An Improved and Versatile Immunosuppression Protocol for the Development of Tumor Xenograft in Mice

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Abstract. *Background: The objective of the present study was to evaluate the efficacy of a simple, versatile and cost-effective immunosuppression protocol, using cyclosporine, ketoconazole and cyclophosphamide drug regimen to develop human tumor xenograft in mice. Materials and Methods: Cyclosporine, ketoconazole and cyclophosphamide drug regimen was administered to C57BL/6 mice to induce immunosuppression. Five million A549, LNCaP and KB cells were injected subcutaneously in the immunocompromised mice for the development of tumor xenograft. Tumor volume was calculated every week. Histopathology of tumor tissue was analyzed. Results: Prolong immunosuppression was achieved by this combination treatment. The average tumor volume was found to be greater than 600 mm^3. Histopathology of tumor tissue revealed the presence of large and irregular nucleus and scanty cytoplasm, which are characteristic of malignant cells. Conclusion: A versatile immunosuppression protocol was developed which was validated for xenograft development using three different cell lines, with a 100% take rate and no mortality.*

The International Agency for Research on Cancer (IARC) states that over 10 million new cases of cancer occur each year and over six million deaths annually occur from cancer. The IARC also estimates that by 2030, the cancer burden will increase to 27 million new cases and 17 million cancer-related deaths globally (1). In response to these statistics, there is immense research in the field of medicinal chemistry for synthesis of novel anticancer agents (2), isolation and screening of natural product-based anticancer compounds (3), designing of novel drug delivery systems for chemotherapeutics (4) and biological drugs such as Small interfering RNA/Small hairpin RNA (si/shRNA) (5), to cure or manage the disease.

In vivo efficacy study of these anticancer agents or drug delivery systems majorly employs tumor xenograft model in mice. Tumor xenograft models are not only useful to establish the efficacy of anticancer agents in terms of tumor reduction but are also valuable in studying the effect of anticancer agents on key hallmarks of cancer, such as tumor angiogenesis (6), and metastasis (5).

All such studies utilize genetically-immunodeficient athymic nude mice for the development of tumor xenografts. Eventhough the nude mice xenograft model is widely employed, there are serious drawbacks associated with this model. Disadvantages include high cost; difficult inavailability, especially for developing countries; difficulty in transportation and aseptic maintenance; tendency for graft rejection; high mortality rate, *etc* (7-9). Additionally, nude mice may not accurately reflect true disease progression because they lack immune cells which play a critical role in tumorigenesis (10, 11).

In light of these drawbacks of nude mice, many researchers have proposed different immunosuppression protocols for the development of pharmacological immunosuppression with the help of appropriate methods such as total-body irradiation, neonatal thymectomy, and immunosuppressive drugs. For instance, Steel *et al*. proposed an immunosuppressive model by thymectomy and total-body irradiation combined with syngeneic bone marrow transplantation or cytosine arabinoside pre-treatment (12). Floersheim *et al*. developed a xenograft model of human tumors in mice after short-term immunosuppression with procarbazine, cyclophosphamide and antilymphocyte serum (13). However, the technical requirements of these protocols are expensive, prolonged and make animals moribund, which compromise their use for large-scale screening procedures. For instance, Floersheim reported a 33% mortality rate from thymectomy and a further 39% mortality rate within 60 days of irradiation with 9 Gy of megavoltage X-rays (14). After the discovery of cyclosporin

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A, displaying potent immunosuppressive effects against the allograft response in animals (15) and Man (16), cyclosporine A has become a drug of choice for developing pharmacologically immunocompromised models for the development of tumor xenograft.

Cyclosporine is a polypeptide derived from the fungus *Tolypocaladium inflatum* Gams. It has been reported that cyclosporine acts mainly by suppressing the release of interleukin-1 from macrophages, required for the activation of T-lymphocytes. It also inhibits the release of interleukin-2, which is essential for the proliferation of activated T-lymphocytes (17). Floersheim initiated the use of cyclosporine for the development of tumor xenografts in C3H mice (14). However, Floersheim reported minimal tumor development after administration of cyclosporine for 30 days. After this initial success, many researchers reported development of tumor xenograft by modifying the dose of cyclosporine, route of administration, duration of treatment and combining with other drug regimen in several strains of rat (8, 10, 18-20). Even though these models showed considerable success in the development of tumor xenograft, they have several limitations. These limitations include long duration of cyclosporine treatment, requirement for a large number of tumor cells, and validation with only a single cell line. Moreover, all these models have been developed in rats, which are more difficult to maintain and handle for the development of tumor xenograft compared to mice. Due to these limitations, these models are difficult to scale up and use to screen large number of anticancer agents. Hence, an immunosuppressive mouse model which is easy to develop, causes little or no mortality and can be exploited to develop xenograft from any cancerous cell lines with a 100% take rate is needed.

