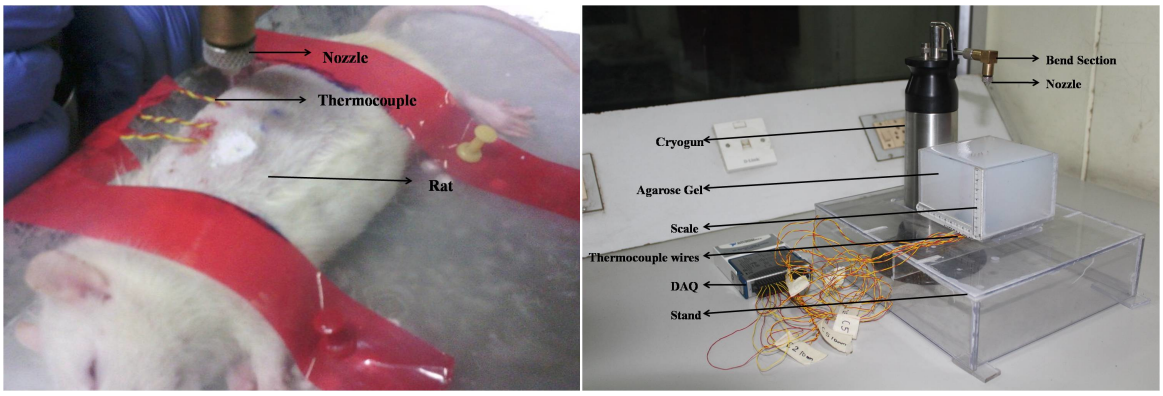
CHAPTER 4

Establishing relation between in-vivo and in-vitro Cryospray experiments through thermal characteristics

Any treatment modality cannot be approved directly for clinical trials to test its feasibility in actual conditions. Each parameter should be evaluated under in-vitro condition before proceeding for in-vivo conditions. In-vitro experiments are substantial because they provide control over physical and chemical conditions, economical and offers detailed analysis of individual parameters under controlled environment. However, the results of in-vitro experiments may not fully replicate in actual conditions. It requires the confirmation of in-vitro results with in-vivo results before proceeding for clinical trails. In this perspective cryospray experiments are conducted on rats to verify the results of in-vitro condition with in-vivo conditions. Charles Foster rats are used in this study for in-vivo experiments because their skin is structurally similar to the human skin [2]. Several in-vivo and in-vitro studies of drug penetration through rat skin have been carried out in the past few years [35, 79, 143, 171]. The aim of these studies is to estimate the rate of drug absorbtion through transdermal route. Moreover, avilability and docile nature of rats make them suitable for in-vivo experiments.

In order to increase the efficacy of cryospray (in the treatment of both the cases), modification in spraying devices is suggested by different authors [3, 30, 65, 167]. They suggested such modifications to increase the scope of cryospray in the treatment of larger lesions with improved cooling rate. Thus, another aspect of present study is to analyse the impact of multihole nozzle on cryoablation. This approach is similar to multiprobe



(a) In-vivo experiment (b) In-vitro experiment

Figure 4.1: Experimental Setup

method of cryosurgery in which multiple cryoprobes are inserted simultaneously to increase the ablation rate of larger and irregular lesions [93, 115].

4.1 Materials and Method

In-vivo experiments are performed on healthy male rats (Charles Foster rats) weighing about 150-200 grams. Cryogun (CS-1), is used to spray cryogen on dorsal area of the rat while carefully removing hairs in that particular area for better observation. Liquid nitrogen is used as a cryogen for the study. Hematoxylin and eosin, procured from Sisco Research Laboratories Pvt. Ltd. (SRL) India, are used in histopathology. Glass slides (50 mm x 20 mm x 2 mm) and cover slips (30 mm x 20 mm x 0.2 mm) are purchased from Blue star. Paraffin wax, xylene, and 4 % paraformaldehyde are purchased from Loba Chemie Pvt. Ltd. Absolute ethanol of 99.9 % high analytical grade and distilled water are used in the in-vivo experiments. Single hole nozzle with outlet hole diameter of 0.8 mm is used to spray cryogen. Prior to experiment, rats are anesthetised via diethyl ether to reduce their locomotion and pain. Rats are further euthanized by cervical dislocation which ensures no pain to the animal. For in-vitro experiments all the material are same as explained in Chapter 3.

