We studied the direct and filtered effect of ultraviolet rays (UVR) on growth of different bacteria. This was followed by in-vivo experimental study on infected excisional wound in albino Wistar rats. On confirmation of wound infection by laboratory investigation, wounds were irradiated with specific durations of UVR (254nm) depending on the results of in-vitro study. Outcome measures studied were percentage of wound contraction, culture by semi quantitative method, quantitative bacterial count and histopathological analysis.

6.1. In-vitro study of direct effect of ultraviolet rays (254 nm) on common wound infecting bacteria:

Our study demonstrated excellent bactericidal effect (100% eradication of bacteria inoculated) of ultraviolet rays (254nm) on eight common wound infecting bacteria by 5-25 seconds. Two studies using UVR (254nm, 15.54mW/cm²) exposure from 1-inch distance have shown 99-100% bactericidal efficacy with exposure duration ranging from 4-120 seconds. In our study, 5mJ/s/cm² (=5mW/cm²) of the UVR energy was delivered from a distance of 10 cm.

The intensity of illumination varies directly with square of the distance between the lamp and treatment surface. Thus reducing the distance between the treating surface and the lamp by 50% will increase the delivered dose by 400%. Even though the distance from the source to the culture plate was 10 centimeters as prescribed by the manufacturer, our results were comparable to other researchers. The study conducted by Sullivan and Conner-Kerr using UVC at 1 inch distance (2.54cms) on procaryotic and eucaryotic organisms, concluded that short exposure times (3-5 seconds 99.9% kill rate and by 30-120 seconds 100% kill rate) of UVC were detrimental to procaryotic (Pseudomonas aeruginosa) and simple unicellular eucaryotic organisms while sparing more complex multicellular organisms (Aspergillus Fumigatus). In our study, there was a more effective response (100% eradication of Pseudomonas...
aeruginosa within 20 seconds at 5mJ/s/cm² UVR energy from 10 cm distance) than reported by Sullivan and Conner Kerr.

The primary mechanism by which UVC has been postulated to cause bacterial cell destruction involves structural changes in DNA. DNA damage induced by UVR is wavelength dependent. UV-A (320 to 400 nm) causes only indirect damage to DNA, proteins, and lipids through reactive oxygen intermediates. On the other hand, UV-B (280 to 320 nm) and UV-C (100 to 280 nm) cause both indirect and direct damage because of the strong absorption at wavelengths below 320 nm by the DNA molecule. The most abundant products formed by irradiation with UV-B and UV-C are cyclobutane pyrimidine dimers (CPD). Sullivan et al suggested that the faster replication rate and DNA synthesis of bacteria renders them more sensitive than mammalian cells to UVC. Furthermore, these researchers noted that in procaryotes (bacteria), the genetic material is located freely in the cytoplasm; whereas, in eucaryotic cells, the genetic material is surrounded by both the plasma membrane and the nuclear membrane of the nucleus. It has been proposed that because bacteria do not have an additional physical barrier to protect their genetic material from UV irradiation, they are more vulnerable to the effects of UVC than mammalian cells. Conner-Kerr et al observed that UVC was able to selectively kill antibiotic-resistant bacteria such as MRSA present in the wound tissue of rats without detrimentally affecting healthy granulating tissue.

**Effect on gram-positive cocci:**

UVR (254nm) has been reported in previous in-vitro study to have the ability to kill bacteria, including antibiotic-resistant bacteria such as MRSA. Our results of the in-vitro study on MRSA showed better bactericidal efficacy with lesser exposure duration compared to that reported by Conner et al. This could be because of small proportion (5%) of UVB delivered by our equipment, which causes both indirect and direct damage because of the strong absorption at wavelengths below 320 nm by the DNA molecule. Our study, demonstrated sensitivity to UVR (254nm) was maximum for MSCONS and minimum for MSSA. The order of UVR sensitivity was (MSCONS =
MRSA > MSSA. In contrast to our study, Conner et al have demonstrated equal sensitivity of MRSA and MSSA to UVR exposure.

Our study demonstrated 100% eradication of *Streptococcus pyogenes* by 5 seconds irrespective of their susceptibility patterns. Further studies are needed to observe the efficacy of irradiation with less than 5 seconds, which may minimize the side effects on host cells. Sullivan et al\textsuperscript{19} in their in-vitro study showed 99.9% kill rate at 4 seconds of UVC exposure on *Streptococcus pyogenes* (*Group A Streptococcus-GAS*). Due to technical limitations of our equipment, we could not study the effect of UVR exposure at less than 5 seconds duration.