In the present study, a new, versatile and a reproducible immunosuppression protocol was developed by using cyclosporine, ketoconazole and cyclophosphamide drug regimen to develop human tumor xenograft in mice.

Materials and Methods

Materials. Cell culture mediums, Roswell Park Memorial Institute medium (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco, Grand Island, NY, USA. Similarly, fetal bovine serum (FBS), sodium bicarbonate, sodium pyruvate, trypan blue and trypsin were obtained from Gibco. Cyclosporine (Sandimmune) and Ketoconazole (Nizral) were purchased from, Novartis, Basel, Switzerland and Johnson & Johnson, New Brunswick, New Jersey, USA respectively. Cyclophosphamide (Endoxan) was purchased from Baxter, Halle, Germany. Ampoxin was purchased from Unichem laboratories Ltd., Mumbai, India. Rodent diet was obtained from VRK nutrition, Pune, India.

Animals. Healthy mice C57 BL/6 were purchased from Mahaveera Enterprises, Hyderabad, India. All the mice were kept in individually ventilated cages, with a relative humidity of 60±5% and a temperature of 25±2˚C was maintained. A 12:12 h light:dark cycle was also regulated for these animals. Balanced rodent food pellet and water was provided *ad libitum*. All experimental protocols were reviewed and accepted (PERD/IAEC/2013/014) by the Institutional Animal Ethics Committee prior to initiation of the experiment.

Cell lines. All the human cancer cell lines [A549 (human lung adenocarcinoma), LNCaP (human prostate adenocarcinoma), and KB (cervical adenocarcinoma)] were procured from NCCS, Pune, India. A549 and LNCaP cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1.0 mM Na pyruvate, whereas KB cell line was maintained in DMEM supplemented with 10% heat-inactivated FBS. The cells were grown in 75 cm^2 flasks and maintained in a standard tissue culture incubator at 37˚C with 5% CO_2.

Immunosuppression. Healthy male mice (C57 BL/6), 4-6 weeks old, were divided into seven groups (n=6). Groups 1, 3 and 5 were administered 5 mg/kg ketoconazole and groups 2, 4 and 6 were administered 10 mg/kg ketoconazole by oral route every day for 7 days. Groups 1 and 2 were administered 10 mg/kg, groups 3 and 4 20 mg/kg, and groups 5 and 6 30 mg/kg cyclosporine by intraperitoneal route every day for seven days. No treatment was given to the control group. All the animals were provided autoclaved rodent food pellet and water *ad libitum*. Animals were given ampoxin (0.1 μg/ml) by drinking water during the study. After completion of the study, hematology was carried out to determine the total white blood cells (WBC) and lymphocyte count to confirm immunosuppression. Cylophosphamide was injected subcutaneously at a dose of 60 mg/kg on days 3 and 1 before tumor cell injection in groups of mice showing the highest immunosuppression.

Total WBC and lymphocyte count. Blood samples were collected from all the animals from retro orbital sinus under isoflurane anesthesia in a heparinized 1.5 ml microcentrifuge tube. Total WBC and lymphocyte counts were then performed in an automated hematology analyzer (VetScan HM-5; Abaxis Inc.,Union City, CA, USA).

Preparation of tumor cells. Semi-confluent cells (A549, LNCaP and KB) were trypsinized by using 0.25% trypsin to detach the cells. Cells were centrifuged at 200 × *g* for 7 min at 4˚C, resuspended and washed in their respective growth medium *i.e.* RPMI-1640 and DMEM. After washing, cells were again resuspended in their respective growth medium. The cells were counted using a Neubaur chamber and viability was determined by trypan blue exclusion test. Viable cells were stored on ice and injected immediately.