Fig. 4.1a and Fig. 4.1b show the experimental setup used for the in-vivo and in-vitro studies respectively. For temperature measurements, three thermocouples are placed at a distance of 2 mm from the surface in in-vitro and in-vivo experiments. Since the case is symmetric so all the 3 thermocouples are placed on the left side of the centre of spray. Distance of the thermocouples with respect to the centre of spray (CS) is 0 mm, 10 mm and 20 mm. Nomenclature of thermocouples on the basis of their orientation is depicted in fig.

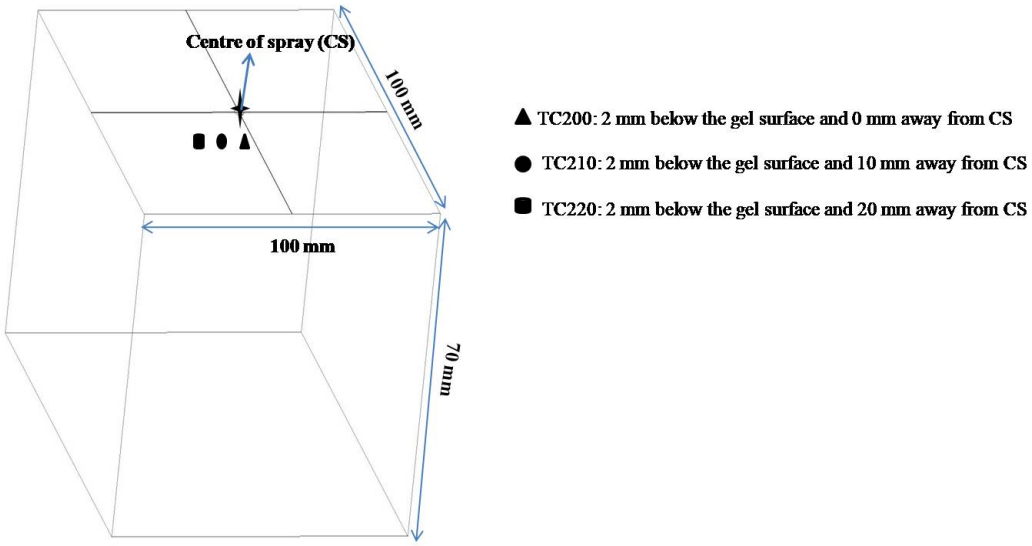


Figure 4.2: Positions of thermocouple

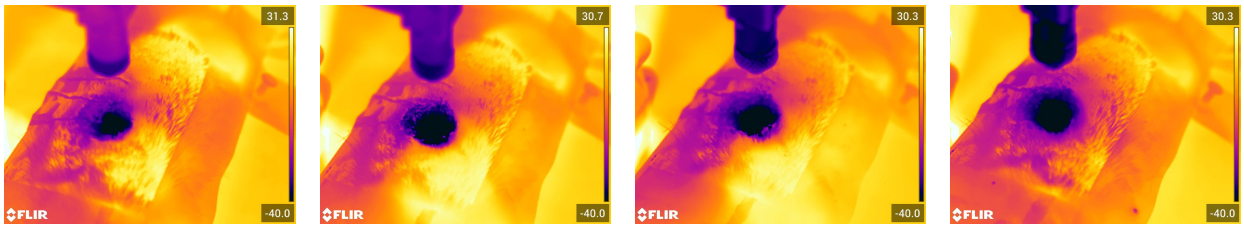
4.2. Spraying distance is selected as z = 18 mm. Cryogen is sprayed through commercial SHN (procured for SMT Praha) and customised MHN. Dimensions of MHN and its spraying distance are selected on the basis of results obtained through the experimental study discussed in section 3.2.3 of chapter 3.

