TUMOR PROGRESSION DECREASED LIFE SPAN OF MALE MICE AS COMPARE TO FEMALE MICE

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Abstract:

Aims of the Study: *The effect of gender differences in human development, aging, and disease isvery common. There are measurable differences between men and women in cancer prevalence, mortality, and progression.The latest analysis of the National Cancer Institute Surveillance Epidemiology and End Results (SEER) database showed that the lifetime probability of developing cancer is 44.85% for males as compared to 38.08% for females. It was also noted that there are several cancers that are more frequent in male as compare to female and shows high mortality rate in male. Therefore, in the present study, the effects of gender variation on the development of Dalton's lymphoma (DL), a type of TransplantableT-cell lymphoma of spontaneous origin, mimic human T cell lymphomawere investigated.*

Methods and Material: *Groups of normal and DL-bearing BALB/c mice of different days were taken to measure the IFN-γ, survival rate and different physiological changes in the body such as body weight and belly sizes of male and female mice. Cryosectioning of the*

reproductive organ of different days of male and female was also done to see the effect of the tumor progression.

***Result**: It was found that the development of Dalton's lymphoma in mice was gender dependent and male mice were more prone towards DL progression. As a result, the male dies after 21 days, whereas female dies after 31 days of DL transplantation.The release of IFN-γ more in female as compare to male mice.*

***Conclusion:**From the above findings, it can be concluded that development of cancer shows gender dimorphism. As a result, female mice shows less vigor of tumor progression as compare to male mice. Although the precise mechanism(s) underlying the observed gender dimorphism of the differential induction of tumor progression.*

***Keywords:** Tumor; Longevity; Cytokine; reproductive organ.*

Abbreviation: SF= Seminiferous tubules, ST= Spermatogonia, SC= sertoli cell, TE= Theca Externa, TI= Theca Interna, Gf= Graafian follicle, An= Antrum, CR= Corona radiate, ZP= Zona pellucida, OO= oocyte, O.C.T= Optimum cutting temperature, IFN-γ= Interferon gamma- γ, DL= Dalton's Lymphoma.

1. INTRODUCTION

The effects of gender differences in human development, aging, and disease are very common.There are considerable differences between men and women in terms of cancer prevalence,progression, and mortality. Understanding the effects of sex differences, it is required to examine the differences between male and female at the molecular, cellular, tissue, organismal and evolutionary levels, because the study of sex differences can provide a novel view for particular disease of interest. Moreover, it has the potential to put lights on the hidden facts of pathobiology of a disease. In order to affect disease phenotype, sex must affect fundamental mechanisms of risk and progression of that particular disease. Genderemploys its effects through differences in sex chromosome, action of sex hormone, and sex specific maintenance or reprogramming of maternal and paternal genetic profile. As a result, males and females shows different normal physiologies as well as different responseto developmental, metabolic, and genotoxic stressors.These compensatory reactions produce further gender specific changes in the genetic profile to widen the differences between males and females for a disease risk.

The effect of gender difference in primary cancer susceptibility can be measure by comparison of incidence rates in males and females. The analysis of the National Cancer Institute Surveillance Epidemiology and End Results (SEER) database showed that despite a short life expectancy, male has higher probability of developing cancer (44%) as compare to the female with 38.08% only. The same analysis also showed that the cancer mortality rates were higher in males: 223.0 versus 153.2. Most importantly, the common cancers have the highest male-to-female (M:F) ratio like, colorectal cancer(1.35), lung and bronchus (1.52), non-Hodgkin lymphoma (1.44), urinary bladder (4.0). Except breast cancer, which rarely occurs in males, only a few cancers like Gall bladder, anal, and thyroid tumors with M:F ratio less than 1.0 are more common in females (Cook et al., 2009).

Studies show that the increased male to female ratio for incidenceofcancer is not distinctive to a particular country, population, or region. The data analysis of age and sex-specific cancer in five continents provided by the International Agency for Research on Cancer (IARC) revealed the universal nature of the gender disparity in cancer (Edgren et al., 2012). In numerous cancer sites across the geographical regions, maleswere found with higher incidence rates consistently with three exceptions of thyroid, gallbladder, and anal cancer that had higher incidence rates in females.Even in the childhood, gender differential play an important role in the incidence of cancer, and it is well established that males Childs are always at a higher risk than females and it is consistent worldwide (Pearce and Parker, 2001; Cartwright et al., 2002; Desandes et al., 2004).

