



**Original Study**

# Effects of Hydrocortisone on the Growth of Cultured Equine Fibroblasts Isolated from Distal Aspects of the Limb

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## Abstract

**Objective-** To determine the effects of the Hydrocortisone on “in vitro” growth of fibroblasts isolated from the distal aspects of the horses limbs.

**Design-** Experimental in vitro study.

**Animals-** A total of 4 Caspian miniature and 4 mixed thoroughbred horses. **Procedures-** Under general anesthesia and aseptic condition, a full thickness of skin incision was created on the lateral aspects of the metacarpal mid-third and 3 grams of subcutaneous tissue was harvested and placed on culture medium (RPMI-1640) in an incubator at 37°C in 5%CO<sub>2</sub>. The Hydrocortisone was added in 3 different concentrations

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[treatment groups A (10<sup>1</sup>/<sub>4</sub> g/ml), B (100<sup>1</sup>/<sub>4</sub>g/ml), and C (300<sup>1</sup>/<sub>4</sub>g/ml)] into the culture medium and cell counting was carried out after 4 days. The effects of Hydrocortisone added to control media were compared with a control media on fibroblast growth and viability. The data were analyzed by one way ANOVA and Tukey test.

**Results-** There were no significant differences in fibroblast growth rate and viability percentage ( $p>0.05$ ) between the groups control and A, but the groups B and C suppressed significantly the fibroblast growth and decreased viability percentage in compared with groups control and A ( $p<0.05$ ), and also there were no significant differences between groups B and C in fibroblast growth rate and viability percentage ( $p>0.05$ ).

**Conclusion and Clinical Relevance-** The effect of Hydrocortisone on cultured fibroblast growth is dose-dependant. Application of this agent can inhibit fibroblast growth; therefore it can be used for treatment of exuberant granulation tissue and improving wound healing processes, especially in the distal limb wounds of the horses.

**Key words-** hydrocortisone, fibroblast, growth, horse

## Introduction

Cutaneous wounds occur commonly in horses and often require expensive and prolong treatments<sup>1,2,3,4</sup>. Second- intention wound healing in horses has inter-related species-specific complications, such as the formation of exuberant granulation tissue (proud flesh) and subsequent retardation of epithelization and contraction, especially when the wounds are located on the distal aspect of the limb<sup>1,2,3,4,5,6,7</sup>.

Granulation tissue consists primarily of developing blood vessels, fibroblasts and their protein products, which form the surrounding matrix. Exuberant granulation tissue is unsightly, prone to abrasion and secondary infection, and may produce a mechanical restriction to normal movement<sup>1,2,3,4,5,6,7,8</sup>.

A specific cause for development of exuberant granulation tissue is unknown<sup>3</sup>. Mechanisms possibly involved in production of exuberant granulation tissue in horses include infections or foreign body response, excessive motion or tension on the surrounding skin, reduced blood supply, with resultant hypoxia and imbalance of collagen synthesis, deposition, and lysis<sup>1,3,4,5,7,9</sup>.

Many treatments have been reported to promote equine wound healing by controlling excessive fibroplasias<sup>1,3</sup>. None of these treatments has consistently eliminated exuberant granulation tissue formation in equine limb wound<sup>1,3,10</sup>.

Steroids have been reported to affect wound healing by inhibiting the inflammatory phases, suppressing the angiogenesis, inhibiting the epithelization, retardation of wound contraction and decreasing wound tensile strength. Corticosteroids are effective on reducing granulation tissue production in many species by reducing fibroplasias<sup>1,3,11</sup>. It seems that, in horses with exuberant granulation tissue, these suppression effects may override other negative effects that steroids may have on wound healing<sup>1</sup>. In some clinical studies, application of topical steroids was recommended in treatment of exuberant granulation tissue in horses<sup>1,10,11</sup>.

Recently, many researches have been conducted on the effects of steroids on the proliferative activity of various vertebrate fibroblast-like cell lines, human cell lines, and primary fibroblast culture<sup>12</sup>, but there is no document about the effects of Hydrocortisone on growth characteristics of equine dermal fibroblasts.

Hydrocortisone is one of the most important glucocorticoids and it has extensive effects, such as anti-inflammatory, immunosuppressive and also antimitotic and antigrowth effects on epidermal cells<sup>13,14</sup>.