Tumor implantation. Immunocompromised male C57 BL/6 mice (4-6 weeks old) were used (n=6 for each cell line). Hairs were removed by waxing from the shoulder blade of each animal one day before injection of 0.1ml of cells (approximately 5×10^6 A549, LNCaP and KB cells) subcutaneously into the right shoulder blade of mice. Tumor growth was observed at the site of injection. Tumor volume was measured every week externally by digital caliper using following formula (21):

Volume (mm^3)=(A) × (B^2)/2, where A was the largest diameter (mm) and B the smallest (mm).

At the end of the study, tumors were excised and histopathological analysis was performed.

Histopathological analysis. At the end of the study, tumors were excised from the animals and maintained in 10% neutral buffered formalin. Tumor samples were cut into 5 μm sections and stained with hematoxylin and eosin. The slices were observed and photodocumented by optical microscopy (IX 51; Olympus, Tokyo, Japan) equipped with a digital camera (TL4) in order to confirm the presence of malignant cells.

Statistical analysis. All the data are given as the mean±SD. One-way ANOVA followed by posthoc Bonferroni correction was applied to determine the significance of differences among groups. Probability values with $p \leq 0.05$ were considered to be significant.

Results

Immunosuppression. Combination of cyclosporine and ketoconazole induced significant immunosuppression in a dose-dependent manner when compared to control animals. Figure 1 shows the mean WBC and lymphocyte count in different groups of mice at the end of treatment. From the graph, it can be seen that efficient immunosuppression was found in the animals of group 6, which were administered cyclosporine (30 mg/kg) and ketoconazole (10 mg/kg), when compared to control animals. Total WBC and lymphocyte counts were significantly decreased in animals of group 6 when compared to control animals (Figure 1). Hence, animals from group 6 were selected for the tumor xenograft development.

Subsequently, cyclophosphamide was injected into animals of group 6 subcutaneously at a dose of 60 mg/kg on days 3 and 1 before tumor cell injection. Figure 2 shows the mean WBC, lymphocyte and neutrophil count in animals of group 6 after cyclophosphamide treatment. Administration of cyclophosphamide significantly reduced neutrophil and residual WBC and lymphocyte count in the animals of group 6. Moreover, none of the mice showed any signs of toxicity or premature death due to drug treatment. Hence, cyclosporine, ketoconazole and cyclophosphamide induced severe immunosuppression in the treated C57BL/6 mice.

Tumor implantation. Approximately 5×10^6 cells from each cell line (A549, LNCaP and KB) were injected subcutaneously into the shoulder blade of immunocompromised mice. In all injected animals, a palpable tumor was found on the third day after tumor injection, with a 100% take rate. Figure 3 shows the mean tumor volume each week after tumor implantation. It can be observed from the graph that the mean tumor volume increased radically every week until the fourth week for each cell line. Subsequently, the tumor volume was found to increase steadily until the eighth week. Thereafter it reached a plateau and maintained a steady state. The mean tumor volume of A549, LNCaP and KB xenograft was found to be 720 mm^3, 626 mm^3 and 668 mm^3, respectively. Subsequently, the tumor volume started to decrease in some animals. Growth of A549 and KB xenografts was found to be more aggressive than that of LNCaP xenografts. Figure 4 shows C57BL/6 mice bearing tumor xenograft eight weeks after tumor implantation (Figure 4). Hence, with this protocol, tumor xenografts successfully developed using three different cell lines, *i.e*. A549, LNCaP and KB, and were maintained for more than two months.

Histopathological analysis. The presence of malignant tumor was confirmed by histopathology. Tumors were excised, sectioned and stained with standard hematoxylin and eosin. Figure 5 shows hematoxylin and eosin-stained sections of A549, LNCaP and KB xenografts. The section shows cells with large and irregular nuclei and scant cytoplasm, which are characteristic of malignant cells (Figure 5B). Histopathological analysis also revealed the presence of angiogenically-activated blood vessels, suggesting the induction of angiogenesis (Figure 5Ai). Additionally, it also shows malignant cells invading adjacent stromal tissue (Figure 5Aii and iii).

Discussion

Tumor xenograft models are primarily used to evaluate the *in vivo* efficacy of anticancer agents (3-5). These studies employed athymic nude mice for the development of tumor xenograft. However, due to several limitations of nude mice, many researchers have developed immunocompromised models which can accept tumor xenograft and subsequently proliferate to produce larger tumor. These models employ total body irradiation, neonatal thymectomy, and immunosuppressive drugs (12, 13). However, these protocols are expensive and cause huge mortality.