However, existence of sex differential or gender dimorphism and the mechanisms associated in the progressivegrowth of several types of tumors remains unclear.Although, nearly 18% of total human malignancies are lymphocytic origin (Nakamura, 2004), very little information is available in the literature regarding the mechanism behind this. It is also not deciphered whether gender-specific hormones have any role in the regulation of suchlymphocytic tumors. Recently, lymphoma is considered as one of the most complicated neoplasm for clinicalmanagement (Moll et al., 2004;Zhou et al., 2005). Dalton's lymphoma (DL) is a transplantable T cell lymphoma that originates inthymus of DBA strain (H-2d) of mice (Klein, 1951; Goldie et al., 1951). DLcan grow as anascitic or a solidtumor (Udaychander et al., 1987)and has been reported to possess chromosomal aberrations(Khynriam et al., 2003).Earlier, it has been observed that in comparison to DBA

mice progression of DL growth is rapid in syngenicBALB/c (H-2d) mice causing death of the host in a relatively short time (Vivekanand et al., 2008).

Therefore, in the present study, using a murine model of Dalton's lymphoma, we have investigated several aspects of host–tumor relationship. Our results show that DL progression is gender dependent and it was found more aggressive in male mice as compared to female mice. Results also indicate that expression of IFN-γ was gender specific and its production was more prominent in female mice. To the best of our knowledge, this is the first report of its kind to show that progression of Dalton's lymphoma in mice is gender dependent.

2.MATERIALS AND METHODS

2.1 Mice and tumor system

Inbreed adult mice of either sex at 8–10 weeks of age were used. The mice received food and water *ad libitum* and were treated with almost human care in an approved animal room facility. For tumor system, Dalton's lymphoma (DL-cells), a type of T-cell lymphoma were maintained in ascitic form by serial transplantation in BALB/c mice, and stock of DLcells is also maintained in a cryopreserved state for reference. In all the experiments, peritoneal macrophages and DL cells as applicable were obtained, where the yield of cells is higher. To induce T-cell lymphoma, mice were injected intra-peritoneally with 1.5 X 10^6 cell/mouse in 1ml phosphate buffer saline (PBS).

2.2. Reagents

Tissue culture medium RPMI-1640 from Hi Media (India) and fetal calf serum (FCS) was purchased from Hyclone (Logan, Utah), Phosphate buffer saline (PBS) from HiMedia, IFN-γ ELISA kit (ELISA MAX™ Deluxe Sets) was purchased from BioLegend, India and most of other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo) and O.C.T compound was purchased from Sakura company and plastic wares purchased from Tarson, India.

2.3. Serum preparation

For serum preparation, blood from adult DL– bearing male and female mice were collected at different time interval by retro-orbital bleeding. The blood was kept at room temperature for 45-60 min and then after separating the clot from eppendorfwall, kept overnight at 4°C, Serum was then collected, centrifuged at 1500 rpm for 15 min to separate hemolysed cells.

After centrifugation, serum was finally collected and stored at –20°C for further experiments.

2.4. Measurement of body weight and belly thickness

Pathogen free inbreed populations of BALB/c (H-2^d) strain of mice (N=3) of different sex and 8–12weeks age of mice were injected with 1.5 × 10^6 Dalton's lymphoma cellin 1 ml phosphate buffer saline (PBS) for tumor model.After DL transplantation, male and female group of mice were rear for 18 days for full growth of Dalton's lymphoma. In the meantime, body weight and belly thickness (or volume of the belly) of each mice was measures at interval 0, 6, 12 and 18 days upon DL transplantation.

2.5. Host Survival Assay

Group of healthy male and female mice transplanted with 1.5 × 10^6 Dalton's lymphoma cellin 1 ml PBSwere analyzed their rate of survival. Both groups of mice were kept under observation for tumor growth reared until death. Mice survived for number of the days was plotted against their respective group.

2.6. IFN-γ measurement by ELISA

Expression of IFN-γ level in the serum of DL-bearing male and female mice at different time intervals was measured by Sandwich ELISA. Briefly, 100 µl capture antibody was added in each well of 96 well flat bottoms ELISA plate (Tarson, India) incubated overnight at 4ºC. After three wash, 200 µl washing buffer was added for 1 hr to block non-specific binding followed by addition of 100 µl diluted standard and sample in each wells, shake gently and incubated for 2 hrs at room temperature. Wells were added with 100µl detection antibody and incubated for 1. Washed the wells twice and incubate with 100µl Avidin-HRP solution for 30 min at room temperature. Finally, 100µl TMB substrate buffer was added in dark condition and after 20 to 30 min absorbance was taken at 450 nm with the help of ELISA plate reader (Biorad, Hercules, CA, USA).