The purpose of the present study was to determine the direct effects of hydrocortisone on growth of fibroblasts, which had been harvested from distal limb of horses, because fibroblasts have a significant role in producing of exuberant granulation tissue and controlling fibroblasts growth may provide a means to regulate the production of exuberant granulation tissue.



## Materials and Methods

A total 4 Caspian miniature (2 males and 2 females) and 4 mixed thoroughbred horses (1 male and 3 females), ranging from 4 to 10 (mean, 7) years old were used in this study. All animals were sound and found to be healthy thorough clinical examination before the start of the experiment. Under general anesthesia, full-thickness skin incision was created in the lateral aspect of mid-third of left metacarpi using aseptic technique and 3 grams of dermal and subcutaneous tissues were harvested. Then, these tissues were minced and placed in ventilated flasks 25cm<sup>2</sup> (Nunclon<sup>TM</sup> Cell Culture, No: 163371) containing RPMI-1640 culture medium (Roswell Park Memorial Institute, Sigma-Aldrich, Inc, St. Louis, USA) in an incubator at 37°C in 5%CO<sub>2</sub>. The explant culture method was used for separating the fibroblasts from the tissue. Fibroblasts were grown to confluence in RPMI supplemented with 10% fetal bovine serum (FBS), antimicrobial agents [penicillin-streptomycin (Bio Gene, Cat: AP 110), 100 IU/ml, 100 µg/ml, respectively], L-glutamine and bicarbonate buffer. The culture medium was changed 3 times, weekly.

After confluence stage of fibroblasts, the cells were removed by trypsin treatment (Trypsin 1:250, T-4799, Sigma-Aldrich, Inc, St. Louis, USA) with EDTA (1mM) approximately 3 minutes. Passage of fibroblasts was then carried out and the cells were placed in a new ventilated flask contained 4.5cc culture medium plus 0.5cc FBS. Passage of cells was performed 2 times, during this study. When the cells grew and filled almost 90% of the flask floor, they were removed by trypsin with EDTA and then were suspended in RPMI. After cell counting, a concentration of 10000 cells/ml was placed into each well of a 24-well plate (Greiner bio-one, Cellstar, No: 662160). Hydrocortisone (Hydrocortisone-water soluble powder, 100mg/gr, H0396, Sigma-Aldrich, Inc, St. Louis, USA) in 3 different concentrations was added to culture medium of treatment groups in day 0.

The samples of each animal were placed in 12 wells. The wells were distributed in four groups (3 wells in each), the control group without treatment and A, B, C groups with 10, 100, 300 µg/ml Hydrocortisone, respectively.

The fibroblasts were cultured in the wells for 4 days and then were detached with Trypsin in EDTA solution for 5 minutes. After addition of RPMI with FBS (10%) the cells were centrifuged (Eppendorf, centrifuge, 5810 R) in 10000 rpm for 3 minutes and counting of fibroblasts was carried out. The mean number of fibroblasts in 3 wells was recorded. The viability of fibroblasts was determined by trypan blue exclusion<sup>3,15,16</sup>. Cell counting was performed by the Invert light microscope (Olympus IX 70) and standard hemocytometer.

To statistical analysis of data, one way ANOVA test was performed and Tukey HSD test was used to detect significant differences between the groups. The significant level was set at  $P < 0.05$ .

## Results

Fibroblasts in 8 cultured tissues from 7 cases started to grow at the first time and only in one horse the fibroblasts failed to grow and tissue sampling was performed again. There were no significant differences in growth rate and viability percentage between Caspian horses and mixed thoroughbred horses ( $p > 0.05$ ). The mean  $\pm$  SD time between the cultures of the tissues in ventilated flasks till fibroblast confluency (formation of big colonies of fibroblasts around the cultured tissues), was  $17.5 \pm 0.725$  days.

Statistical analysis demonstrated that there was no significant difference in mean fibroblast growth rate (table 1) between groups Control ( $42750 \pm 11187$ ) and A ( $41387 \pm 11737$ ) ( $p > 0.05$ ) and also there was no significant difference in Viability percentage (table 2) between groups Control ( $86.31 \pm 4.75$ ) and A ( $83.99 \pm 2.81$ ) ( $p > 0.05$ ).

Fibroblast growth in groups B ( $21575 \pm 5658$ ) and C ( $17512 \pm 4255$ ) was suppressed in compare with groups Control and A, significantly ( $p < 0.05$ ), but fibroblasts growth rate in group C was a little more than B.