Preparation of staining slides

The tissue sample specimens are isolated from the treated area and are fixed in 4 % paraformaldehyde (fixative agent). The samples are fixed and blocks are prepared as defined previously in [1]. Briefly, the specimen is subjected to serial dehydration after the fixation, followed by treatment in the clearing solution to remove unwanted fats/lipid materials out of the specimen and it also acted as an intermediate solvent for ethanol and paraffin wax. The ethanol inside the samples is replaced by molten paraffin wax. Furthermore, the tissue sections are oriented in suitable directions as per the requirement to obtain defined sections. Blocks are clamped into the sectioning machine and are sliced in four μm sections followed by slides preparation.

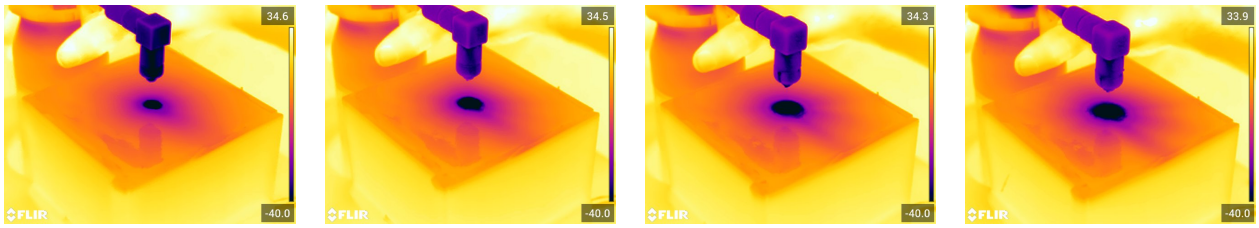
Hematoxylin and Eosin staining

One of the most common tissue stains used in histology is the hematoxylin and eosin stain (often abbreviated as H&E stain or HE stain). These staining techniques are majorly used



(a) After 30 s (b) After 60 s (c) After 90 s (d) After 120 s

Figure 4.3: Thermal images of in-vivo experiments with SHN



(a) After 30 s (b) After 60 s (c) After 90 s (d) After 120 s

Figure 4.4: Thermal images in-vitro experiments with SHN

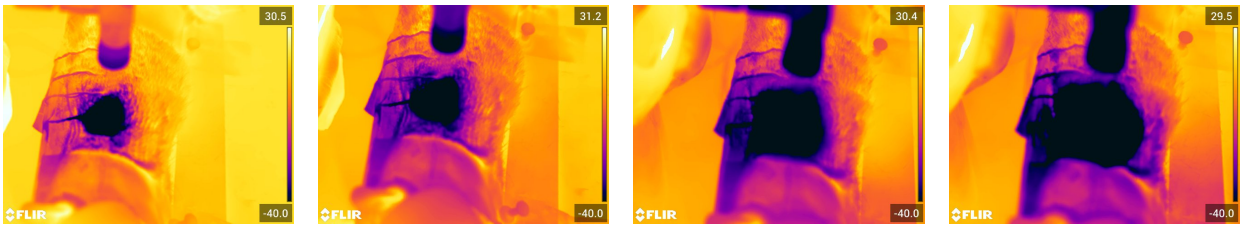
in medical diagnosis, and used in almost all pathology labs because of its wide applicability. H&E staining enables the morphological identification of various tissue types, particularly in the diagnosis of cancer. Hematoxylin has a deep blue-purple color and stains nucleic acids by forming a complex. Eosin stains are pink and are used to stain proteins non specifically. In natural tissue, nuclei look blue, while the extracellular matrix and cytoplasm can be darker or lighter in various shades of pink. The sections of tissue samples are collected from different areas of the pretreated rat skin. The tissue sections are subjected to H&E staining to observe the affected areas and the degree of damage occurred because of cryotreatment.

4.2 Results and discussions

In the current study, variation in the outcomes of in-vivo and in-vitro experiments is examined. Each experiment is repeated thrice to ensure accuracy in results. Experiments are conducted at room temperature and initial temperature of gel is kept same as the dermis temperature of rat (i.e., 30 ^{0}C) to minimise the errors.