2.7.Staining of Testing and Ovary

Testis and Ovary were dissect out from normal and DL bearing mice of different days(0, 6, 12, 18).These were fixed in fixative 4% paraformaldehyde overnight at 4°C and after that rinse in different concentration of Sucrose solution and organs were fixed in O.C.T compound for cryosectioning. Section was stained with hematoxylin for 3 min at RT after rehydration. Further tissue section slide were washed thrice with water and dehydrate by

different alcoholic solution 30%, 50%, 70%, & 100%. Eosin staining was used after 70% of serial dehydration and slide was mounting in DPX and oil immersion imaging was done.

2.8. Statistical Analysis

Simple Student's t test was used to test the significance of the data obtained in experimental settings for IFN-γ production. All the experiments were performed at least three times and data were taken as significant at $p<0.05$. All statistical analysis wereperformed on Sigma Plot Version 11 (Systat Software, San Jose, CA, USA).

3. RESULTS

3.1. Effect of Tumor progression on body weight and belly thickness of mice

Group of adult male and female mice of same age and body weight were transplanted with 1.5×10^6DLcells per mice in 1ml PBS and increase in their body weight and belly size was measured at different time intervals. Result shows that as the tumor progress, body weight and belly size of both groups of mice was increases in time dependent manner. After 6 days of DL transplantation, increase in the body weight and belly size was not so evident in any group of mice, as the day progresses, weight and belly size of mice were also increases. However, it was interesting to observe that increase in the body weight was more evident in male mice as compare to the female **(Fig. 1.A**). Further, increases in the belly size was also follow the similar patter to body weight and male mice shows more swelling of belly as compare to female mice**(Fig.1.B**).Day 0 to day 18 belly size of male were increase upto 3.1 cm from 1.4 cm and female have also increase but less than the male i.e., 1.6 to 2.8 cm shown in (**Fig. 2).**

3.2. Effect of tumor progression on male and female mice as days increase

As the day increases after DL transplant, male and female mice's physiology as well as number of DL cell count was also changed. The graph showed that male mice had more number of DL count as comparison to female mice. These are probably due to female have more number of hormones and it also have different reproductive cycle whereas male has less number of hormone (**Fig. 3**).

3.3. Effect of Tumor progression on the development of testes

Effect of tumor progression on testeswas studied with the help of Histochemistry. Briefly, the cryosection of testes was mounted on a slide and observed under microscope. It was found that on 0 days (day of tumor transplantation) no any effect of tumor on the internal

structure of seminiferous tubule. All cells such as spermatogonia, sperm cells and sertoli cells are compactly arranged.Whereas, after 6 days deformed structure of seminiferous tubules was found. The spermatogonia are loosely arranged, as a result, less number of sperm cells can be seen in the tubules. Further, outer layer of seminiferous tubules become more deform and sperm cells starts degenerated after 12 days of DL transplantation. Complete degeneration of seminiferous tubules and spermatogoniawas observed after 18 days of tumor transplantation (**Fig. 4**).

3.4. Effect of Tumor progression on the development of ovary

Further, effect of tumor progression on the development of ovary was studied by histochemical analysis of cryosection of ovary of a DL-bearing mouse. It was observed that tumor progression has immense impact on oocyte development. On 0 day of DL transplantation female ovary shows no such effect of tumor on the internal structure of graafian follicle. All the cells are compactly arranged their order such as circular shaped oocyte surrounded by corona radiata grow and form membrane granulosa cells. After 6 days of tumor transplantation, deformed structure of membrane granulosa cells was observed in ovary. It is loosely arranged, outer and inner layer become perforated and oocyte shows some deformed structure. But after 12 days degeneration of oocytes become more prominent and it completely degenerated after 18 days of DL transplantation (**Fig.5**).

