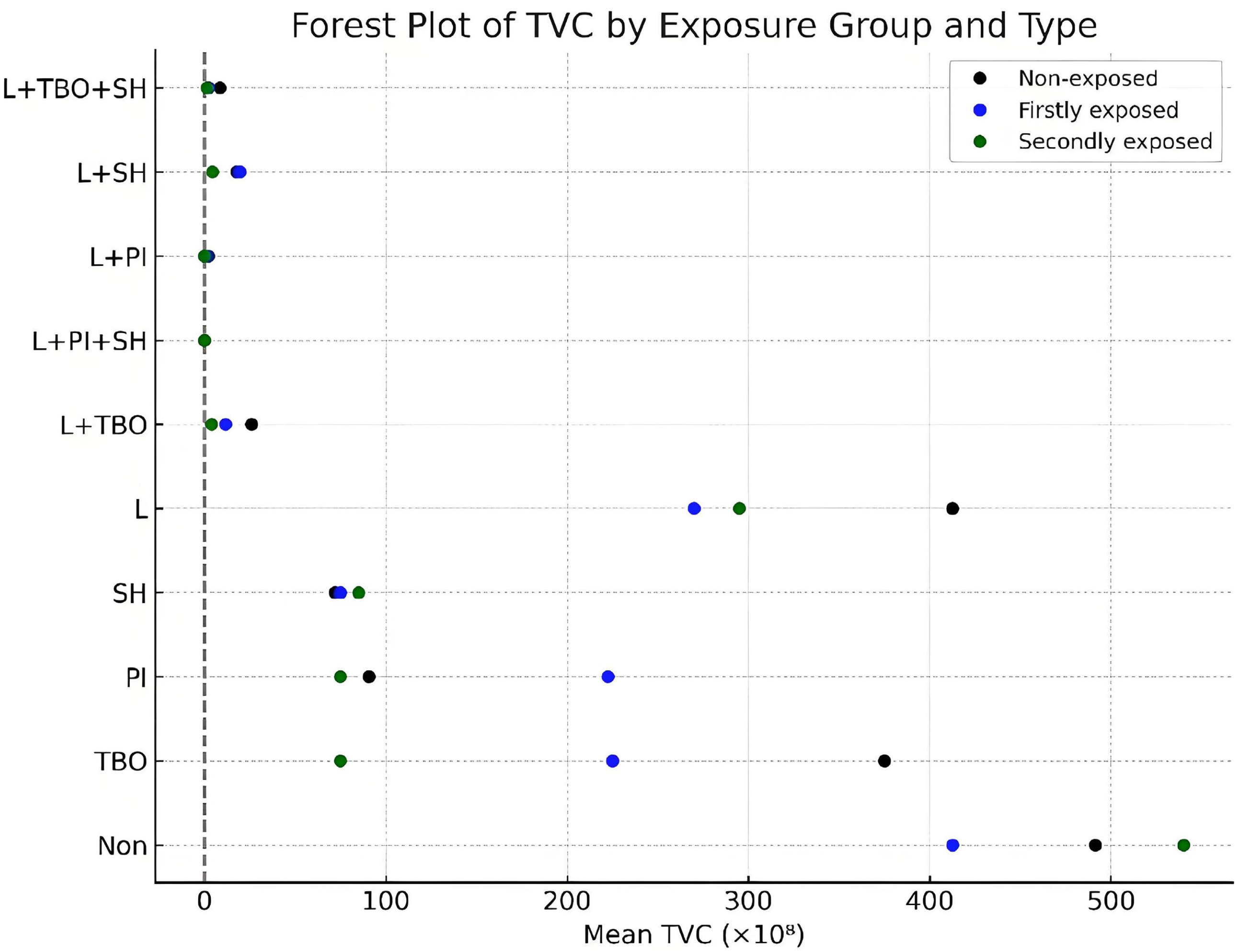

Figure 6. The forest plot reveals the mean Total Viable Count (TVC) ($\times 10^6$) across different exposure types and exposure times.