The mean viability percentage in groups B ( $79.78 \pm 3.48$ ) and C ( $76.28 \pm 4.75$ ) was less than groups Control and A ( $p < 0.05$ ). (Figs 1,2,3,4)



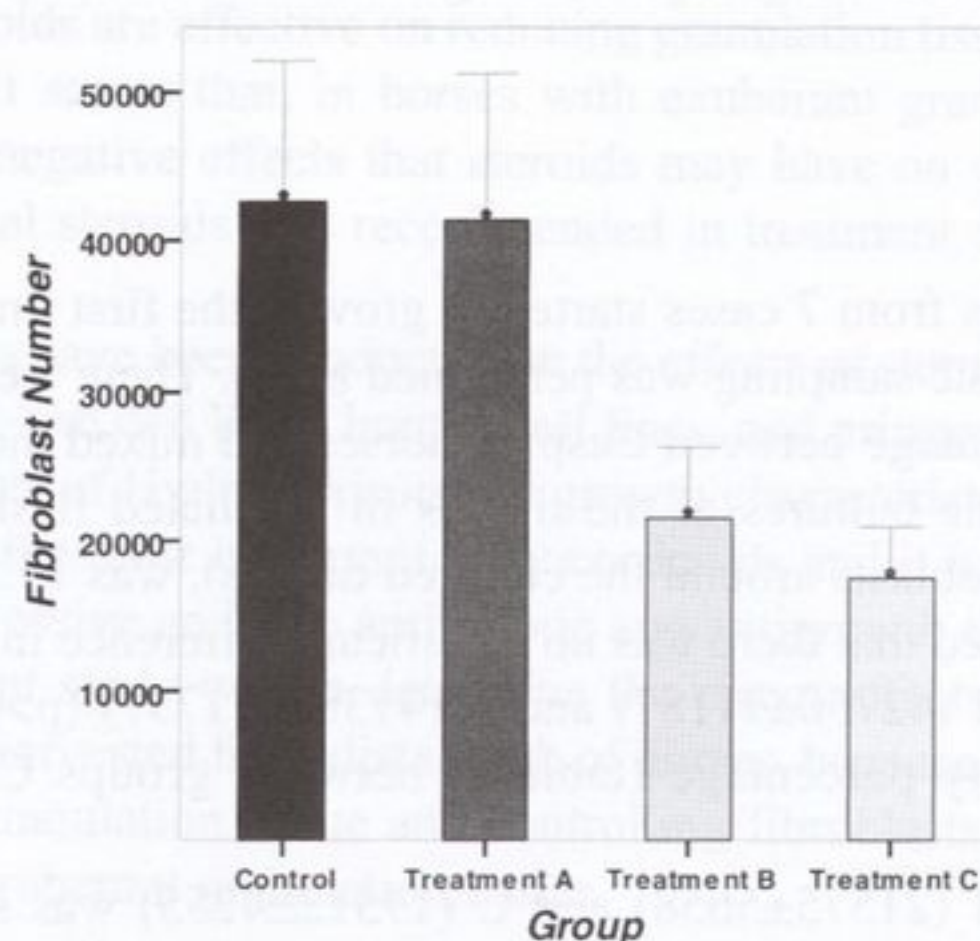
There was no significant difference in fibroblasts growth rate between groups B and C ( $p>0.05$ ). Although the number of cells in group B was a little more than C. There was no significant difference between viability percentage of fibroblasts between groups B and C ( $p>0.05$ ).

**Table 1-** Fibroblasts number of horses in control and treatment groups

Groups Fibroblast Number	Control Group	Treatment Group:A Hydrocortisone (10 µg/ml)	Treatment Group:B Hydrocortisone (100 µg/ml)	Treatment Group:C Hydrocortisone (300 µg/ml)
Case 1	24900	26500	15100	12500
Case 2	43200	38700	23000	17700
Case 3	57500	60000	32000	25400
Case 4	42000	35000	18300	18100
Case 5	34800	31700	17700	12400
Case 6	34100	36000	18500	16000
Case 7	53500	48700	27500	19400
Case 8	52000	54500	20500	18600
<i>Mean ± SD</i>	<b>42750±11187</b>	<b>41387.5±11737</b>	<b>21575±5608</b>	<b>17512±4155</b>