3.5. Effect of Tumor progression on IFN-γ production

Production of IFN-γ in tumor bearing mice was studied with the help of sandwich ELISA. Briefly, serum was isolated from DL-bearing male and female mice after different time intervals, coated with monoclonal antibody and process as described in material and methods. Result shows that DL progression significantly affects the expression of IFN-γ in male and female mice. As tumor progresses, expression of IFN-γ increases (till 6^{th} day of DL transplantation, but after that tumor cells tries to neutralize immune system of the host and as result secretion of IFN-γ decreases in the serum of male and female mice. However, it was surprising to see that on 18^{th} day of DL transplantation, expression of IFN-γ increases abruptly, probably due to final attempt to overcome from the tumor burden in both cases (**Fig. 6**). It was also noted that at any time point, expression of IFN-γ in female was always higher than male.

3.6. Effect of Tumor progression on survival rate of mice

Group of male and female mice transplanted with 1.5×10^5 Dalton's lymphoma cellin 1 ml PBSwere analyzed their rate of survival. Result shows that female mice were survived for longer time (31 days) as compare to male mice that survived only for 23 days (5 days more than the time of full growth of Dalton's lymphoma). Data clearly show that male mice are more prone to cancer development than female mice and therefore, show less survival rate after tumor development (**Fig.7**).

4. DISCUSSION

Our results showed that during tumor progression, male and female mice gained body weight in time dependent manner. As the tumor progresses, body weight of male and female affected differently as male gain morebody weight as compared to female, as a result the belly size of male is much larger than that of the female mice. This result clearly indicates that gender dimorphism play important role in progression of T cell lymphomaowing the expression of proteins regulating tumor growth and cell death. Although the precise mechanism(s)underlying the gender dependent tumor progression and cell deathis remains unclear. Previously, the apoptotic pathwaysin the cells was shown to be regulated by gender-specifichormones like testosterone, androgen, estrogen,progesterone and other pro-apoptotic factors likecytokine and reactive free radicals (Hatzoglou et al., 2005; Lewis et al., 2005;Kirschenbaum et al., 2006; Zhang et al., 2004). Therefore, it is also required to investigate the involvement of other factors known to regulate cell death and proliferation through these pathways.

We further investigate the effect of tumor progression on gonadal development in male and female mice. Results show that development of tumor in male and female mice greatly affect the etiology of testes and ovary. On day 0, there were no any changes in either of the organs at any level, but after 6 day, deformity in the structure of testes and ovary was observed. Result clearly indicate that after 12 days of DL transplantation, seminiferous tubules of testes and inner layer of oocytes were loosely bound and respective cells were also show sign of significant deformity in their structure; but, after 18 days, the seminiferous tubules in male testes and membrane of graafian follicle are completely deformed. From our results, it is very clear that tumor developments equally affects the deformation of male and female gonads and have no any significant difference in the time of deformation.

Further, effect of tumor progression on the development of ovary was studied by histochemical analysis of cryosection of ovary of a DL-bearing mouse. It was observed that tumor progression has immense impact on oocyte development. On 0 day of DL transplantation female ovary shows no such effect of tumor on the internal structure of Graafian follicle. All the cells are compactly arranged their order such as circular shaped oocyte surrounded by corona radiata grow and form membrane granulosa cells. After 6 days of tumor transplantation, deformed structure of membrane granulosa cells was observed in ovary. It is loosely arranged, outer and inner layer become perforated and oocyte shows some deformed structure. But after 12 days degeneration of oocytes become more prominent and it completely degenerated after 18 days of DL transplantation (Fig. 3).

Further, effect of tumor progression on IFN-γ production was studied and it was observed that the level of production of IFN-γ in male and female mice serum was different. After 18 days of DL transplantation, female mice show higher production of IFN-γ as compared to male mice. Earlier, it has been established that IFN-γsecreted by T cells and NK cells directly regulate apoptosis in lymphoma cells (Nitsu, 2002). Therefore, increase in IFN-γ level at the advanced stage of tumor development clearly indicates the role of hormone-dependent cytokinesin regulation of gender dimorphism of tumorcell death. Findings of the study clearly corroborate the previous findings that male are more prone towards cancer development as compare to the female, and provide better understanding regarding the involvement of gender and their specific hormones in the progression of T cells lymphomas.