4. Discussion

It was shown that the exposure of *S. aureus* to laser, laser-toluidine blue O combination, and laser-providine iodine combination leads to a decrease in its total viable counts, but this decrease was very high when the above photochemical treatments were combined with sodium hypochlorite (Tables 2-7). For example, the TVCs of strain no.1 were 505 $\times 10^8$ before exposure, then became 455, 17, and 0 $\times 10^8$ after exposure to laser, laser-toluidine blue O, and laser-providine iodine combinations for eight minutes. The present data revealed that a reduction in the

TVCs was time-dependent, *i.e.*, as long as the exposure time continues, the total viable counts are reduced. Wilson et al. indicated that the exposure of a bacterial suspension of *S. aureus* to the He/Ne laser light (35 mW) for 60 seconds in the absence of TBO caused a small reduction in the viable count; they also demonstrated in other studies that exposure of S. aureus to the same laser type (7.3 mW) in the absence of the dye resulted in a decrease in the viable count, but not statistically significant [26]. On the other hand, Al-Jebouri and Al-Obaidy demonstrated that the TVCs for one strain of *S. aureus* were 550×10^8 before exposure and became 300, 250, 400, and 420×10^8 after exposure to laser light for 32, 16, 8, and 4 minutes, in the presence of dye, respectively [22]. In contrast, Hardee et al. found that there were no significant differences observed in the reduction of colony-forming units among groups of *Bacillus stearothermophilus* when pulsed Nd: YAG laser radiation was used with and without 0.5% sodium hypochlorite combination [27]. In the presence of 12.5 μg/ml of Toluidine Blue O (TBO) and sodium hypochlorite combination, the TVCs of strain no.1 decreased from 500×10^8 to 0, 4, 10, and 20×10^8 after exposure to laser-TBO-sodium hypochlorite combination. Wilson [11] demonstrated that methicillin-resistant *S. aureus* could be killed by short-term exposure (15 s) to He/Ne laser light (35 mW) in the presence of toluidine blue O (12.5 μg), and he found a decrease in the TVCs with exposure times of 45, 30, and 15, and almost similar results were found elsewhere [28]-[30]. Moreover, Wilson found that there were statistically significant reductions in the viability of *S. aureus* of 37%, 55%, and 45%, which were obtained following exposure to the He/Ne laser light (7.3 mW) for 120, 240, and 480 seconds, respectively [11]. However, the decrease in the TVCs with different exposure times to laser light in the presence of photosensitizers was almost similar to results concluded elsewhere [25] [26] [31] [32]. The ability of certain chemicals like Toluidine Blue O and Providine-Iodine (PI) to sensitize bacteria to killing by laser light was markedly species-dependent, since lethal photosensitization requires binding of the sensitizer to the cytoplasmic membrane of the target cell [33]. Such variation in the susceptibility might be attributed, at least in part, to differences in membrane binding and/or cell wall permeability [34]. Furthermore, surface components such as capsules, fimbriae, and fibrils, if they are capable of binding the sensitizer, may protect the species possessing such structures. This lethal photosensitization is thought to be due to membrane-induced damage arising from free radicals generated by photoactivated sensitizers and also membrane and wall damage induced by sodium hypochlorite disinfectant [35] [36]. In the case of radicals generated by sensitizer molecules bound to peripheral structures, quenching by neighboring molecules without concomitant damage to essential cell structures would be more likely eventually than their interaction with the distinct cytoplasmic membrane. Qualitative and quantitative differences in the surface components of the three target organisms may also have contributed to their varying susceptibilities to lethal photosensitization [31]. The ability of TBO and PI to sensitize mammalian cells with respect to killing by light has obvious implications with respect to their use in the lethal photosensitization of bacteria *in vivo* if extensive killing of adjacent host cells were to occur. However, no data are available regarding the relative concentration required to kill bacteria that are found to be less susceptible to TBO and PI-sensitized bacteria and mammalian cells to He/Ne laser light. If the light energy doses and sensitizer concentration required to kill bacteria are found to be lower than those needed to kill mammalian cells, then this therapeutic window could be opened to kill bacteria selectively without damaging the adjacent host cell *in vivo* [26]. It was found that the Nd:YAG laser was able to eradicate about 97% of the *Enterococcus faecalis* in vitro by using a laser beam on the root canal walls, and the efficiency of the low-power laser in killing viable bacterial cells was better than that of the sodium hypochlorite solution. Laser has been tested against multi-species bacterial cells and showed different levels of reaction to irradiation.