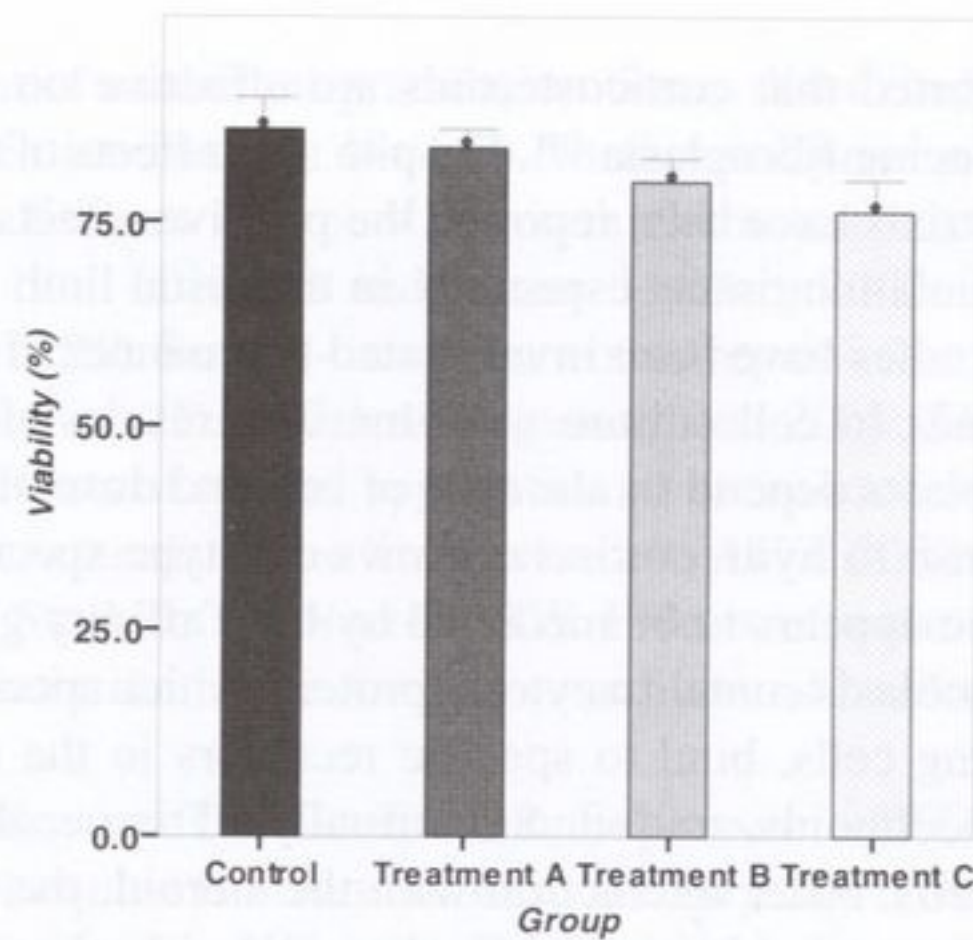
**Table 2-** The viability percentage of fibroblast of horses in control and treatment groups

Groups Viability (%)	Control Group	Treatment Group:A Hydrocortisone (10 µg/ml)	Treatment Group:B Hydrocortisone (100 µg/ml)	Treatment Group:C Hydrocortisone (300 µg/ml)
Case 1	84.6	85.40	83	77.5
Case 2	82	81	78	81
Case 3	86	80	75	70.5
Case 4	93	85	82.30	75
Case 5	88.30	83.5	80	76
Case 6	90	89	85	79.5
Case 7	78.60	85	79	82
Case 8	88	83	76	68.80
<i>Mean ± SD</i>	<b>86.31±4.57</b>	<b>83.98±2.81</b>	<b>79.78±3.48</b>	<b>76.28±4.75</b>