Cyclosporine, a potent immunosuppressant, selectively inhibits the activation of T-cells. Cyclosporine binds to the cytosolic protein cyclophilin of T-cells. This complex inhibits calcineurin, which, under normal circumstances, is responsible for activating the transcription of interleukin 2, which promotes T-cell activation and proliferation (22, 23).

Floersheim first reported the use of cyclosporine for the development of tumor xenograft (14). However, Floersheim reported minimal tumor development after daily administration of cyclosporine (100 mg/kg) for 30 days. In 1983 Hoogenhout *et al*. reported a combination of total lymphoid irradiation, cyclophosphamide and cyclosporine A for immunosuppression and achieved a 100% take rate with mouse osteosarcoma. However, with this protocol they achieved only a 63% take rate with human colonic adenocarcinoma (18). Similarly, Goodman *et al*. reported the growth of human melanoma section in Lewis rats given cyclosporine at 15-50 mg/kg with a 85% take rate. However, under the same protocol they were unable to grow tumors when human melanoma cell suspension injected subcutaneously (8). Akhter *et al*. reported a 100% take rate of the human colonic adenocarcinoma cells in Sprague

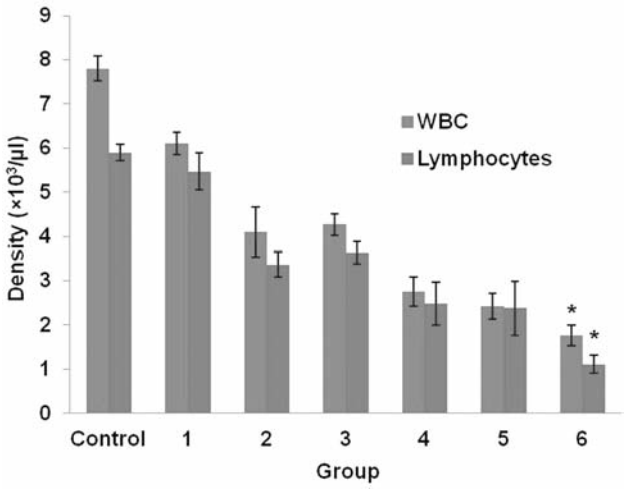

Figure 1. *Graph showing the mean white blood cells (WBC) and lymphocyte count of different groups of mice at the end of cyclosporine and ketoconazole treatment (n=6) (*p<0.05). Groups 1, 3 and 5 were administered 5 mg/kg ketoconazole and groups 2, 4 and 6 were administered 10 mg/kg ketoconazole by oral route every day for 7 days. Groups 1 and 2 were administered 10 mg/kg, groups 3 and 4 20mg/kg, and groups 5 and 6 30 mg/kg cyclosporine by intraperitoneal route every day for seven days. No treatment was given to the control group.*

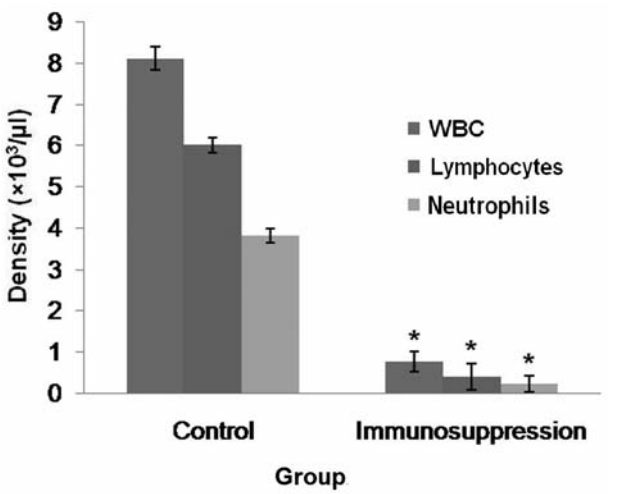

Figure 2. *Graph showing mean white blood cells (WBC), lymphocyte and neutrophil count of control and of immunosuppressed mice after cyclophosphamide treatment (n=6) (*p<0.05).*