Our study indicates that resistant strains (>6 antibiotics) of *Enterococcus species* was eradicated with shorter duration (10 seconds) of exposure as compared to that of sensitive strains (15 seconds). Experiment conducted by Conner et al\textsuperscript{193} on *Enterococcus faecalis* (VRE and VSE) showed that VSE were eradicated by 8 seconds while VRE were eradicated by 45 seconds.

**Effect on gram-negative bacilli:**

Our study on exposure duration for 100% eradication of gram-negative bacilli revealed that *Escherichia coli* were most sensitive to UVR exposure. (*Escherichia coli* -15 seconds > *Pseudomonas aeruginosa* -20 seconds > *Klebsiella pneumoniae* -25 seconds). Johnson\textsuperscript{271} has studied the effect on *Escherichia coli* and *Pseudomonas aeruginosa*. His findings were similar to our study.

Our study on *Pseudomonas aeruginosa*, 100% eradication was achieved (20 seconds) with source at 10 cm distance and 5mW/cm\textsuperscript{2} UVR energy. Sullivan et al\textsuperscript{227} reported 100% eradication of *Pseudomonas aeruginosa* by 30 seconds (average output, 15.54mW/cm\textsuperscript{2} at 1 inch distance). Different antibiotic susceptibility patterns of *Pseudomonas aeruginosa* did not have any influence on UVR sensitivity.

The time required to obtain 100% bactericidal effect on *Klebsiella pneumoniae* was 25 seconds. Our study showed that longer duration of exposure was required for 100% eradication of pan drug resistant *Klebsiella pneumoniae* than their sensitive strain. This
indicated increased UVR resistance of the antibiotic resistant *Klebsiella pneumoniae*. Similar finding was reported by Marchese A272. His study showed that UV radiation (exposure duration of 20 seconds) reducing the number of viable cells was 30%-62% for resistant *K. pneumoniae* and 43%-66% reduction for the parent drug-susceptible strains. There was no mention about the wavelength and intensity of UVR in their study.

We have obtained bactericidal effect for *Escherichia coli*, which varied, from 5-15 seconds for different antibiotic susceptibility patterns. Our results indicate that the antibiotic resistant organisms were also resistant to UVR exposure. This is similar to findings of Marchese A272 who reported with 20 seconds of irradiation with UV light, a 14-69% reduction in the number of viable cells for resistant E. coli strains, compared with 56-79% reduction for susceptible strains.  High and High17 studied the effect of Kromayer (UVR) lamp model 10, (254-436nm) on *Pseudomonas pyocyanea* and *Escherichia coli* with dosage varying from E2, E3, E4 at 10 cm distance. They concluded these bacterial strains were not viable beyond E4 dose. However, in this study authors have not described duration of exposure and percentage emission of different wavelengths of UVR.

In our study, gram-positive cocci were more sensitive to UVR than gram-negative bacilli (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) which are not consistent with results of Chang et al273. The probable reason for the different UVR sensitivity between gram-positive and gram-negative bacteria could be ascribed to the morphological differences between these microorganisms. Gram-negative cells have a complex additional outer barrier that may be responsible for increased resistance to UVR274.

### 6.2. In-vitro direct effect of ultraviolet rays (400nm, UVA) on common wound infecting bacteria

Bactericidal efficacy of UVA with long duration of exposure has been examined under various conditions in combination with titanium oxide275, psoralen276,277, salt278. UVA can promote generation of reactive oxygen species (ROS) which are known to damage lipid, proteins and DNA278. UVA is a known agent of immune suppression279 and is a
suspect in cutaneous carcinogenesis with longer duration of exposure. Understanding the adverse effect of longer exposure, we intended to study the beneficial effect with shorter duration. In the present study, we investigated the effect of UVR (400nm) for duration varying from 5-30 seconds. In our study, we did not obtain any bactericidal effect of ultraviolet rays (400nm) on both gram-positive cocci and gram-negative bacilli. Ultraviolet rays (400nm) had disadvantage of production of excessive heat 49°C that did not enhance any bactericidal effect in-vitro.

6.3. In-vitro study of effect of filtered ultraviolet rays (254nm, 400nm) through plastic sheet on common wound infecting bacteria

In our study, there was no bactericidal effect of filtered rays in both wavelengths 254nm and 400nm. We conducted an experiment to see the amount of radiation passing through the plastic sheet by UV photometer (UV 1700 series) at Department of Biotechnology Manipal University. Experiment showed that there was no transmission of UVR (254nm) but transmission of UVR (400nm) was 100%.

