Research Article

Amifostine Has Chemopreventive Effects in a Mouse Skin Carcinogenesis Model

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Purpose: Amifostine (WR-2721) is dephosphorylated and converted into an active free radical scavenger (WR-1065) by the enzyme alkaline phosphatase, which is found at high levels in normal tissues but at low levels in tumor cells. However, although there are studies on the fibrosis-healing effect of Amifostine, there is no study on preventing secondary cancerization. We aimed to investigate the chemopreventive properties of WR-1065, the active metabolite of WR-2721 by using Amifostine at different stages of carcinogenesis in the skin carcinogenesis model to shed light on the question of whether it will protect only the normal cells and prevent the formation of secondary cancers.

Material and Methods: 5-6week old female, 160 CD-1 mice weighing 22-24 g were purchased from the laboratory of Charles River Breeding, Germany. Forty CD-1 mice were used twice weekly for the toxicity study to find the practicable dose. Skin carcinogenesis control and experimental groups were formed with 120 CD-1 mice. Control group; DMBA (100 nmol) day 0. + TPA (10nmol twice a week, 22 weeks), Experiment 1; to measure the promotional effect, it was applied twice a week for 22 weeks before TPA application, Experiment 2; To measure the effect of initiation, Amifostine was applied 2 days before and 5 days after the DMBA application, Experiment 3; Amifostine was administered before both DMBA and TPA to measure the effect of both initiation and promotion. The number of tumors per week (incidence) and the number of mice with tumors (multiplicity) were noted up to week 33rd. Tumor samples were stored in formalin solution for histopathological analysis. Statistical comparisons for normal data among groups were performed using the one-way ANOVA test, then the Dunnet test was used for non-normal data among groups was performed by using the Kruskal Walls test and then Bonferroni correction was used for comparison of the

experimental groups with the control group (p<0.016 was considered as statistically significant for Bonferroni correction).

Results: Papillomas were first seen during the 6th week in the control group. Incidence and multiplicity values for the week recorded for the control group were compared with each group of Experiment-1, Experiment-2, and Experiment-3. At the end of the 22nd week, tumor mean values for control and experimental groups were 40.81±18.26, 13.00 ± 11.99, 18.04 ±20.94, 4.82 ± 5.93 (*p<;.001,*p<;.000*, p<;.001), tumor multiplicity respectively; 26 (100%), Experimental-1; 22 (91.7%), Odds Ratio (OR): 2.18, Experimental-2; 25 (100%), OR:1, Experimental-3; 17 (77.3%), OR:2.52, (*p=.225*, p=1, p=.015) were found. As a result, Amifostine showed the most chemoprevention properties in both tumor number and tumor multiplicity when used together before initiation and promotion.

Conclusions: Amifostine was shown to have chemoprevention properties in the chemical carcinogenesis model. Amifostine is abandoned due to its side effects such as nausea, vomiting, and hypotension. However, as we used in our experiment, studies for clinical use at low doses can be triggered. The ability to prevent secondary malignancies, especially from late effects that may develop due to chemo-radiotherapy, should not be ignored.

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Introduction

Amifostine (WR-2721) is a selective cytoprotective agent for normal tissues against chemotherapy and radiotherapy-associated toxicity. Amifostine is dephosphorylated and converted into an active free radical scavenger by the enzyme alkaline phosphatase, which is found at high levels in normal tissues but at low levels in tumor cells.[1][2][3][4][5] It received U.S. Food and Drug Administration (FDA) approval in 1996 because of its ability to reduce the cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian cancer and non-small cell lung cancer.[6],[7] Additionally, it received FDA approval for radiation-induced xerostomia in head and neck cancers in 1999.[8] It was reported that while amifostine showed greater ability to protect normal tissue than tumor cells, it does not have a significant effect on the sensitivity of tumor cells to radiation. Improvement in acute radiation toxicity has been observed in studies that frequently used several types of treatment intensification, such as concomitant chemotherapy or accelerated

radiotherapy. When the literature was examined, we found that there are very few studies on fibrosis with Amifostine.[9], [10] However, to our knowledge, there are no studies on late morbidities such as secondary cancerization.

Amifostine was discovered by the Walter Reed Army Research Institute as a radioprotector because of its acceptable level of toxicity among similar sulfhydryl compounds during drug development to protect soldiers from nuclear weapons.[11] It was approved by the FDA for its subsequent pre-clinical and clinical studies, suggesting that normal tissue is selectively protected from damage caused by irradiation and chemotherapy. However, FDA approval did not increase the popularity of the drug because whether it protected the tumor cells as well was a matter of concern.[3], [5]

With the increasing number of cancer survivors, the risk of secondary malignancies as a result of radiotherapy and chemotherapeutics becomes alarming. Leukemia accounts for approximately 20% of secondary malignancies, with the remainder appearing as sarcomas in and around the previously irradiated area. Children, young adults, those with a genetic predisposition to cancer, and immunocompromised individuals who have previously been treated for cancer are at higher risk. Epidemiological studies require careful monitoring because of the increased risk of breast and lung cancers in Hodgkin lymphoma survivors, leukemia and sarcomas in cervical cancer survivors, and sarcomas in childhood retinoblastoma survivors.[12][13][14][15][16]

Curative chemoradiotherapy has an important place in the treatment of many tumors such as head and neck, esophageal, lung, gastric, rectal, anal, and cervical carcinomas. It promotes the organ preservation approach in modern oncology treatment management and makes neoadjuvant treatment options possible. Amifostine is slowly being abandoned due to its high toxicity. Chemotherapy is already a highly toxic treatment, therefore its combination with amifostine is becoming less preferable. Additionally, intensity-modulated radiation therapy is an option that can effectively protect normal tissues.[17][18][19][20][21][22][23][24,][25]

One of the best-proven in vivo models for observing and studying the development of tumors is the mouse skin model of multi-stage chemical carcinogenesis. In this model, the transformation of benign epidermal papillomas to squamous cell carcinoma (SCC) as a result of topical application of 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) can be monitored with the naked eye. It is also possible to test natural or synthetic agents to determine

whether they are chemo-preventive or not, and which stage of carcinogenesis they inhibit.[26][27][28][29][30][31]

We aimed to investigate the chemopreventive properties of WR-1065, the active metabolite of WR-2721 by using Amifostine at different stages of carcinogenesis in the skin carcinogenesis model to shed light on the question of whether it will protect only the normal cells and prevent the formation of secondary cancers.

Material and Method

Chemicals and applied medicine

DMBA and TPA were purchased from Sigma-Aldrich (St Louis, MO, USA). Application doses were 100 nmol DMBA and 10 nmol TPA dissolved in 200 μl acetone. DMBA and TPA were applied to the hair-free back skin of the mice with a precision pipette. Amifostine (Ethyol®) was available as 500 mg sterile lyophilized powder vials from Cumberland Pharmaceuticals (Nashville, TN, USA) for use. It was dissolved in water and administered to mice subcutaneously (sc).

Animals

5-6 week old female, 160 CD-1 mice weighing 22-24 g were purchased from the laboratory of Charles River Breeding, Germany. All procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association. The animal study protocol was approved by the Trakya University Faculty of Medicine Institutional Animal Care and Use Committee. The mice were placed in an air-conditioned room at 25 °C with a 12-hour dark/light cycle. They were fed a commercial pellet diet and provided with unlimited drinking water. After the mice were quarantined for one week, the back skin was shed at least 2 days before treatment. The back hairs of our mice were shaved using a depilatory cream approximately every 4 weeks as the tuular grew. During the experiment, no anesthesia method was used for our mice, as there was no need for it. In the last stage of our experiment, the cervical dislocation method was used before sacrification. This study is reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines[32].