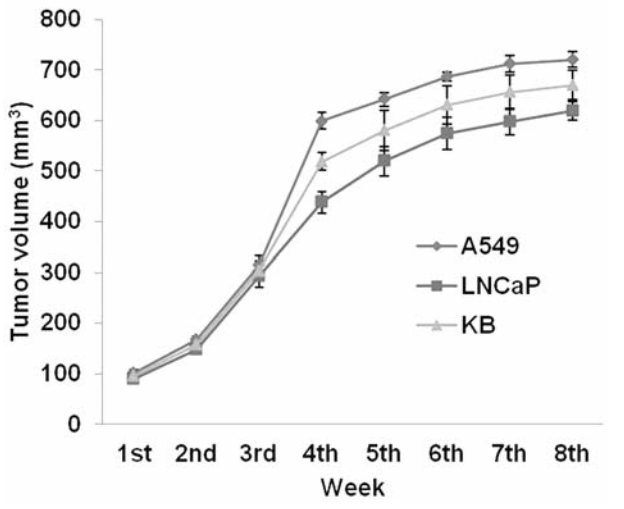

Figure 3. *Graphs showing mean tumor volume of A549, LNCaP and KB xenograft (n=6).*

Dawley rats when administered 35 mg/kg daily cyclosporine until the end of study. However, the inoculum size injected in this protocol was quite high, ranging from 50-100×10^6 cells per rat (10). Recently in 2011, Cunha *et al*. reported xenotransplantation of human glioblastoma cells in immunosuppressed rats induced by orogastric cyclosporine at a dose of 5 mg/kg until the end of study (20).

All the above discussed immunosuppression protocols were validated with only single cancer cell line or tumor xenograft. Moreover, to achieve prolonged immuno-suppression, cyclosporine was administered daily until the end of study. Along with its potent immunosuppressive activity, cyclosporine is also reported to have an anticancer activity (24, 25). Hence, the poor take rate of xenograft with the protocols discussed above may be attributed to anticancer activity of cyclosporine. Prolonged immunosuppression protocol, high inoculum size, and variable take rate limit the use of such protocols.

In the present study, a simple, versatile and cost-effective immunosuppression protocol was developed using a cyclosporine, ketoconazole and cyclophosphamide drug regimen to develop human tumor xenograft in mice. Ketoconazole is an antifungal agent which interfere with synthesis of ergosterol, a constitute of fungal cell membrane. Moreover, it also inhibits cytochrome p450 enzyme which metabolizes cyclosporine (26-28). In this way, ketoconazole helps in prolonging circulation of cyclosporine and simultaneously protects from probable fungal infection, which is very common with cyclosporine treatment. Cyclophosphamide is an alkylating agent that interferes with DNA replication. It also reduces the number of neutrophils, B- and T-cells and natural killer cells to a significant extent (29-32).

Immunosuppression of animals receiving cyclosporine and ketoconazole was evident by significant reduction in total WBC and lymphocyte count. Administration of cyclophosphamide to these animals suppressed neutrophils and residual B- and

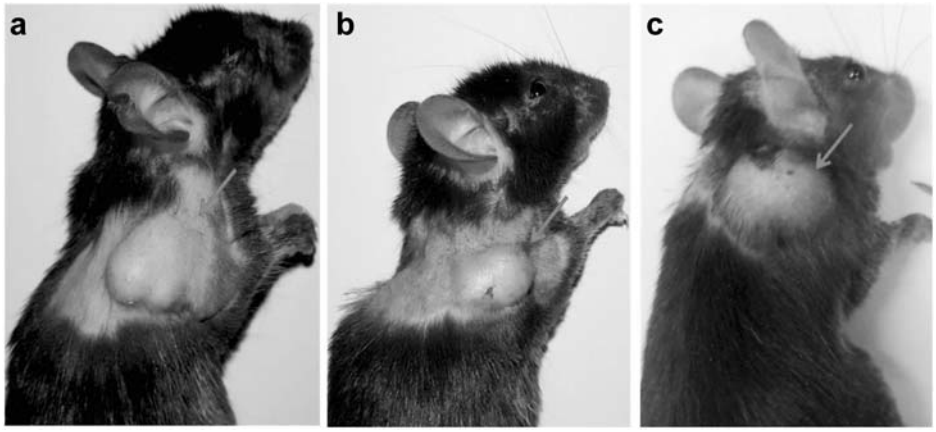

Figure 4. *Immunocompromised C57BL/6 mice bearing tumor xenograft of A549(a), LNCaP(b), and KB(c) as indicated by arrows.*