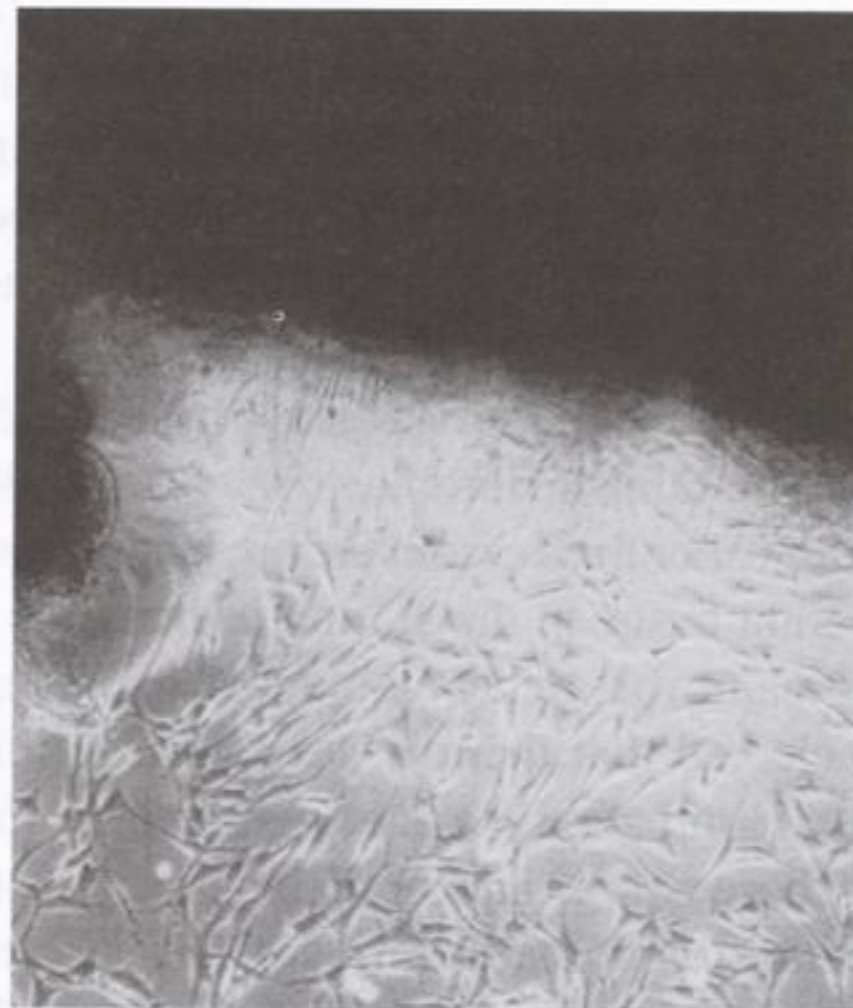


**Fig. 1.** Mean  $\pm$ SD fibroblast growth rate isolated from distal aspect of limb of horses that cultured in control media with 3 different concentrations of hydrocortisone (A: 10, B: 100 and C: 300 µg/ml )