5. CONCLUSION

The gender difference in cancer susceptibility is one of the most consistent findings in cancer epidemiology. Hematologic malignancies are generally more common in males and this can be generalized to most other cancers. In human population, worldwide, few cancers are more common in females, but overall, males have higher susceptibility. Therefore, the present investigation was undertaken to study the effect of gender on the development of transplantable murine T cell lymphoma, designated as Dalton's lymphoma (DL). From the result, it can be concluded that development of cancer shows gender dimorphism. As a result, female mice shows less vigor of tumor progression as compare to male mice. Although the precise mechanism(s) underlying the observed gender dimorphism of the differential induction of tumor progression remains unclear, present study has clinical

significance as these results will helps in understandingthe involvement of gender and their specific hormones with respect to the progression of Tcells lymphomas.

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Figure and Figure legend

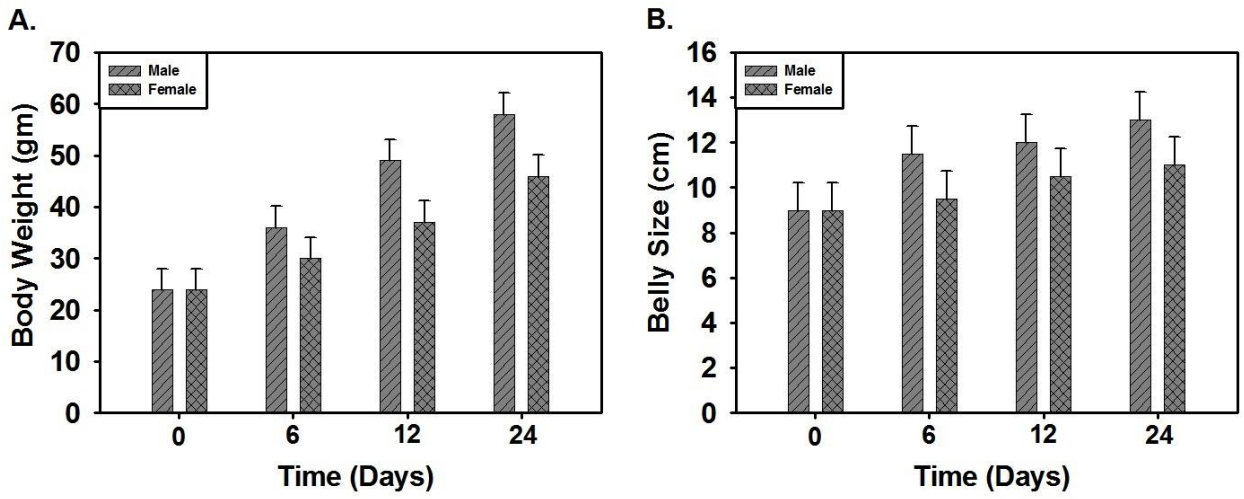

Figure 1.Effect of DL progression on body weight and belly size. Group of male and female mice were transplanted with 1.5×10^6 DL cells per mice and their effect on body weight and belly size was measured at different time intervals. Figure (**A)** shows comparative analysis of body weight of male and female mice in which male gain more weight as compare to female during tumor progression, whereas (**B)** shows significant increase in belly size of male mice as compare to female mice during tumor progression. Data represent the mean body weight and belly size ± SEM of three independent experiments in triplicate.