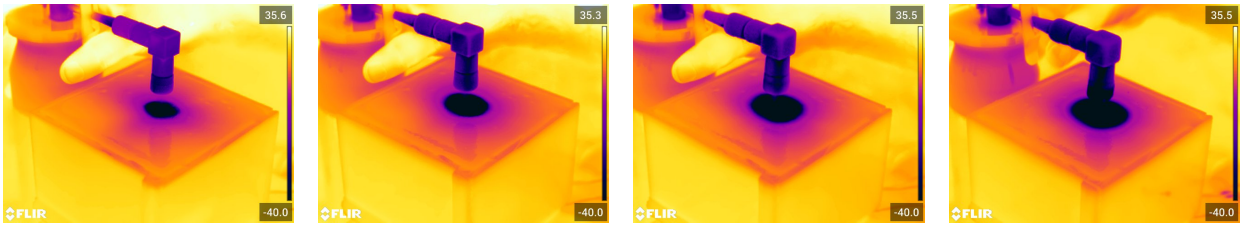
4.2.1 Radial temperature distribution

Fig. 4.3 and Fig. 4.4 depict temperature contours on the rat skin and gel surface



(a) After 30s (b) After 60s (c) After 90s (d) After 120s

Figure 4.5: Thermal images of in-vivo experiments with MHN

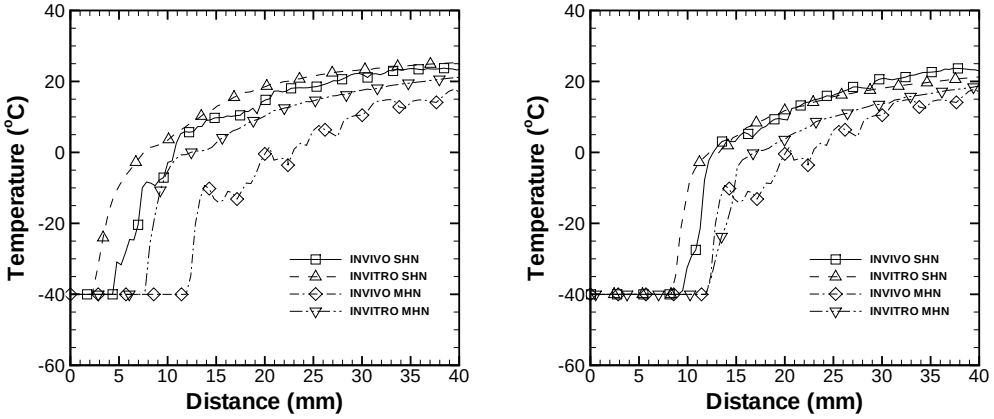


(a) After 30s (b) After 60s (c) After 90s (d) After 120s

Figure 4.6: Thermal images of in-vitro experiments with MHN

respectively when SHN is used to spray the cryogen. It can be observed from the images that formation of ice ball is circular in both the cases and the temperature increases as the distance from CS increases. Goff et al. [64] have also used thermal images for the estimation of cryoablation. Images are captured after an interval of 10 s and plots of every quarter with respect to duration of spray is shown in Fig. 4.7. The graph is plotted in such a way that abscissa represents the radial distance from CS and ordinate represents corresponding temperature at that location. The radius of lethal front (i.e., -40 ^{0}C) and freezing front (i.e., 0 ^{0}C) is higher upto 30 s of spray in in-vivo experiments than in in-vitro experiments. It might be due to the response of rat's body towards the sudden cold which causes heat to diffuse more on the surface in order to protect the inner core. However, as time progresses the radius of lethal front and freezing front becomes almost equal in both the experiments. The radius of lethal front is 9.5 mm in in-vivo case and 10 mm in in-vitro case after the end of spray while freezing front is 16.5 mm and 15 mm in the respective cases. It can be inferred from this observation that biological response of rat becomes ineffective as the time progresses. It also reflects that other significant factors like blood perfusion and metabolic heat generation are not influencing the heat sink on the surface.