Application of the carcinogenesis model

Toxicity studies for the Lethal Dose (LD50), Maximum Tolerance Dose (MTD), and repeated application dose for 22 weeks of Amifostine were done using 40 CD-1 mice prior to the commencement of the carcinogenesis trial to avoid potential toxicity. LD50 was determined as 1 gm/kg-400 mg/kg and MTD was determined as 200 mg/kg. Repeated Application Dose (RAD) was determined as 40 mg/kg.

Next, control and experimental groups were formed. Prior to the experiment, the back hairs of all mice were shed with depilatory cream. The skin carcinogenesis model was initiated with a single topical application of 100 nmol DMBA (dissolved in 200 µl acetone) to shaved back skin, marking day 0 of the experiment. One week after DMBA application, 10 nmol TPA was applied to the back skin twice a week for 22 weeks. The experiment was concluded by the termination of the mice at the end of the 33rd week. Skin tumors 1 mm in diameter were counted and recorded every week from the time of their appearance until week 33. The percentage of mice with tumors (tumor multiplicity) and the number of skin tumors per mouse (tumor burden) were recorded.

Amifostine administration was started in Experimental-2 and Experimental-3 groups 2 days before day 0. In the Experimental-2 group, Amifostine was administered daily sc at a dose of 40 mg/kg two days before DMBA administration, on the day of DMBA administration, and until 5 days after DMBA administration. In this group, TPA application was initiated 1 week after day 0 and continued until Week 22, two days a week.

In the Experimental-3 group, Amifostine application was continued two days before DMBA application, on the day of DMBA application, and 5 days after DMBA application. On the days of TPA administration, amifostine was applied for 30 minutes prior two days a week. Amifostine was administered sc at a dose of 40 mg/kg, twice a week for 22 weeks (Table 1).

Histological Studies

For histological study, dorsal skins were removed and fixed in 10% neutral buffered formalin overnight. Afterwards, the samples were embedded in paraffin and 4 µm thick sections were taken. After the procedures were performed in accordance with the Hematoxylin-Eosin Staining protocol, the slides of the control and experimental groups were examined (H&Ex100).

Statistical Analysis

Normality distribution of the numeric variables was tested by the Shapiro-Wilk test. Statistical comparisons for normal data among groups were performed by using the one-way ANOVA test, and then the Dunnet test was used for comparison of the experimental groups with the control group. Statistical comparisons for non-normal data among groups were performed by using the Kruskal Walls test and then Bonferroni correction was used for comparison of the experimental groups with the control group ($p<0.016$ was considered as statistically significant for Bonferroni correction). Categorical data were compared by the Chi-square test, and then odds ratios were calculated. All data analyses were performed with SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA). P values <0.05 were considered statistically significant

Results

In our experiment, papillomas were first seen in the control group at 6 weeks. After this week, the number of tumors and mice with tumors per mouse was counted and noted once a week Tumor count was not stopped at the end of the 22nd week when DMBA, TPA, and Amifostine applications were stopped, and weekly tumor count was continued until the end of the 33rd week. At the end of the 33rd week, the mice were sacrificed and the experiment was terminated. With the data transferred to the SPSS program, the mean values of tumor burden for each week were calculated for the control, Experimental-1, Experimental-2, and Experimental-3 groups (Table 2). Tumor mean values of the experimental groups were compared with the control group (Table 2, Figure 1a).

The Experimental-1 group was designed to measure the promotional effect of Amifostine. Amifostine delayed the initial tumor formation by up to 12 weeks compared to the control group. In the following weeks, it significantly decreased the tumor incidence compared to the control group. (Table 2, Figure 1a, 2a,2b,2c,2d, 3a,3b,3c,3d).

The Experimental-2 group was designed to measure the effect of Amifostine on initiation. Amifostine was able to delay the first tumor formation for up to 9 weeks in this group. Compared to the control group, the number of tumors formed from the 11th week to the 33rd week was less. However, it was not as effective as it was in the Experimental-1 group (Figure 1a,2a,2b,2c,2d, 4a,4b,4c,4d).

In the Experimental-3 group, the effectiveness of Amifostine in both initiation and promotion was examined. Amifostine was administered sc two days before DMBA application, together with DMBA,

and 5 days after DMBA application. Likewise, it was applied 30 minutes before each TPA application, two days a week for 22 weeks. Amifostine activity in this group was superior to the other two experimental groups. The incidence of tumor formation was the least in this group. Compared to the control group, tumor formation in the Experimental-3 group started in the 9th week, at a statistically significant level, and it was the group with the lowest tumor burden during the entire experimental period (Figure 1a, 2a,2b,2c,2d, 5a,5b,5c,5d).

Mean tumor counts of the control and experimental groups were compared between weeks 1 and 22, weeks 22 and 33, and weeks 1 and 33 to examine the cumulative effect of Amifostine in addition to the weekly tumor count. Comparison of the Experimental-2 group and the control group at weeks 22 and 33 did not bear statistical significance. However, cumulative tumor burden was lower in all experimental groups treated with Amifostine.

Tumor multiplicity was compared between the control and experimental groups. The number and percentage of mice with tumors, p-value, and Odds Ratio (OR) value showing the protective effect of Amifostine are shown in Table 3. Amifostine was the most effective in the Experimental-3 group in reducing multiplicity, similar to incidence. Amifostine administration before both DMBA and TPA reduced tumor incidence and multiplicity. Amifostine was the least protective in the Experimental-2 group (Table 3, Figure 1b).

Histopathological examination

Histological evaluation; we did this by examining the tumor samples from the control and experimental groups. The most aggressive-looking ones on mouse backs were excised while tumor samples were taken. We present two examples of the stages from papilloma to SCC in the control group (Figure 6a, 6b). While tumors with less invasion occurred in the Experimental-1 group compared to the control group (Figure 7a), we saw invasion in the Experimental-2 group almost similar to the control group (Figure 7b). In the Experimental-3 group, where we looked at the effect of amifostine on both initiation and promotion, there was invasive tumor formation in a very small focus (figure 7c).

Discussion

The SCC model formed by the DMBA-TPA carcinogenesis protocol is one of the most frequently used in vivo models that includes the 3 stages of carcinogenesis: initiation, promotion, and progression.

With this model, natural or synthetic agents can be tested to determine whether they have chemopreventive properties[1][2][3], [30]. The mouse model of skin carcinogenesis is very similar to human cancers of the head and neck, esophageal, lung, and cervical SCC. In these cancers, where chemoradiotherapy is most frequently preferred, toxicity on normal cells is as important as treatment success.[17][18][19][20][21][22][23][24][25] Secondary cancerization is among the late morbidities that occur in acute, subacute, and chronic toxicity which negatively affects the success of treatment.[12][13][14][15][16]

The reason why Amifostine was chosen as a chemopreventive agent in our study is its protective effect against both chemotherapy and radiotherapy-induced toxicity, which has been reported by many pre-clinical and clinical studies.[1][2][3][4][5][6][7][8][9][10] While its contribution to late morbidities and fibrosis due to these treatments has been proven, it was seen that its effect on secondary tumor formation has not yet been studied.[8], [31], [33] While the carcinogenesis model used in this study is based on chemical agents, further in vivo studies on radiation-induced carcinogenesis may be necessary. To our knowledge, ours is the first in vivo study in which the chemopreventive effect of Amifostine is shown.

In our study, Amifostine was observed to reduce tumor incidence and multiplicity in DMBA – TPA-induced skin carcinogenesis model, indicating its chemopreventive effect. Amifostine was most effective in the Experimental-3 group, where it was applied both before initiation and during the application of promotional agents. It was least protective in the Experimental-2 group, where it was administrated before initiation only.