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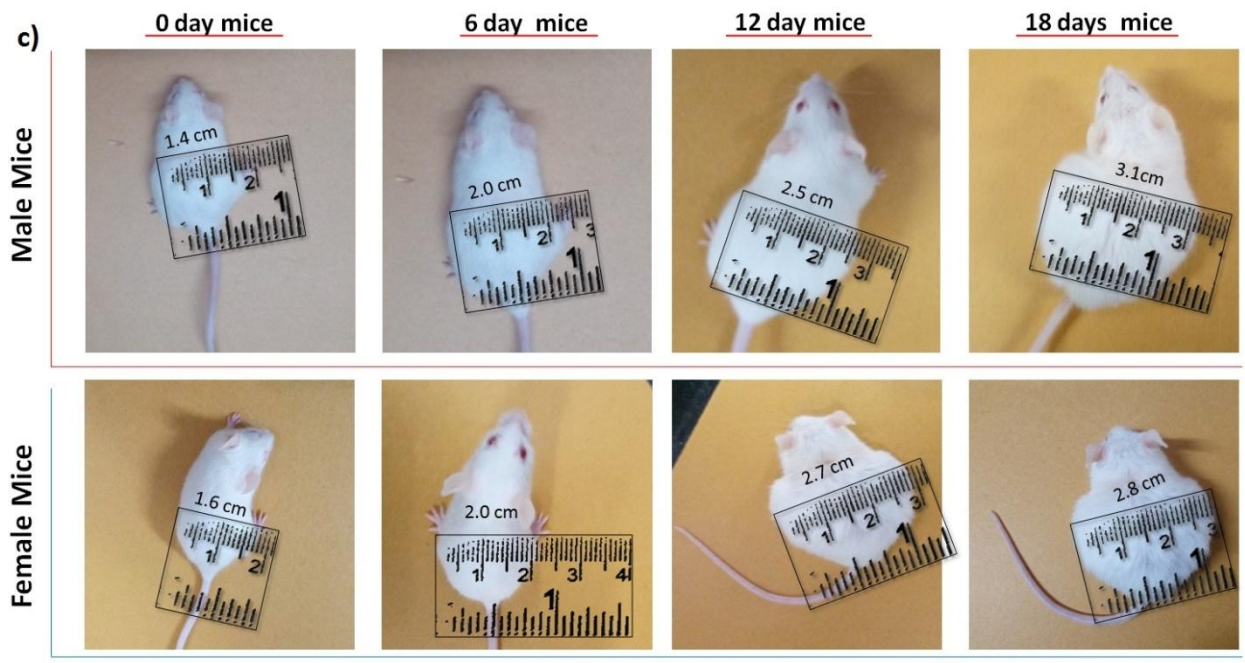

Figure 2.Effect of DL progression on belly size. Group of male and female mice were transplanted with 1.5×10^6 DL cells per mice and their effect on belly size was observed at different time interval (days) (0, 6, 12, 18) and found that male mice were showing increase in belly size as compared to female mice.

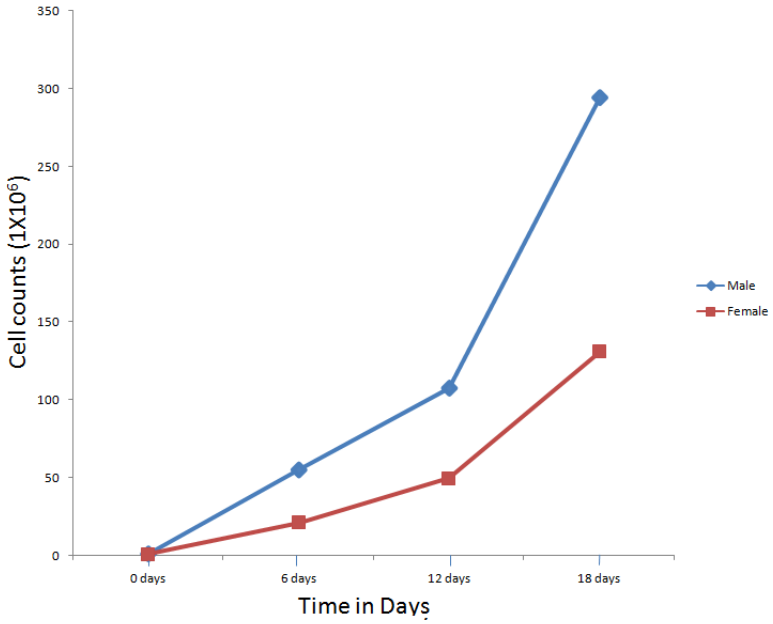

Figure 3. This graph shows DL cells count per ml with respect to the days (0, 6, 12 and 18). Group of male and female mice were transplanted with 1.5×10^6 DL cells per mice and found that male mice have more DL cells count as the days increase in comparison to the female mice.