The dimensions of lethal front and freezing front of in-vivo experiment are always greater than in in-vitro experiment, in case of MHN (refer fig. 4.5 and fig. 4.6). Higher mass flow rate associated with MHN causes the formation of larger (compared to SHN) circular film of cryogen on the phantom surface. However, due to uneven shape of the



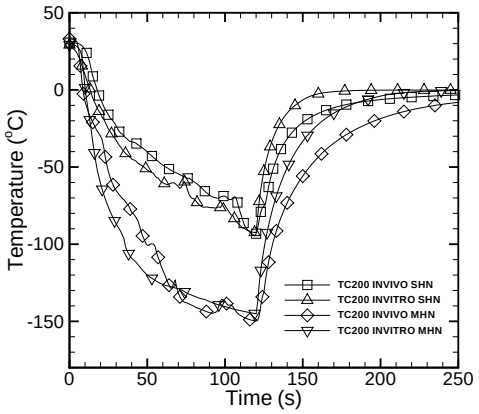
(a) After 30s of spray

(b) After 60s of spray

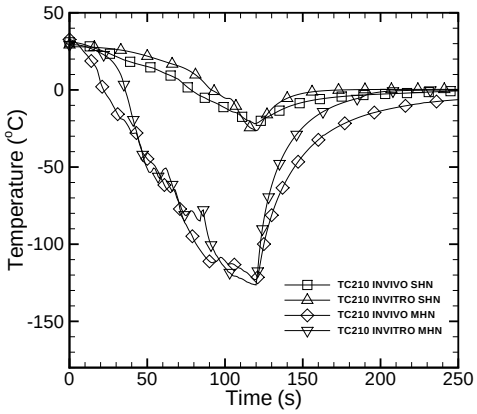
(c) After 90s of spray

(d) After 120s of spray

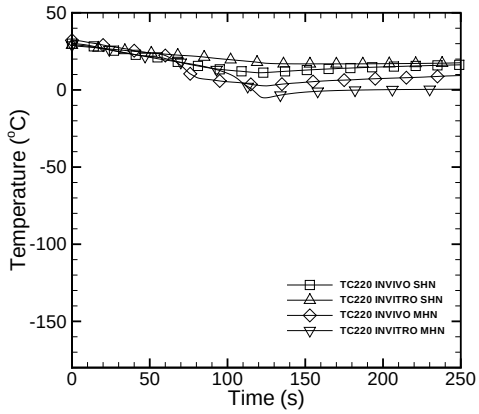
Figure 4.7: Temperature distribution along the surface



(a) 2 mm below gel surface and 0 mm away from CS (TC200)



(b) 2 mm below gel surface and 10 mm away from CS (TC210)



(c) 2 mm below gel surface and 20 mm away from CS (TC220)

Figure 4.8: Transient temperatures at different locations

rat body, the cryogen film becomes rectangular which is responsible for increment in the size of lethal front and freezing front in case of in-vivo experiment. Lethal front in in-vivo experiment is 25 % larger than in in-vitro experiment after 120 s of spray.

4.2.2 Axial temperature distribution

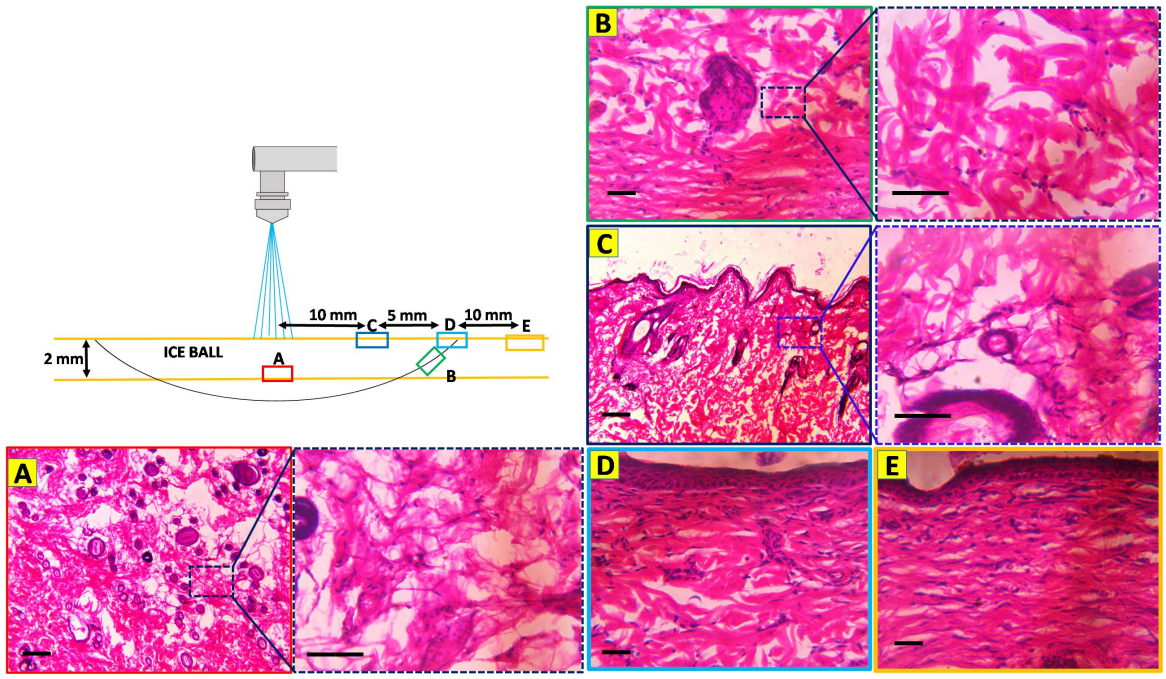
The variation of temperature with respect to time is shown in fig. 4.8. Minimum temperature at TC200 location after 120 s of spray is -93 ^{0}C and -92 ^{0}C for in-vivo and in-vitro experiments respectively in case of SHN. However, in case of MHN, -149 ^{0}C and -145 ^{0}C are the end temperatures after 120 s of spray for the same location. It reflects that bi-