The limitation of our study was that it could not be proven at the molecular marker level, in addition to tumor incidence, multiplicity, and histopathological evaluation. However, our results appear in line with previous studies. Dziegielewski et al reported that WR-1065, the active metabolite of Amifostine, causes an increase in glutathione and cysteine levels and is protective against radiation-induced mutagenesis in vitro.[33] Additionally, they have shown that genomic protection is possible at lower doses. Murley et al reported that this protection increases the resistance to the cytotoxic effects of radiotherapy in the cell by increasing the expression of NFkB and the anti-oxidant gene SOD2.[34] It was also stated that Amifostine could be the primary agent in protecting cells from genomic instability in increasing the release of MnSOD, an antioxidant enzyme located in the mitochondria, against reactive oxygen stress caused by radiation.[35] A similar mechanism may have been involved in our

experiment. In the skin carcinogenesis model, DMBA irreversibly mutates the H-ras region by forming covalent bonds with the DNA of epidermal cells in a single application.[1][2][3] Amifostine, which was first administrated a week before DMBA, may have reduced this binding. In our study, Amifostine reduced tumor formation by 2.13 times in the Experimental-2 group, where only its effect on initiation was observed. TPA activates Wnt/β-catenin signaling via the protein kinase C (PKC) pathway, which may be reversible as a result of repeated and long-term administration and contributes to the transformation of local inflammatory reaction to SCC.[1][2][3], [30] In our study, chemoprevention was stronger in both experimental groups which were given Amifostine before TPA application. Amifostine reduced the risk of tumor formation by 2.44 in the Experimental-1 group and 3.88 times in the Experimental-3 group. In addition, in the histopathological evaluation of the chemoprevention effect we obtained in tumor incidence and multiplicity, we found that Amifostine reduced the degree of invasion in SCC formation in the process from papilloma to SCC. In the Experimental-3 group, Amifostine even prevented the transition from the promotion stage to the progression stage in the skin carcinogenesis model.

Conclusion

Amifostine, which was shown to have chemoprevention properties in the chemical carcinogenesis model, could also show partial prevention in the radiation carcinogenesis model. Further in vivo studies are required regarding this matter. Amifostine is being abandoned because of its adverse effects such as nausea, vomiting, and hypotension. However, its ability to prevent malignancies secondary to chemo-radiotherapy should not be overlooked, and reconsidering its place in treatment regimens may be in order. Perhaps the dose we used in our experiment, 40 mg/kg, is a tolerable dose in human applications. Instead of discontinuing use due to toxicity, clinical studies can be conducted for a more tolerable dose in humans.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: All authors.

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None.

Data Availability Statements

All data are incorporated into the article and its online supplementary material. The datasets used and analyzed during the study are available upon reasonable request (Qeios).

Tables

Tables are available in the Supplementary data section.

Figures

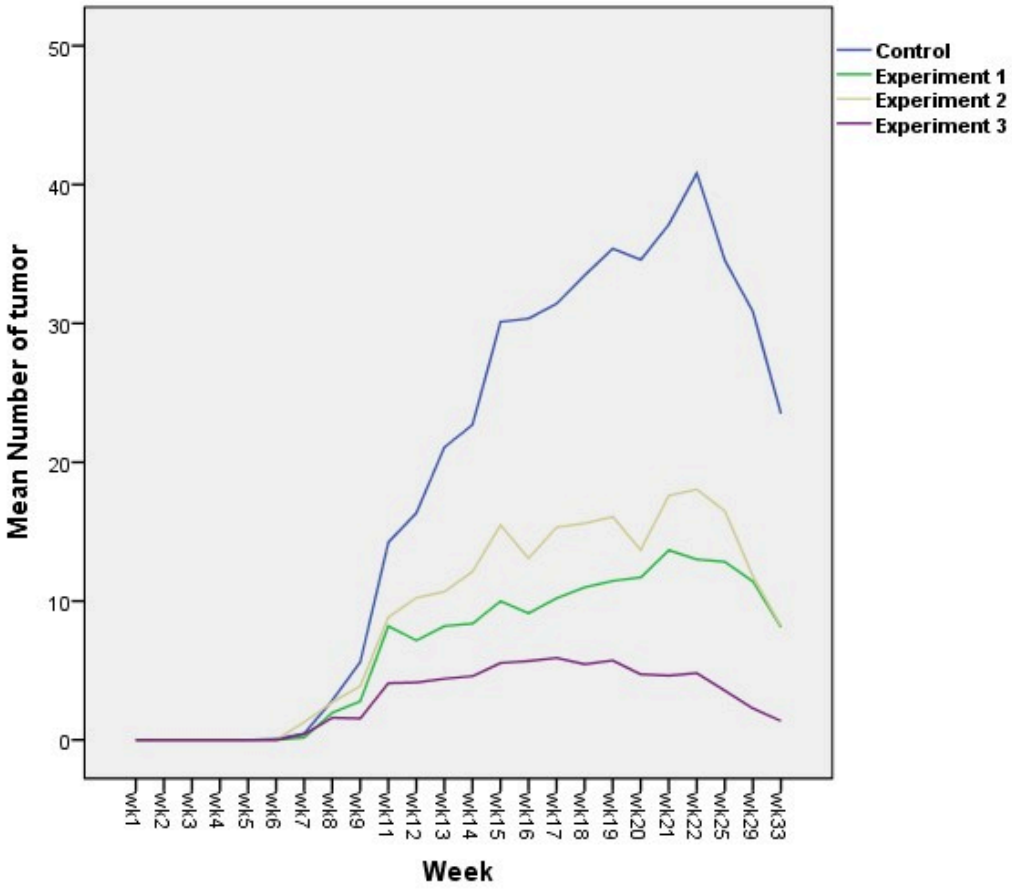

Figure1a. Tumor incidence curve of control and experimental groups.

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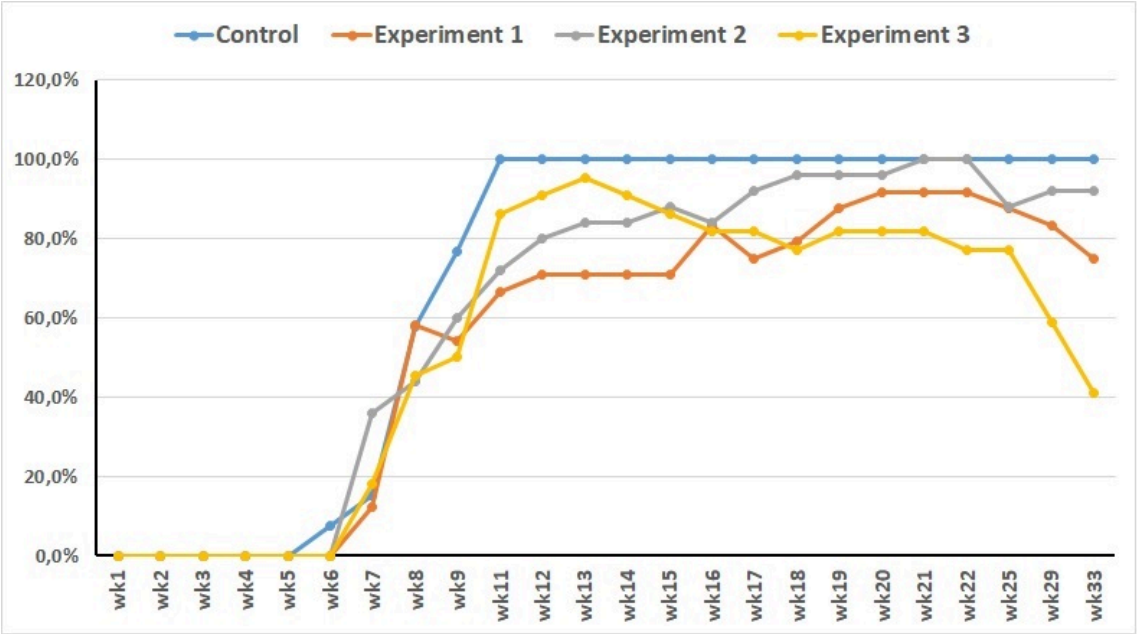

Figure 1b. Tumor multiplicity curve of control and experimental groups.