An alternative strategy would be to target the bacteria by linking the TBO or PI to antibodies against the methicillin-resistant *S. aureus*. This approach, using the sensitizer Sn (IV) chlorine e_6 linked to the monoclonal antibodies against *P. aeruginosa* has been shown to be effective selectively for killing *P. aeruginosa* in the presence of *S. aureus* [32]. Recently, it has been reported that photoactivated methylene blue-antibody conjugates can be prepared [33]. This strategy may be advisable to reduce any possibility of damage to the DNA of host cells, although the cytotoxicity of light-activated toluidine blue appears to result from its effect on the cytoplasmic membrane rather than on DNA, at least in bacteria and eukaryotic microorganisms [34]-[36]. Irradiation with visible light can be utilized to inactivate and/or kill bacteria without the need for any antimicrobial agents, solvents, or additives. The work carried out elsewhere revealed that several types of microbes have been sensitive to these visible light disinfections. In antimicrobial photodynamic therapy, a photosensitizer would be added to facilitate the chemical reaction between light and oxygen. The mechanism of oxidative damage to the microbial cell is due to the Reactive Oxygen Species (ROS) formed during this process. It has been noticed that the utilization of antimicrobial photoinactivation is useful as it kills bacteria with no harm to the host. This new approach has a rapid effect and locally targeted antibacterial action with no harm to the host tissue. Moreover, given the multi-target properties inherent in ROS, the possibility of inducing bacterial resistance is minimized compared to the consequences of antibiotic usage. The cost of using photodynamic therapy is far lower, rapid, and painless [37]. Laser therapy is associated with reduced inflammatory infiltration intensity. The radiation emitted by lasers in the red and infrared range promotes increased fibroblast proliferation and accelerates wound epithelialization. It was found that by the sixth day, wounds that had been irradiated with the laser had a 153% greater area reduction than wounds in a control group. According to Mejía *et al*, it is possible to reduce the healing time of wounds by 40% with a low-intensity laser [38]-[41].

The present results showed that the LPISH combination was more effective than others in killing *S. aureus* in 1 minute as an exposure time, which can exert a considerable bactericidal effect in the presence of an appropriate photosensitizer and sodium hypochlorite. This was obviously an important factor in assessing the clinical applicability of this potential therapeutic approach. Another consideration in this respect would be the period of time required for the sensitizer to render the bacteria susceptible to killing by the laser light. Furthermore, the results of the present study indicated that the laser-providine iodine-sodium hypochlorite (LPISH) combination was the most effective compared to other types of exposure in reducing total viable numbers and/or complete eradication of pathogens causing wound infections among hospitalized patients.

5. Conclusion

Using a Helium-Neon (He/Ne) gas laser emitting light of 632.8 nm wavelength in combination with different disinfectants, including sodium hypochlorite, showed a remarkable bactericidal effect on *S. aureus* isolated from wounds of hospitalized patients. Exposure of bacterial strains to chemical disinfectants at subminimal inhibitory concentrations caused a sharp decrease in total viable counts of disinfectant-resistant *S. aureus*, particularly after exposure to the laser-providine-iodine-sodium hypochlorite combination, with significant statistical differences in viable counts of the pathogen tested ($P < 0.05$). The laser-providine-iodine-sodium hypochlorite combination might be considered a new and promising approach for killing *S. aureus* and other pathogens causing infection which are already exposed to disinfectant in *vivo*. The present study revealed that sodium hypochlorite reduced the TVC's moderately, whereas the combination of laser and sodium hypochlorite showed a dramatic and consistent reduction in TVC's with minimal variation, revealing strong and reliable antimicrobial synergy. Total viable bacteria decreased over time, and the second disinfectant-exposed group revealed the steepest slope, demonstrating the fastest decline. R^2 values were moderate, indicating that a linear model partially explained the decay mode of behavior.

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Consent

Consent was obtained from patients who participated in the present study.

Ethical Approval

All the procedures involving human participation were conducted in strict accordance with the ethical standards of the Institutional Research Committee, Department of Scientific Research, Tikrit University, as well as the 1964 Helsinki Declaration and its subsequent amendments or equivalent ethical norms.

Conflicts of Interest

The authors declare that they have no conflicts of interest, financial or otherwise.

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