ological factors do not have significant impact on cryoablation. At TC210 location, the minimum temperature is found to be -21 ^{0}C for in-vivo experiment while for in-vitro experiment it is recorded to be -26 ^{0}C for SHN. Whereas at TC220 location, freezing is not observed in either case and the minimum temperature in in-vivo experiment is 17 ^{0}C and for in-vitro experiment it records 11 ^{0}C. MHN follows the similar trend as SHN with a much higher temperature drop. The difference in end temperature is found to be 4 ^{0}C and 5 ^{0}C in in-vivo and in-vitro experiments at TC210 and TC220 locations respectively. It can be concluded from the thermal pattern beneath the skin that necrotic zone would be similar in in-vivo and in-vitro experiments upto a depth of 2 mm from the skin surface. Young et al. [181] conducted in-vivo, ex-vivo and in-vitro renal cryosurgery. They concluded that in-vitro and ex-vivo isotherms are much different from in-vivo isotherms thus scope of in-vitro studies are limited. Similar observations are also mentioned by Seifert et al. [144]. It should be noted here that the former cases are related to cryosurgery whereas in the present case cryogen is sprayed on the skin where biological factors have less influence. So, in-vitro experiment can be a good alternative for in-vivo experiments in case of cryospray. However, due to the changes in morphology and thermal properties of the rat skin and gel the necrotic zone might change as the depth from skin surface increases in in-vivo and in-vitro experiments.