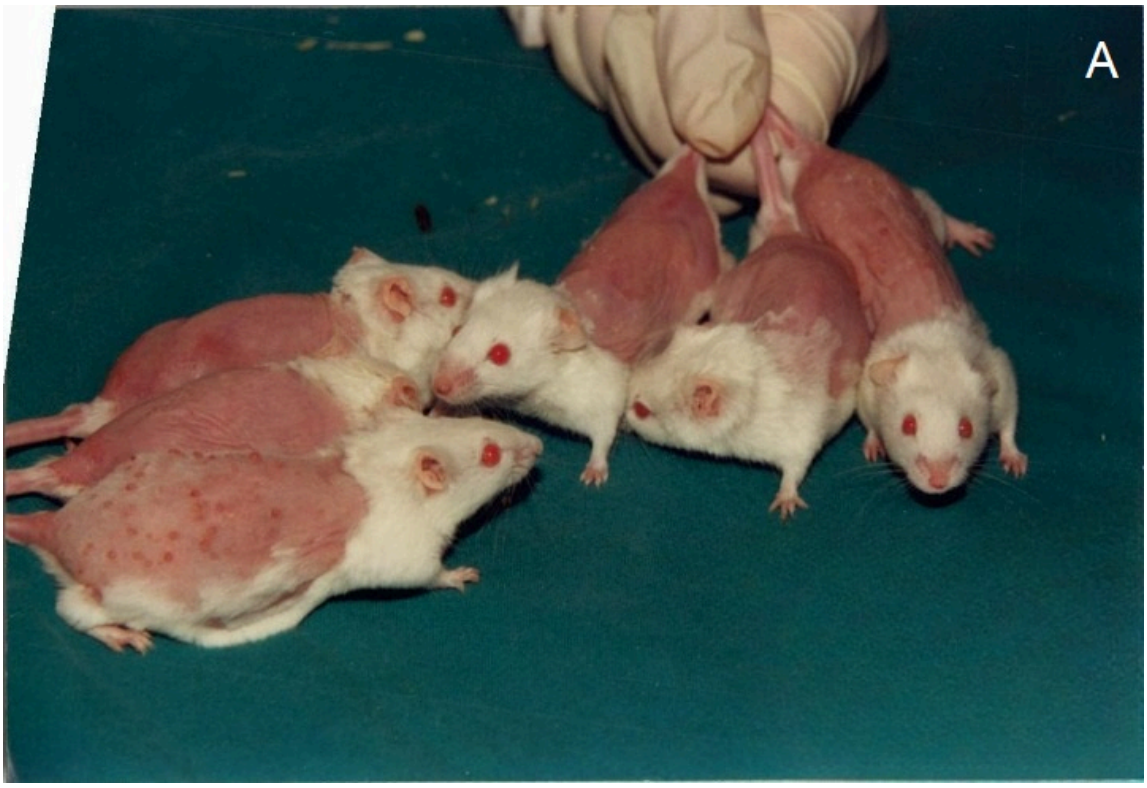

Figure 2a. Control group, week 6, the image in which the first formation of papillomas begins.

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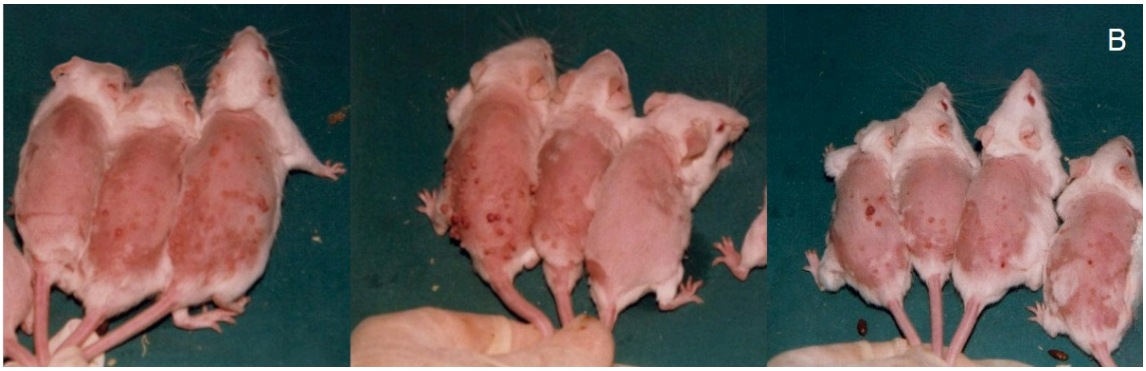

Figure 2b. Control group, week 12, the image in which the number of papillomas continues to increase

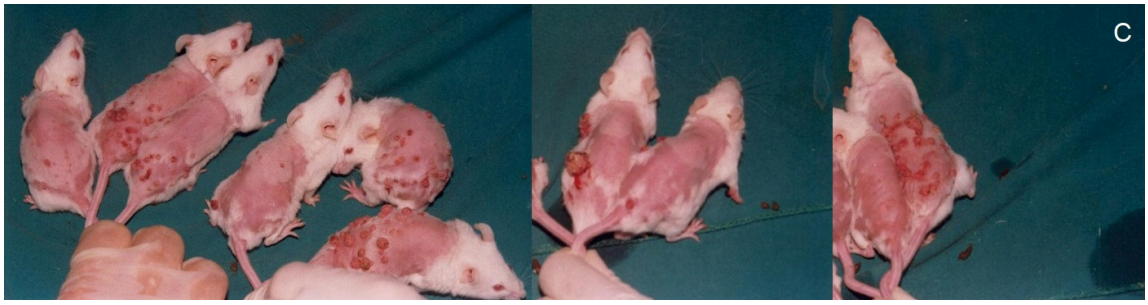

Figure 2c. The control group, week 18, the image where the promotion is fully placed

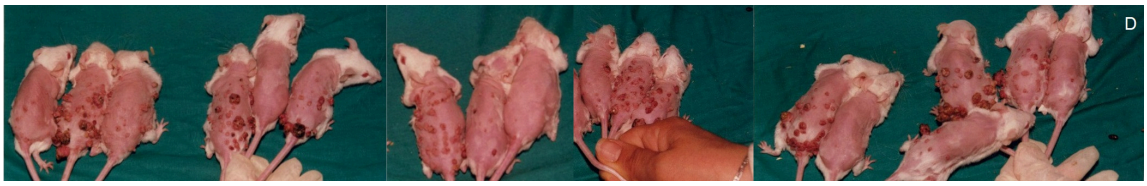

Figure 2d. The control group, week 22, the image where the progression is fully placed