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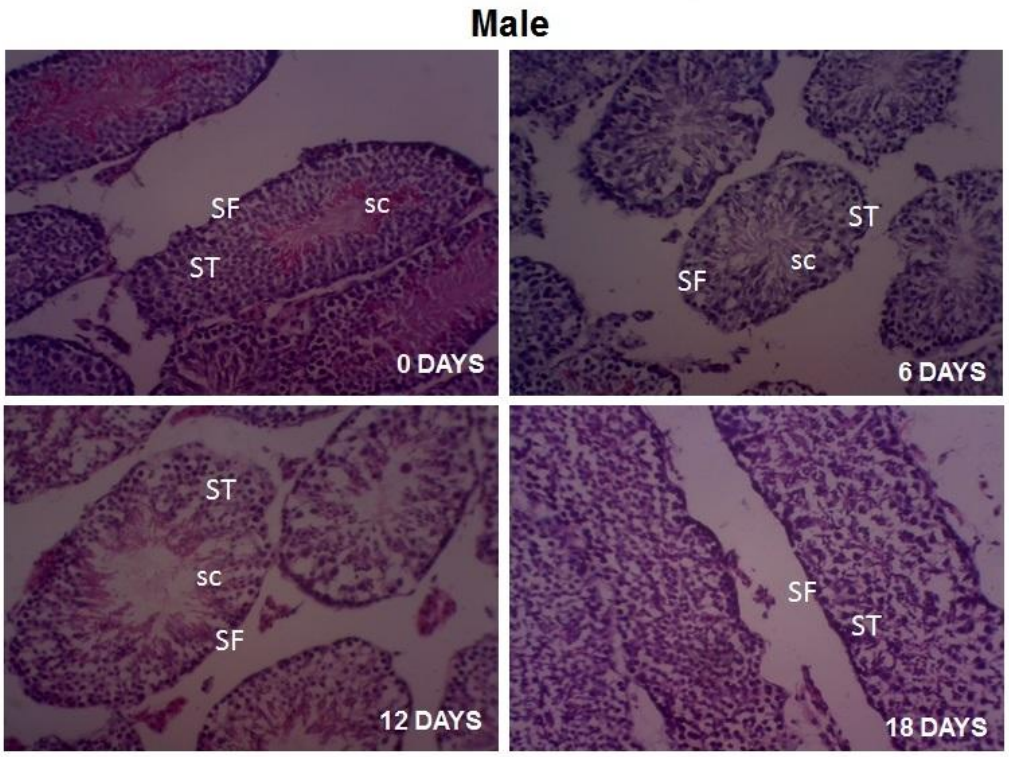

Figure4. Effect of tumor progression on testes development. On 0 day all cells such as spermatogonia, sperm cells and sertoli cells are compactly arranged whereas, after 6 days deformed structure of outer wall of seminiferous tubules was found. After 12days, outer layer of seminiferous tubules become more deform and sperm cells starts degenerated, whereas on 18 days, complete degeneration of seminiferous tubules and spermatogonia was observed.

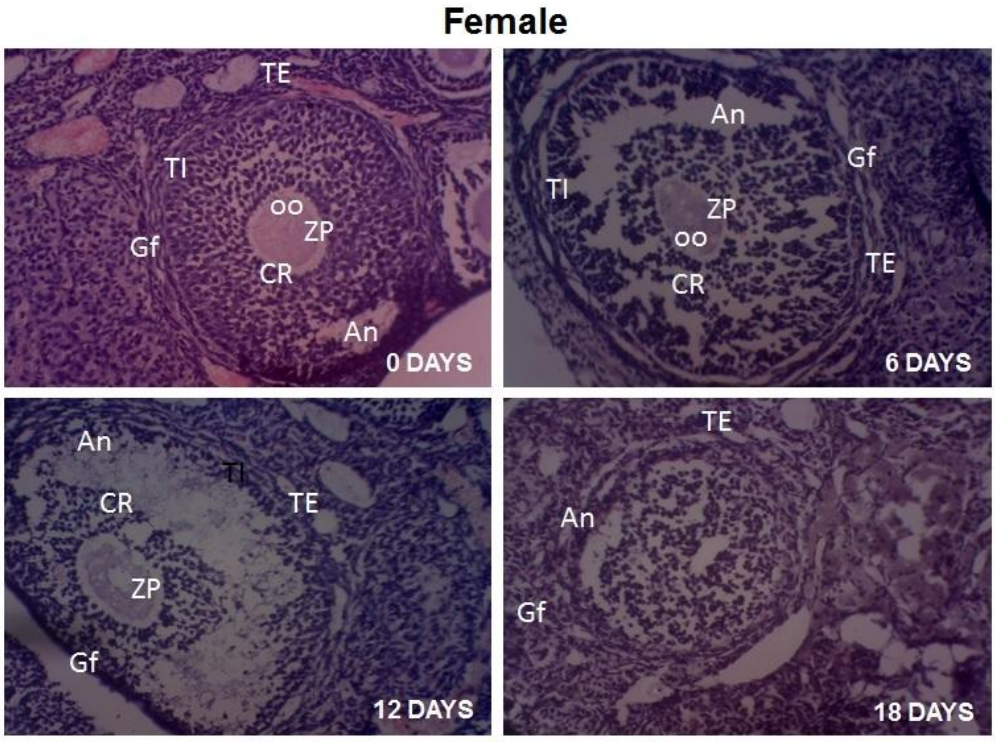

Figure5. Effect of tumor progression on ovary development. On 0 day internal structure of graafian follicle are compact and cells are compactly arranged, but it is loosely arranged, outer and inner layer become perforated and oocyte shows some deformed structure on day 6. After 12 days degeneration of oocytes become more prominent and it completely degenerated after 18 days of DL transplantation.