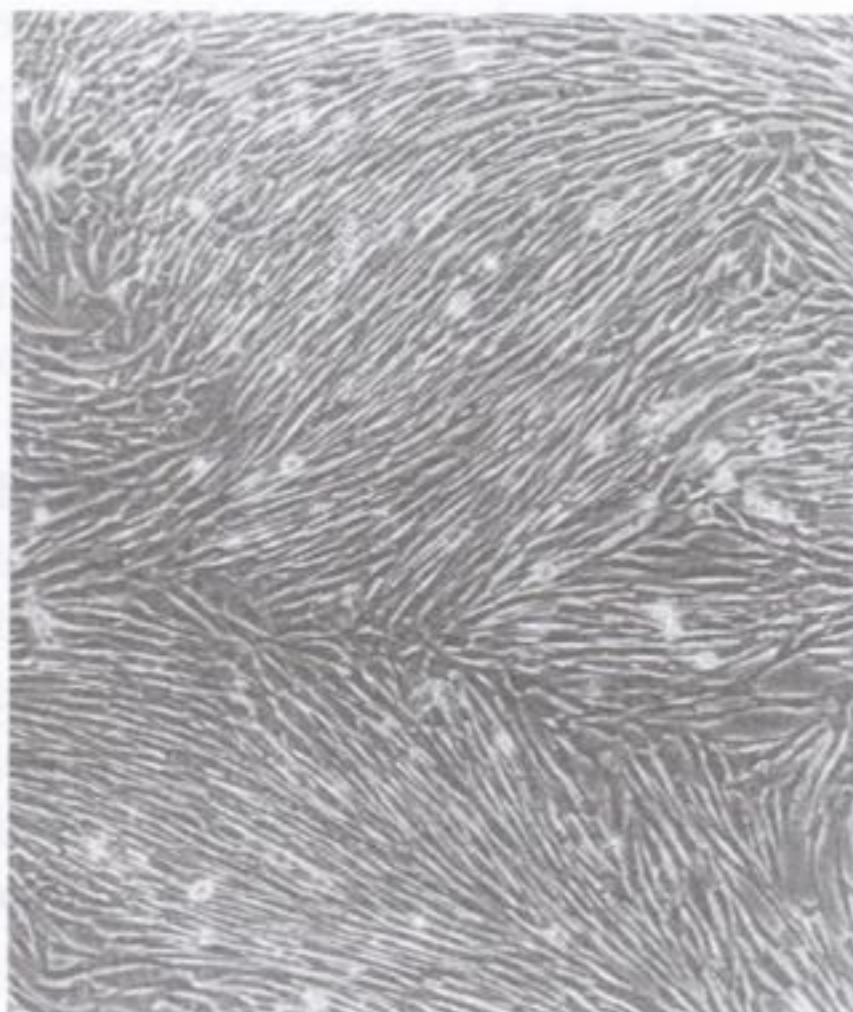




**Fig. 2.** Mean  $\pm$ SD viability percentage of fibroblast isolated from distal aspect of limb of horses that cultured in control media with 3 different concentrations of hydrocortisone (A: 10, B: 100 and C: 300  $\mu$ g/ml )



**Fig. 3** Microscopical appearance of fibroblasts that separated from the harvested subcutaneous tissue from distal limb of horses in culture plate (Mag. 10  $\times$ 10 )



**Fig. 4.** Microscopical appearance of fibroblasts at confluence stage in culture media (Mag. 10)



## Discussion

Clinical studies have been reported that corticosteroids are effective on reducing granulation tissue production in many species by reducing fibroplasia<sup>1,11</sup>. Despite side effects of these medications on wound healing, anecdotes and controlled trials have been reported the positive effects of topical corticosteroids in the management of exuberant granulation tissue, especially in the distal limb wounds of horses<sup>1,3,11</sup>.

In recent years, many in vitro studies have been investigated the manner of effects of corticosteroids on growth characteristics of fibroblasts in cell culture medium. The results of these studies revealed that effects of corticosteroids on fibroblasts depend on the type of cell and dose of agents. Bryon, et al. (1979) suggested that proliferative response to hydrocortisone shows cell-type specificity, as well as a steroids-molecular specificity. The response appears to be mediated by high affinity glucocorticoid to binding site<sup>12</sup>. The subcultured human skin fibroblasts contain a cytosol protein, which specifically binds glucocorticoids<sup>13,14,17</sup>. Glucocorticoids after entering cells, bind to specific receptors in the cytoplasm. These receptors, which have high affinity for Glucocorticoids, are found in virtually all tissue, about 3000 to 10000 per cells, the number varying in different cells. After interaction with the steroid, the receptors become activated, then steroid-receptor complex move to nucleous and binds to steroid –response elements in DNA. The effect is on repressing (preventing transcription of) or inducing (initiating transcription of) particular genes<sup>13,14</sup>.

Until the present time, there has not been any documented data about direct effects of Hydrocortisone on growth rate of cultured fibroblast of horses. This study evaluated the effects of Hydrocortisone on growth characteristics of the fibroblasts isolated from limb distal aspects of horses. Several in vitro studies have demonstrated that there were significant differences in the inherent growth of harvested fibroblasts from limb and body of horses in cell culture medium<sup>3,16,18</sup>.

Kondo, et al. (1985) suggested that Hydrocortisone, at physiological concentration, have various effects on different human fibroblasts. The human fibroblasts may be classified into two groups with respect to glucocorticoids binding receptors<sup>19</sup>. For this reason, the present study has focused on distal limb fibroblasts, because the formation of exuberant tissue in distal limb of horses is significantly more than other sites of the body and fibroblasts play a major role in developing of this complication<sup>1,2,3,5,16,20</sup>.

Our previous studies have cleared that there is no significant difference in growth characteristics of fibroblasts isolated from the distal limb of mixed thoroughbred and Caspian miniature horses (Unpublished data). With respect to this fact, these two groups of animals were considered as one group in this study.

The results of this study demonstrated that Hydrocortisone decreases fibroblast growth in cell culture medium and this effect is dose-dependant. Addition of 10µg/ml of Hydrocortisone in cell culture medium was ineffective, meanwhile addition of 100 and 300µg/ml of Hydrocortisone suppressed cell growth.