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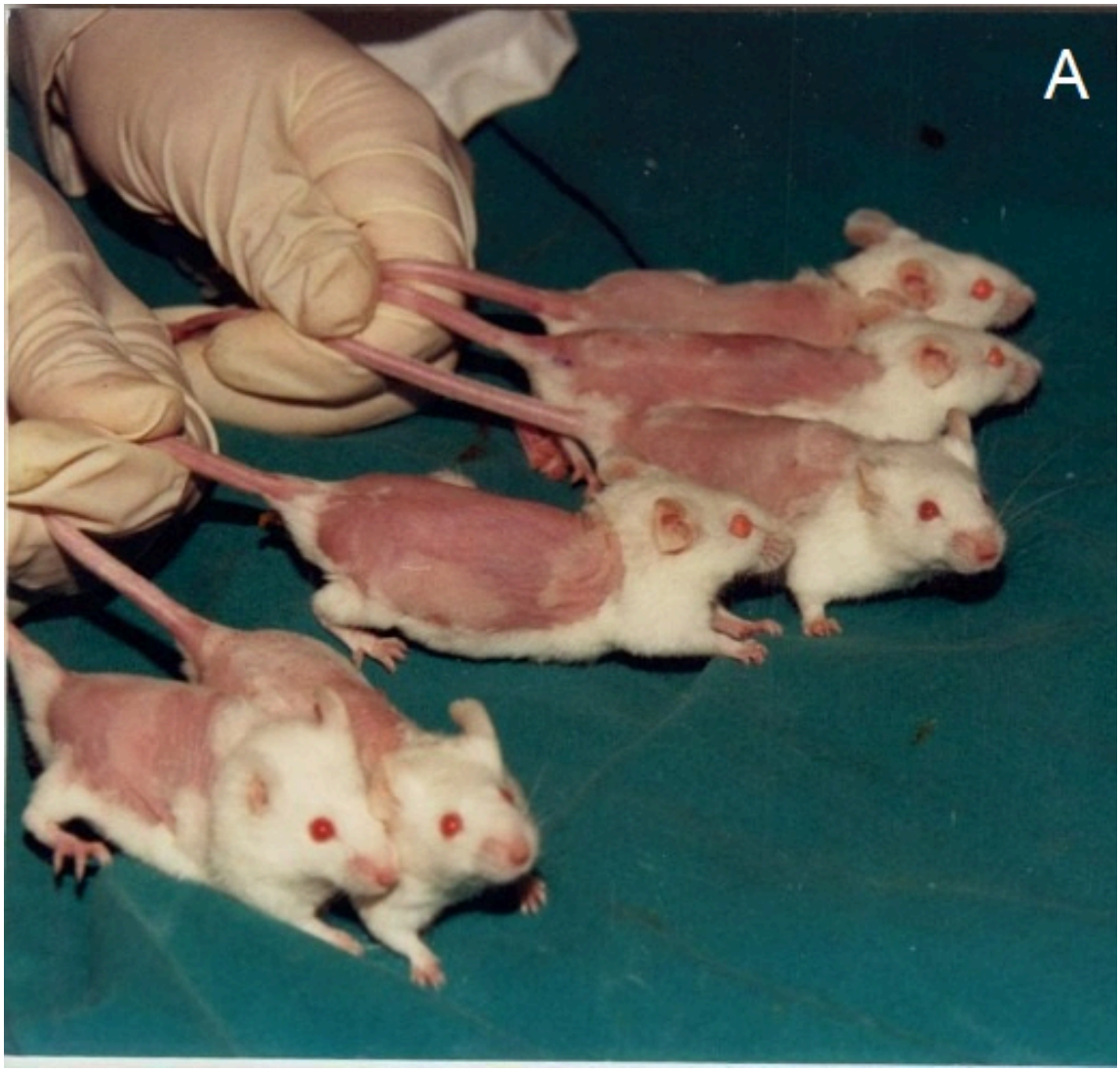

Figure 3a. Experiment 1, week 6, tumor formation has not yet started

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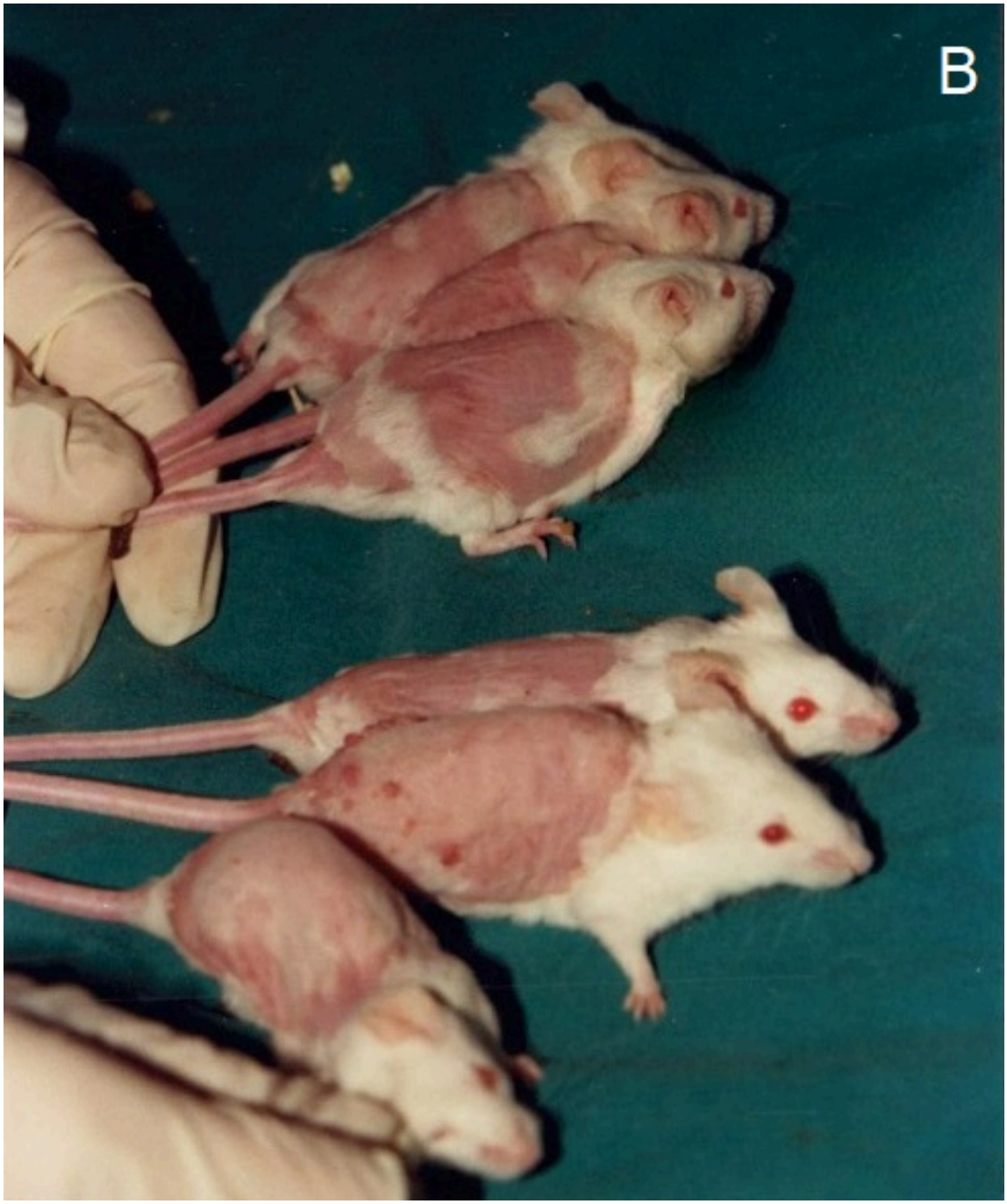

Figure 3b. Experiment 1, week 12, image where tumor formation has just begun

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Figure 3c. Experiment 1, week 18, image of the promotion phase of the experimental group

Figure 3d. Experiment 1, week 22, image of the progression phase of the experimental group