Since UVR 254 nm was not transmitted through the plastic sheet under the study, there was no effect of filtered UVR 254nm. Though our study showed UVR 400nm was transmitted through the plastic sheet, there was no effect of filtered UVR 400nm because even direct UVR 400nm had no effect on bacterial growth. In addition, UVR (400nm) had disadvantage of production of excessive heat (49°C) which deformed the plastic with longer duration.

In 1984, empirical study by MacKinnon and Cleek have reported UVR can be applied through the transparent dressing. However, in 2001 Conner et al reported UVR did not show any filtered effect. In our experiment, we have shown that UVR 254nm does not cross the plastic sheet of 0.15mm thickness used during LAD to cover the wound. Our results were consistent with reports by Conner et al. However, at this stage, we can safely conclude that the plastic sheet of 0.15mm thickness may be used to protect the surrounding skin from exposure (below 297nm) during therapy. For using UVR with the LAD, there is a need to develop/construct a specialized UVC delivery system in LAD similar to the prototype device designed by Dai et al. They constructed a special
prototype device of UVC to prevent catheter related infections. This device consisted of a UVC cold cathode fluorescent lamp (UVC-CCFL) in a quartz tube connected to a piezoelectric inverter that turns 12 V DC into the 600 V, 5 mA current to create the electric discharge. A dimmer (PhotoGlow) was also fitted to the circuit to be able to control the amount of emission from the UVC-CCFL. Emission from the UVC-CCFL was in a cylindrical pattern that is ideal for insertion into the lumen of a catheter. The diameter of the UVC-CCFL was 6 mm and the length 45 mm. The outer diameter of the catheter was 8 mm with the catheter thickness of 0.95 mm. The irradiance of the UVC-CCFL achieved 0.4 mW cm\(^2\) at the lamp surface by tuning the dimmer in the electric circuit.

6.4: In-vivo study of direct effect of Ultraviolet rays (254nm) on dynamics of excision wound healing in albino Wistar rats:
Since UVR (254nm) equipment has 5% UVB and 2.5% of UVA, in order to minimize acute effects of UVR on epidermis and dermis of surrounding normal tissue as well as wound bed, the distance of 10 cm was used as suggested by the manufacturer. Considering the inverse square law and poor transmission ability of UVC, if we reduce the distance less than 10 cm, then the time required to obtain the bactericidal effect will also reduce. Since our machine has small proportion of UVA and UVB along with UVC, we did not consider for conducting the experiment with lesser distance because that would have resulted in increased exposure to UVA and UVB. Similar to our study, Johnson had suggested treatment parameters for wounds with UVC with distance of ¼ to 3 inches, intensity of 5-20mW/cm\(^2\) and duration of exposure from 5 seconds to 1 minute but preferred duration being 5-20 seconds\(^2\)\(^7\). We have used UVR exposure over the excision wound for maximum of 30 seconds which is less than the permissible exposure limits (1-3 minutes) for germicidal UVR (UVC)\(^2\)\(^1\) (Table 2.4). Johnson has highlighted the need for increasing duration of treatment depending upon the type and character of the wound, microorganism to be eliminated and the intensity and position of the UVC source.

In many studies\(^2\)\(^2\)\(^9\),\(^2\)\(^3\)\(^3\), including that of Dai et al\(^2\)\(^2\)\(^3\), researchers have used UVC on immediately or within 24 hours. However, in our study we have left the wound open for
7 days and induced wound infection. This type of wound model was used to mimic closely the type of wound dealt in routine clinical practice. It is difficult to extrapolate the result of our in-vivo study in Wistar rats to human wounds but can be taken as basis for intervention in wound management. This may navigate the surgeon to take up the wound for further wound closure technique early and may reduce the hospital stay and cost.

The depth of penetration of UVR in human skin and the ability to produce physiological changes is wavelength dependent. Skin absorption reduces from UVA to UVB and is least for UVC\textsuperscript{135}. UVC does not penetrate beyond upper layers of the epidermis of human skin. In order to have physiological changes ultraviolet radiation has to be absorbed (clinically demonstrated as erythema). Erythemal effectiveness lowers at 297nm rapidly decays from 305-320nm. To minimize the damage to host cell, based on the penetration power at different wavelengths, Conner et al\textsuperscript{135} has suggested that selection of UVC (254nm) exposure time on the basis of susceptibility results rather than erythemal dose (selection in case of UVA and UVB is based on erythemal dose).