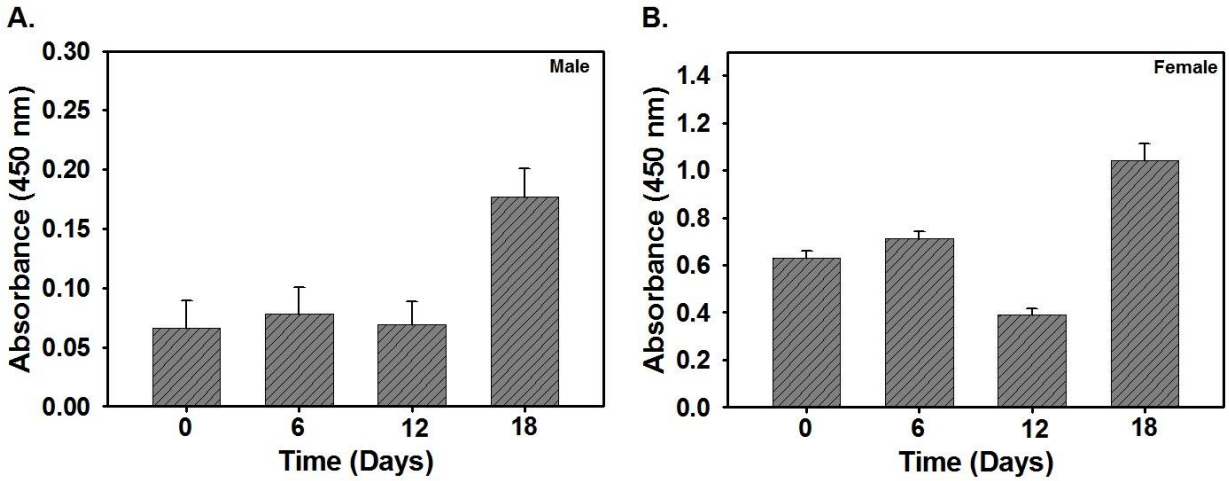

Figure6. Effect of tumor progression on IFN-γ expression. Serum isolated from DL-bearing male and female mice were treated with monoclonal anti-mouse IFN-γ antibody in a 96 well flat bottom ELISA plate and absorbance was taken at 450 nm. Figure A, shows concentration of IFN-γ in male serum, whereas B, shows concentration of IFN-γ in female serum.Data represent the mean concentration of IFN-γ ± SEM of three independent experiments in triplicate.

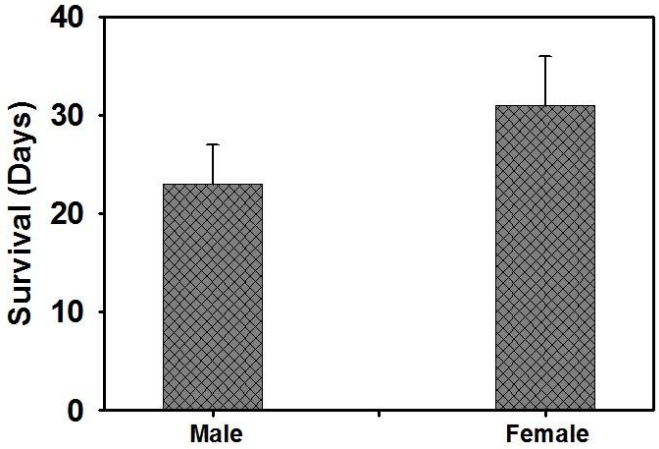

Figure7. .Effect of tumor progression on the survival of mice. Group of male and female mice were injected with 1.5 × 10^5 Dalton's lymphoma cell in 1 ml PBS were analyzed their rate of survival. Figure shows that female survive for 31 days after DL transplantation as compare to the male for 23 days only.