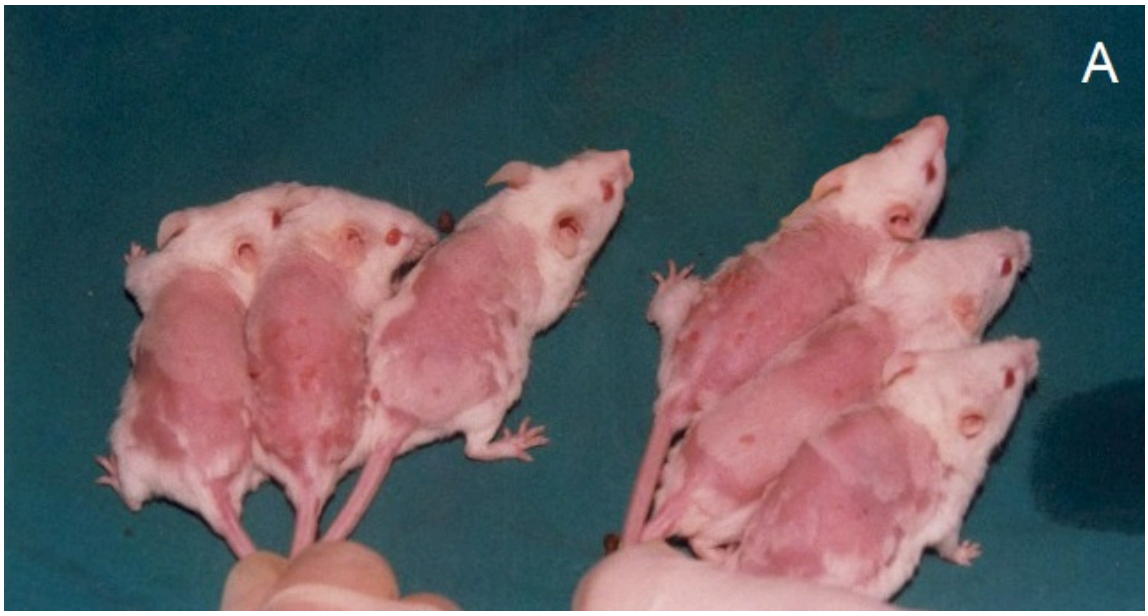

Figure 4a. Experiment 2, week 6, there are signs that papillomas will begin to form

Figure 4b. Experiment 2, week 12, image where papillomas are seen more clearly

Figure 4c. Experiment 2, week 18, image of the experimental group's promotion progressing

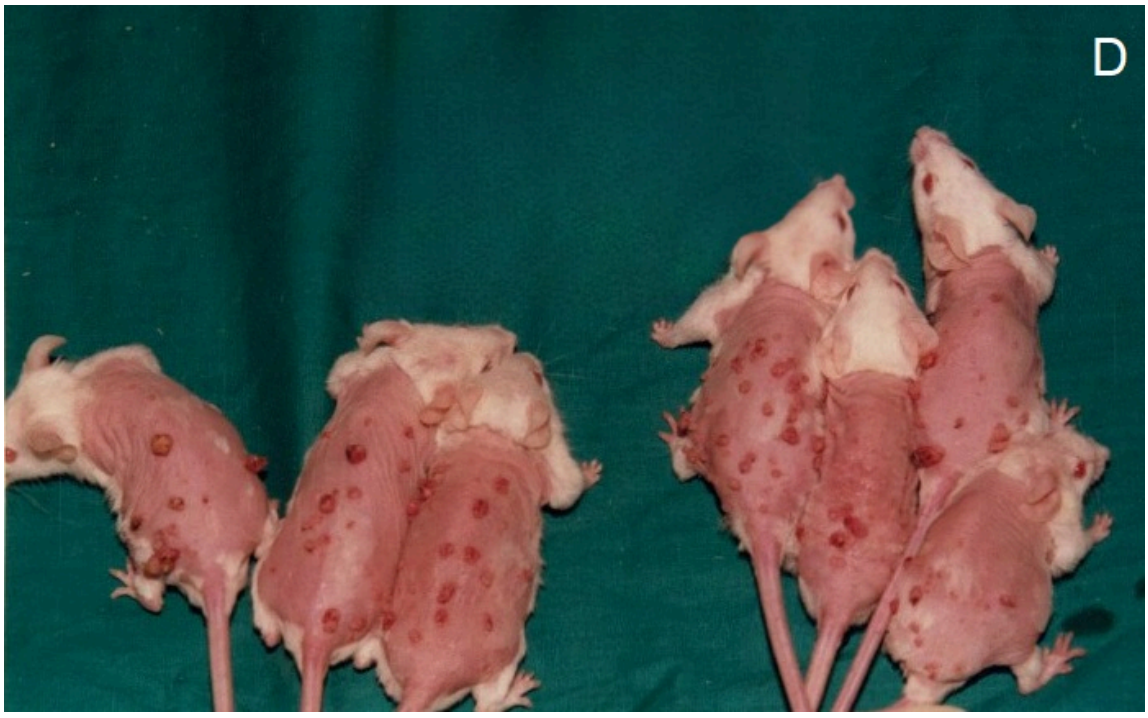

Figure 4d. Experiment 2, week 22, image of the experimental group's progression progressing

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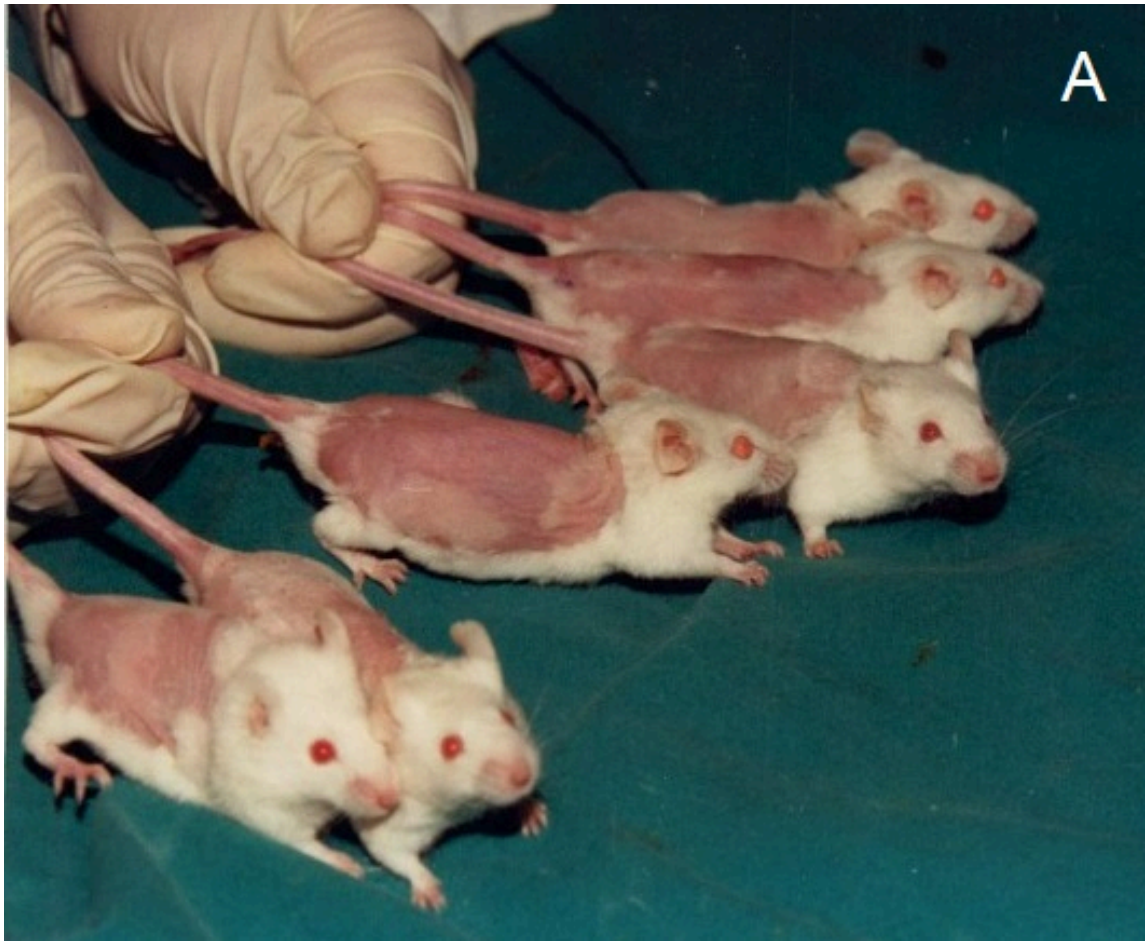