Since wound does not have superficial covering layers, even UVC can produce erythema. In the literature there is contradicting report whether UVC produces erythema\textsuperscript{21,135} or not\textsuperscript{283}. Since erythema is produced after varying interval of exposure, it may be justified to use in-vitro bactericidal dose as a guide for selection of dose in clinical setting. Hence, based on the in-vitro study with UVR (254nm, Table5.9) we decided the in-vivo exposure dose (Table 4.1).

6.4.1: Percentage of wound contraction:
Percentage wound contraction from 8\textsuperscript{th} day to 18\textsuperscript{th} day showed that increasing doses of radiation was associated with higher rate of contraction in all the organisms except in \textit{Klebsiella pneumoniae} where there was a reverse relation between percentage wound contraction and radiation dose. On 18\textsuperscript{th} day, wounds infected with gram-negative bacilli showed better percentage wound contraction than their sham controls. In contrast, wounds infected with gram-positive cocci showed lesser percentage of wound contraction on 18\textsuperscript{th} day than their sham controls. We could not find any similar literature to compare our results.
Literature review showed that UV mediated wound contraction is probably due to increased fibronectin (an extracellular matrix protein) secretion. Morykaw et al\textsuperscript{234} conducted an in-vitro study using cultured fibroblasts, demonstrated increased secretion of fibronectin into the culture medium after UVC irradiation.

Possible mechanism of stimulated wound healing by UVR is frequently cited in the literature\textsuperscript{17,21,142,271}. UVR induces inflammation, which manifests as erythema\textsuperscript{226}. Within minutes, due to release of inflammatory mediators (histamine, serotonin, prostaglandins, kinins) vasopermeability increases. A delayed inflammatory erythema may be observed within hours and a late-phase tissue response after few days of UVR exposure. After an initial decrease in DNA, RNA and protein synthesis, the rate of synthesis increases which is followed by thickening of skin. This phenomenon (increased synthesis of DNA, RNA and protein synthesis and increased skin thickening) is not only considered reparative after initial damage from UVR, but also protective against further UV radiation. This stimulative and the early vasodilative effect is believed to promote wound healing and constitutes a basis for UV therapy\textsuperscript{17}. UV exposure in addition to stimulation of cell proliferation, also helps in wound debridement\textsuperscript{17,142}. Notably UVC exposure does not cause significant permanent skin pigmentation, which is in contrast to application of UVA or UVB. As a result, subsequent UVC radiation treatments need not be started in low dose and then increase in intensity or duration in order to overcome pigmentation effect.

There are only few studies that examined the effect of UVR (254nm) in the animal model\textsuperscript{229,233}. Basford examined the effect of UVC in comparison with laser\textsuperscript{333} and Batouty et al with ultrasound therapy\textsuperscript{229} in non-infected excision wound model. Batouty et al\textsuperscript{229} had used hot quartz lamp (265.2nm) with treatment time starting with 15 seconds and finally reaching to 1 minute(every alternate days for 18 weeks) and Basford\textsuperscript{233} et al used E1 (15seconds) dosage twice daily (E2 equivalent),but both the studies did not mention the energy delivered to the wounds. Moreover, the conclusion drawn by Basford et al\textsuperscript{233} was occluded dressing group healed faster than UVR and laser treated experimental groups. But authors did not study the effect of each modality combined with occlusion since optimum clinical conditions appear to be dependent on
a moist wound surface and when wound is allowed to dry out viable tissue is subjected to secondary desiccation\textsuperscript{21}. They also reported clinically reduced hypertrophic healing in both treated and untreated wounds. However, this phenomenon was lost after first week of wound closure. Batouty et al\textsuperscript{22} concluded ultrasound had better effect on tissue regeneration than ultraviolet irradiation as evidenced by both reduction in surface area of wound and by histological examination. Owing to the methodological differences, we cannot compare the results of our study to theirs, though we obtained nominal improvements in percentage wound contraction.

6.4.2: Color and appearance: Though albino Wistar rats were able to withstand the wound infection\textsuperscript{284}, we could infect all the animals (except \textit{Enterococcus.spp}) in our study. All the animals infected with different bacteria showed signs of acute infection in the wound by seventh day. Among all, the wounds infected with \textit{Pseudomonas aeruginosa}, \textit{Klebsiella pneumoniae} and \textit{Streptococcus pyogenes} showed oozing, pus, greenish/purulent discharge. Only purulent discharge was seen in the other wounds. All the wounds had thick scab. There was significant reduction in the signs of infections by 12\textsuperscript{th} day of study. Similarly, sham control wound also showed reduction in the signs of infection by 12\textsuperscript{th} day of study.