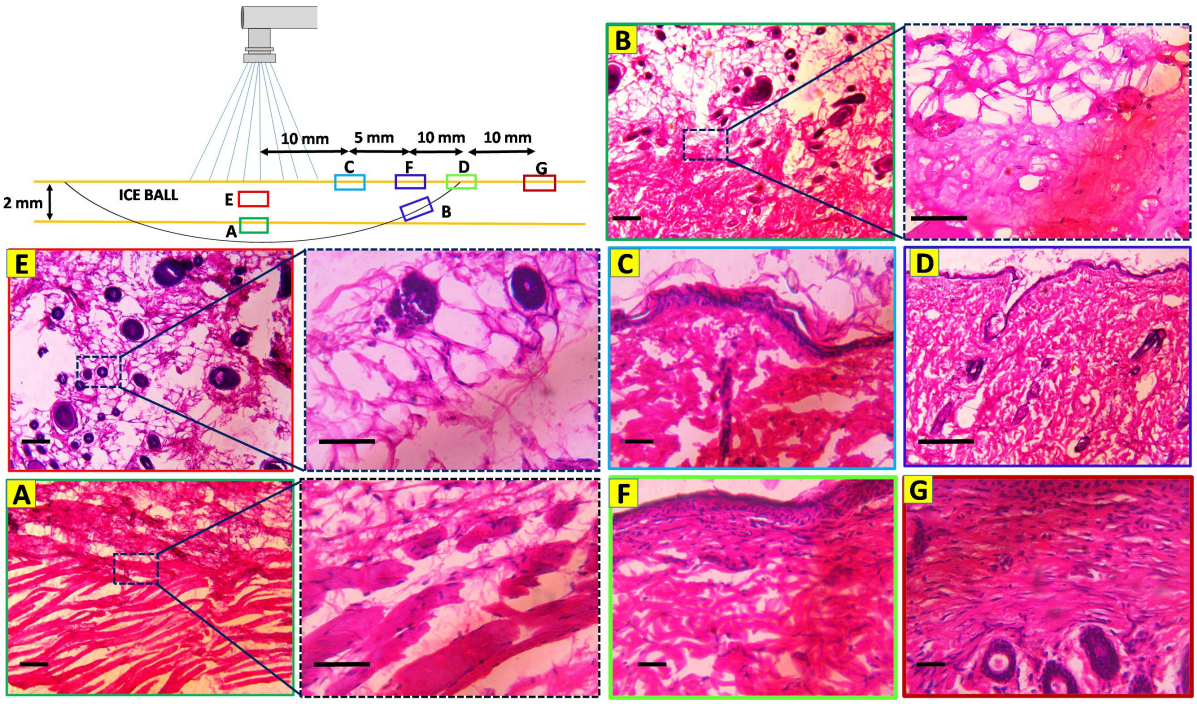
4.2.3 Histology

The results obtained through histopathology provides broader understanding of cryoablation. It is interesting to note here that in in-vitro experiments, necrotic zone is estimated on the basis of temperature readings. Such estimation of necrotic zone provides incomplete data of necrosis because it ignores the cell death achieved during thawing period. Authors have reported that natural thawing is more detrimental than artificial thawing [87]. Therefore, in order to acknowledge the effect of natural thawing, samples after the spray of cryogen are left for 30 minutes to promote natural thawing. Thereafter, the samples are dissected both radially and axially to approximate the amount of destruction. Rectangular boxes in fig. 4.9 represents the locations of dissection. More samples are taken in case of MHN due to the obvious reasons.

In case of SHN, samples collected at TC210 location (represented by box A in fig. 4.9a) record the destruction of tissue whereas in the case of MHN more destruction can be observed at the same location. Readings of thermal images suggest that the lethal front expands upto 10 mm and 20 mm from the CS in case of SHN and MHN respectively after 120 s of spray. It can be attributed to more destruction in case of MHN at the same location.



(a) Histology for singlehole nozzle



(b) Histology for multihole nozzle

Figure 4.9: Histology results

Box D in fig. 4.9a marks the periphery of ice ball in the case of SHN, so less destruction is observed at this location (as temperature ranges between 0 ^{0}C to -10 ^{0}C). The accepted range of necrosis varies from -20 ^{0}C to -40 ^{0}C [38, 91, 139, 154, 195]. Therefore, apoptosis can be the reason of tissue destruction in this case. Apoptosis generally occurs in the peripheral region of spray [61, 109]. Similar results are obtained for MHN at the same location (refer Box D in fig. 4.9b) as its ice ball extends upto 25 mm from CS. The interface of healthy and destructed tissues can be seen in Box B for both the cases. Interface of muscle and skin can be seen in Box A of fig. 4.9b. It can be interpreted from this image that muscles are also getting affected due to the treatment. Box E and Box G in the case of SHN and MHN respectively depict unaffected scaffold showing completely intact tissue for the reference purpose.

4.3 Conclusion

The present study attempts to explore the difference in in-vivo and in-vitro experiments during cryoabalation. It has been observed that cell death during thawing duration is not acknowledged during in-vitro experiments. Metabolic heat generation and blood perfusion have negligible influence on cryoablation. Unlike experiments of cryosurgery, where these parameters have significant influence and results of in-vivo experiments differ significantly with in-vitro experiments, results of in-vivo experiments of cryospray are similar to in-vitro experiments. MHN increases the dimensions of necrotic zone and can be used in the treatment of larger lesions effectively.