Figure 5a. Experiment 3, week 6, tumor formation has not yet started

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Figure 5b. Experiment 3, week 12, tumor formation has not yet started

Figure 5c. Experiment 3, week 18, experimental group in which papillomas are just starting to form

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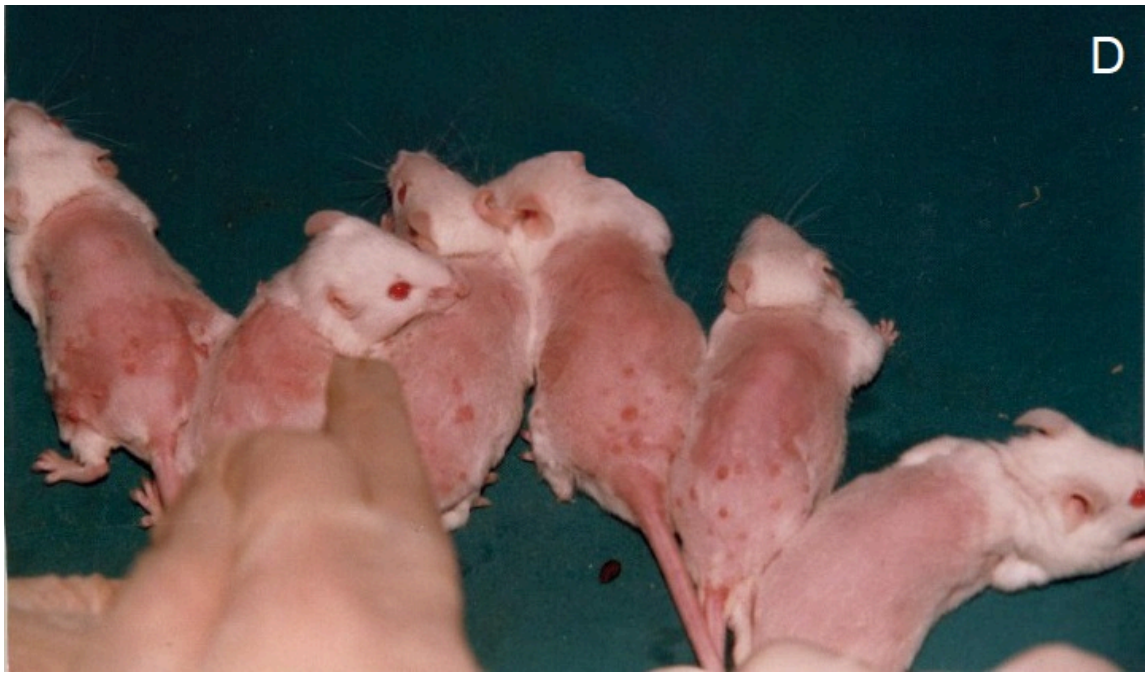

Figure 5d. Experiment 3, week 22, experimental group in which papillomas are just starting to form

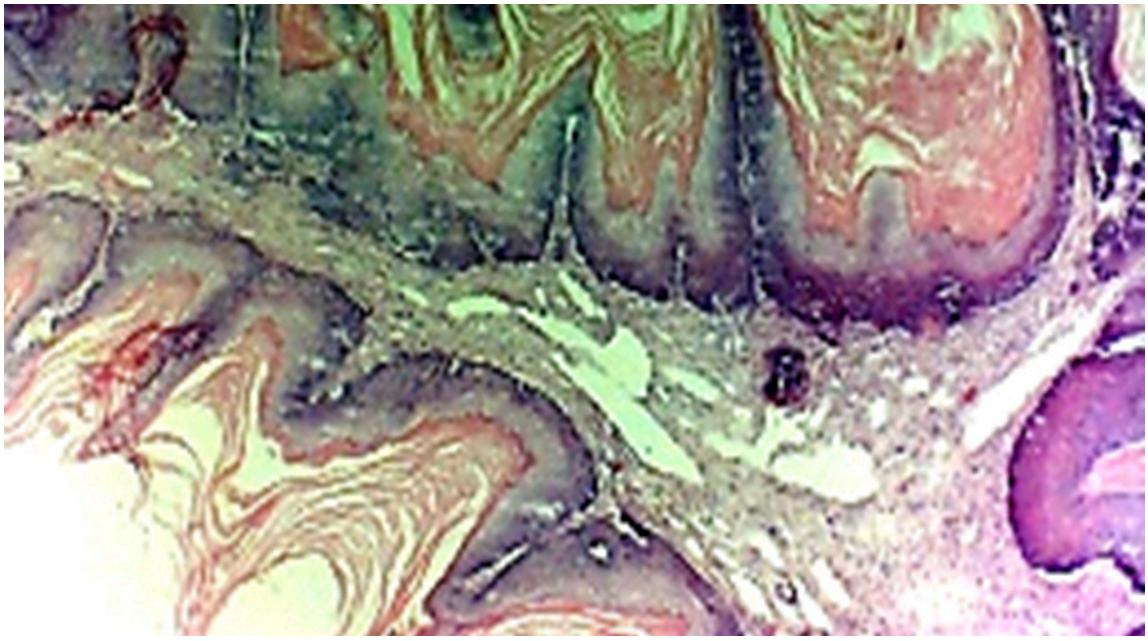

Figure 6a. The control group had minimal invasion in the early period (H&Ex100).

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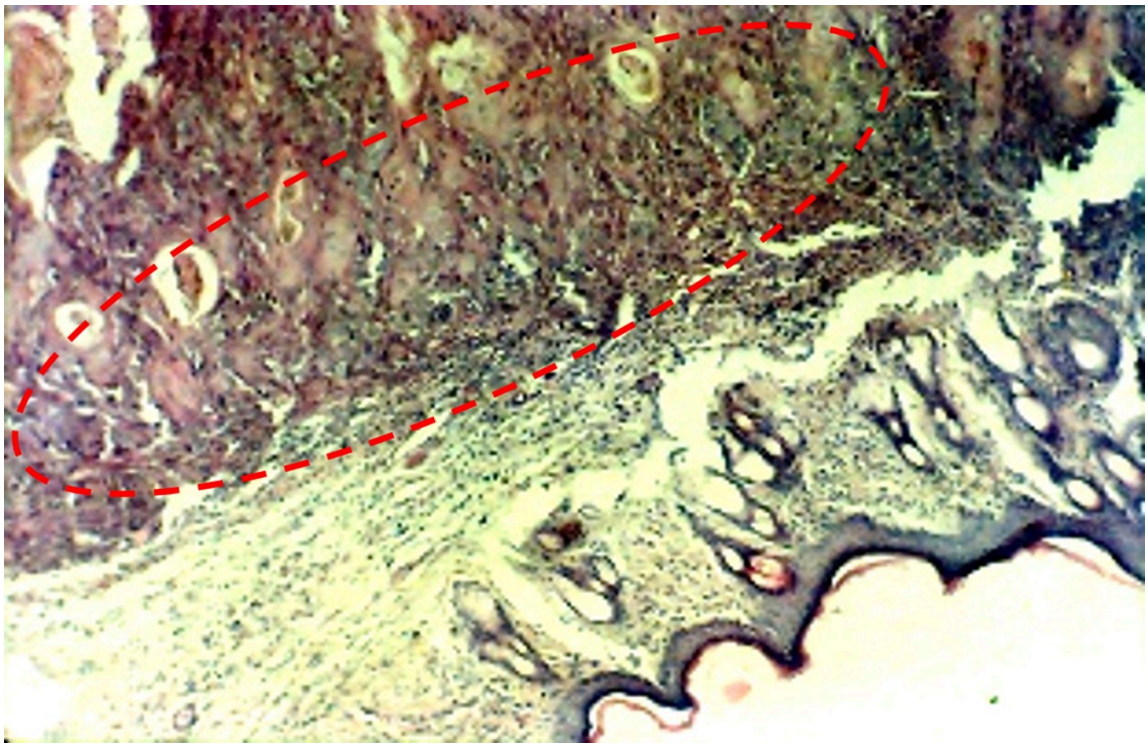

Figure 6b. The control group, prominent invasive tumor area in circle (H&Ex100).