6.4.3: Culture by semi quantitative method and quantitative bacterial count: All the different bacteria studied were able to produce infection, which was confirmed by both culture and QBC by seventh day. However, wounds inoculated with \textit{Enterococcus.spp} were completely replaced by the \textit{Staphylococci} in both culture and QBC by 7\textsuperscript{th} day of study. UVR exposed daily once to all the wounds infected with \textit{MRSA}, \textit{MSSA}, \textit{MSCONS}, \textit{Streptococcus pyogenes}, \textit{Pseudomonas aeruginosa}, \textit{Klebsiella pneumoniae} and \textit{Escherichia coli} in the experimental group for ten days. On 18\textsuperscript{th} day of study, wound specimen did not show the bacteria initially inoculated and the inoculated wound pathogens were completely replaced by normal skin flora (MSSA, β hemolytic colonies, \textit{Diphtheroids} and \textit{Staphylococcus citreous}). On 18\textsuperscript{th} day, there were no signs of wound infection on culture and QBC. These observations imply that there could be bacterial interference from the adjacent skin surfaces or from the environment\textsuperscript{285}. The results of our study it is evident that signs of infections can be reduced by 18\textsuperscript{th} day of wounding.
(10th day of UVR exposure) and bacterial interference from the adjacent flora can occur. Hence, to avoid this interference we need to plan shorter period of study (less than 10 days of UVR exposure).

Except for one study conducted by Conner-Kerr\textsuperscript{135} et al, we could not find any study which used infected albino Wistar rats wound model (with gram - positive cocci and gram - negative bacilli). Conner-Kerr\textsuperscript{135} et al in their experiment have observed ability of UVC to selectively kill antibiotic-resistant bacteria such as MRSA present in the wound tissue of rats without detrimentally affecting healthy granulating tissue. A study conducted by Thai et al\textsuperscript{203} evaluated single exposure(180seconds)of UVC significantly reduced the relative amount of bacteria in several different types of chronic wounds, including pressure ulcers, venous ulcers, diabetic ulcers, and arterial ulcers in humans.

6.4.5: Histopathology: Histopathologically there was no difference in the histopathological score between the experimental and sham control group. All the experimental and sham control groups achieved score of 10-12 by 18th day except wounds inoculated with Klebsiella pneumoniae 3 (Trial 2, Table 5.36) and with Escherichia coli 3 (Trial 1 and 2, Table 5.37) which achieved the score of 7-9 on 18th day.

The subjective assessment of collagen deposition was more with the experimental group than the sham control except in Streptococcus pyogenes. Literature review showed that UVC enhances secretion of fibronectin\textsuperscript{234}. Fibronectin is an extracellular matrix protein that is critical to the laying of collagen, plays a role in wound contraction.

Inflammatory cells were less in the experimental group than the sham control in all groups (i.e. UVR exposure in the present study exhibited anti-inflammatory effect). Our findings of improvement in collagen deposition and reduced inflammatory cells were in contrast with the results of Batouty et al\textsuperscript{229}.
Limitations of study:

1. In-vitro study:
   - Polymicrobial bacterial suspension was not used to simulate the clinical situation.
   - Effect of repeated short duration of UVR exposure was not studied.
   - We could not study the effect of exposure with duration lesser than 5 seconds since the timer of machine was set at five seconds increments.

2. In-vivo study:
   - Longer duration of in-vivo study may lead to fresh episode of infection from different routes (environment, hematogenous)
   - This is an animal study.
   - Effect of UVR was not attempted in polymicrobial wound infection, which would closely mimic the wound in human subjects.

Clinical implications:

1. UVR can be used effectively in all types of wound infections within 25 seconds which is within safety limits(1-3 minutes)

2. UVR does not cross plastic sheet of LAD hence cannot be used in combination with LAD without further modification (fiber optic delivery)

3. This therapeutic option for wound healing may reduce the total cost of treatment to eradicate infection.

Future scope:

1. Use of UVR in prevention of infections and tackling the multi drug resistant organisms in wounds.

2. To observe the beneficial effect in different types of wounds in human subjects.

3. Efficacy of cumulative dose of UVR with different durations of exposure should be investigated.

4. Designing a fiber optic cable to transmit UVR under plastic sheet of LAD.

5. Comparative studies of efficacy of UVR over other therapeutic modalities.

6. Effect of UVR 254nm with lesser than 5 seconds of exposure for Streptococcus pyogenes.