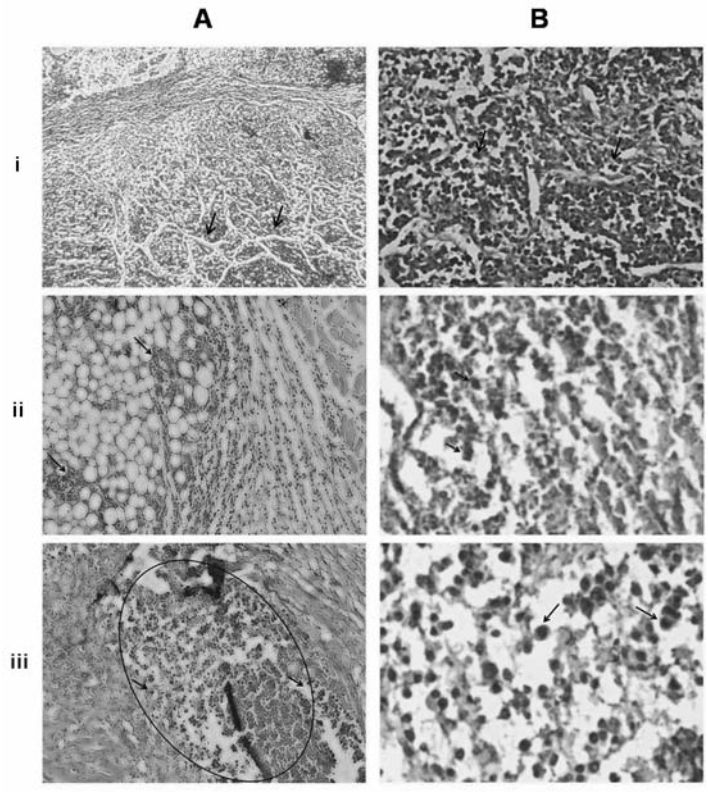

Figure 5. *Light microscopy observation of HE-stained section of A549(i), LNCaP(ii), and KB(iii) tumor xenograft. A: Light microscopy at ×100 magnification. Angiogenesis and tumor cell invasion are indicated by arrows. B: Light microscopy at ×400 magnification. Tumor cells having large nucleus and scanty cytoplasm are indicated by arrows.*

T-cells, which prolonged immunosuppression, as well as achieving a 100% take rate of tumor xenograft. Ketoconazole and ampoxin protected immunosuppressed animals from bacterial and fungal infection, which is a major cause of death in immunosuppression protocols.

Thus, using a combination treatment with cyclosporine, ketoconazole and cyclophosphamide, a 100% take rate was achieved with human lung adenocarcinoma, prostate adenocarcinoma and cervical adenocarcinoma in C57/BL6 mice. Increasing tumor volume was maintained for eight

weeks after tumor implantation with all three xenograft types. Histopathological analysis of tumor xenograft confirmed the presence of malignant tumor cells. It also showed invasion of tumor cells into adjacent stromal tissue; however, the metastatic potential of xenografts was not evaluated in this study. It also revealed the presence of angiogenic blood vessels, which is a prerequisite for tumor formation. Thus, it was confirmed that the xenografts that developed were not the result of simple hyperplasia but was malignant and invasive tumor. However, there is a possibility for neoplastic transformation of host (mouse) stromal cells by the injected human tumor cells (33, 34). Hences further characterization of the tumors is required depending upon the specific use.

In conclusion, a new, versatile, and relatively short immunosuppression protocol was developed using a combination of cyclosporine, ketoconazole and cyclophosphamide drug regimen. The protocol was validated with three different human adenocarcinomatypes, namely lung, prostate and cervical carcinoma for induction of tumor xenograft in C57BL/6 mice. A 100% take rate was achieved by this protocol, with no mortality until the end of study. Moreover, in this protocol, all the immunosuppressive drugs were administered before tumor implantation hence interaction of cyclosporine and cyclophosphamide with any anticancer drug to be evaluated can be avoided. The developed model is cost effective and relatively simple to establish as compared to previously reported models and can be used in place of athymic nude mice to evaluate efficacy of novel anticancer drugs, targeted drug delivery systems and even to study pathophysiology of human tumors. This model will be a boon for developing countries where nude mice are often unavailable for cancer research.

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