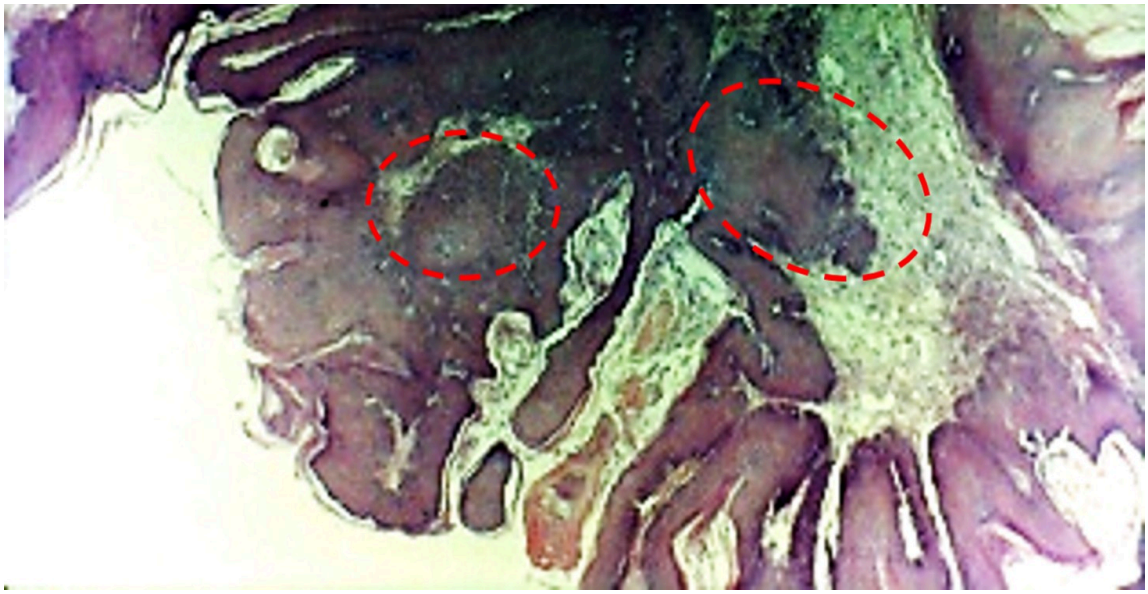

Figure 7a. The Experiment 1; smaller invasive tumor than the control group (H&Ex100).

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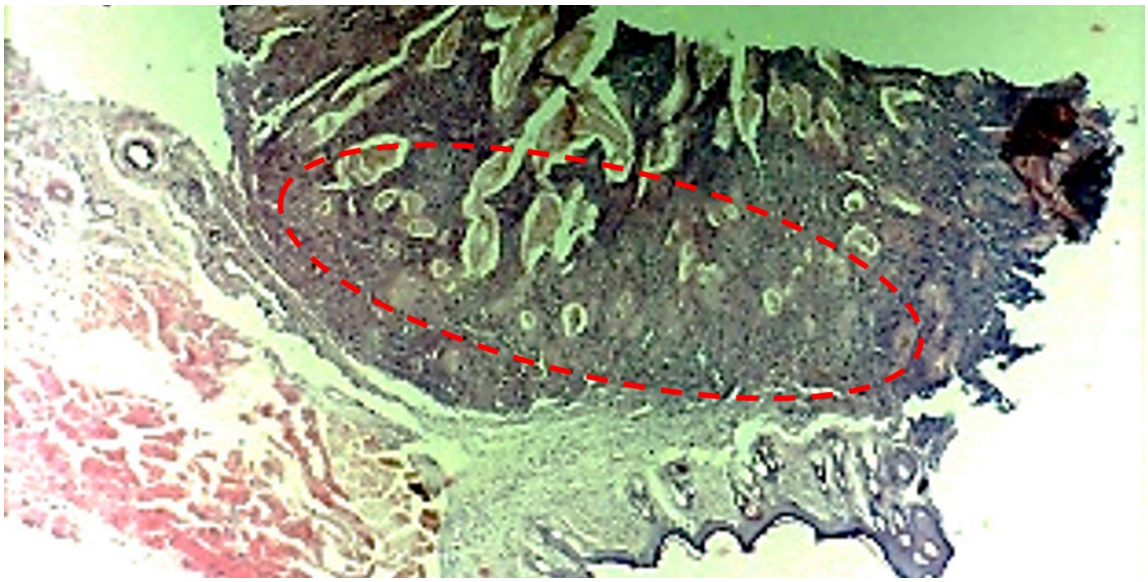

Figure 7b. The Experiment 2; significant invasive tumor similar in size to the control group (circled area), (H&Ex100).

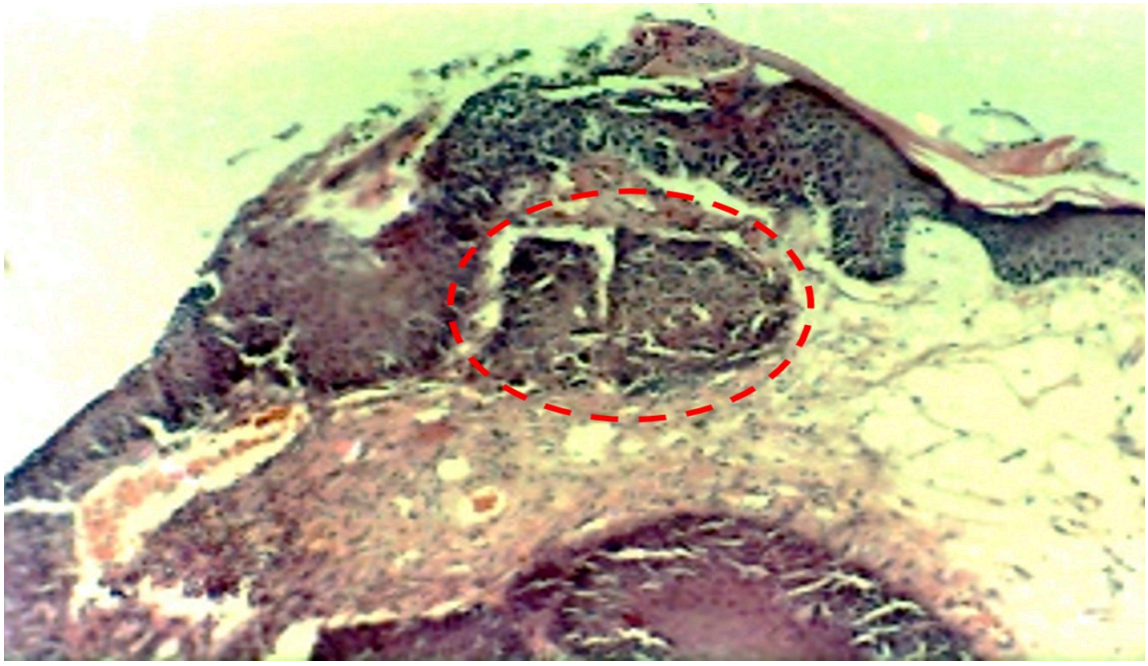

Figure 7c. The Experiment 3; early invasive changes in a small focus (circled area), (H&Ex100).

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