Talbot, et al. (2004) showed that Hydrocortisone treatment on bovine fetal fibroblasts cell line reduced the size of colonies, although the number of colonies formed was not effected<sup>21</sup>. Addition of Hydrocortisone to some vertebrate cell lines, under identical conditions, caused growth inhibition but enhanced the proliferative activity in human cell lines (WI-38) and human fetal foreskin origin<sup>12</sup>. An in vitro study on mouse fibroblasts growth has revealed that glucocorticoides arrested growth of these cells<sup>22</sup>. Verbrugen and salmon (1980) evaluated the effects of Hydrocortisone on primary culture of dermal fibroblast from neonate mice. They noticed that Hydrocortisone produced a dose-dependant inhibition of DNA synthesis in these cells<sup>23</sup>.

The results of this study demonstrated that addition of 100 and 300µg/ml of Hydrocortisone in cell culture medium decrease viability of fibroblast, meanwhile addition of 10µg/ml of this agent was ineffective.

Jung-Testas and baulieu (1984-85) showed that Glucocorticoides decrease adhesiveness of cells to culture plate<sup>22</sup> and also causes cell death in culture medium<sup>24</sup>.

According to the results of the present study, Hydrocortisone suppressed the growth of fibroblasts which were isolated from limb distal aspect of horses, Therefore utilization of topical Hydrocortisone can be reduced the amount of granulation tissue by controlling fibroplasia but further in vivo and in vitro researches are needed to investigate the effects of Hydrocortisone on exuberant granulation tissue treatment in horses.



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## بررسی اثرات هیدروکورتیزون بر روی خصوصیات رشد فیبروبلاستهای جدا شده از نواحی پائینی اندام

### حرکتی اسب در محیط کشت سلول

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**هدف:** بررسی اثر هیدروکورتیزون بر روی سرعت رشد و تکثیر و قابلیت زیستی فیبروبلاست های جدا شده از نواحی پائینی اندام حرکتی اسب در محیط کشت سلول

**طرح:** میدانی و آزمایشگاهی

**حیوانات:** ۴ رأس اسب نژاد تروبرد مخلوط و ۴ رأس اسبچه خزر

**روش کار:** تحت بیهوشی عمومی و با رعایت شرایط آسپتیک، زخم تمام ضخامت در سطح جانبی و ناحیه میانی متاکارپ ایجاد و میزان ۳ گرم بافت زیر جلدی برداشت گشته و کشت و جداسازی و تکثیر فیبروبلاست ها در محیط کشت 1640-RPMI از بافت مذکور در انکوباتور  $37^{\circ}\text{C}$  با غلظت ۵٪ گاز  $\text{CO}_2$  انجام شد. سپس هیدروکورتیزون در غلظت های مختلف [گروههای درمانی A (۱۰ میکروگرم/میلی لیتر)، B (۱۰۰ میکروگرم/میلی لیتر) و C (۳۰۰ میکروگرم/میلی لیتر)] به محیط کشت سلول اضافه و بعد از ۴ روز شمارش سلولی انجام گردید و اثر هیدروکورتیزون در غلظت های مذکور بر سرعت رشد و قابلیت زیستی فیبروبلاست ها در مقایسه با گروه کنترل (فاقد هیدروکورتیزون) مورد ارزیابی قرار گرفت.

**تجزیه و تحلیل آماری:** One way ANOVA و Tukey test

**نتایج:** بعد از شمارش فیبروبلاست ها و تعیین درصد قابلیت زیستی این سلولها و انجام آنالیز آماری بر روی داده های به دست آمده، هیچ اختلاف معنی داری در سرعت رشد و تکثیر فیبروبلاست ها و قابلیت زیستی آنها بین گروههای کنترل و A دیده نشد ( $p > 0.05$ ). در مقایسه با گروههای کنترل و A، در گروههای B و C به طور معنی داری کاهش در سرعت رشد فیبروبلاست ها در محیط کشت سلول مشاهده شده و قابلیت زیستی سلولها نیز به طور معنی داری کاهش یافت ( $p < 0.05$ ). همچنین هیچ اختلاف معنی داری در سرعت رشد و قابلیت زیستی فیبروبلاست ها در گروههای B و C دیده نشد ( $p > 0.05$ ). **نتیجه گیری:** بر اساس نتایج حاصل از این مطالعه، می توان اظهار داشت که استفاده از هیدروکورتیزون سبب مهار رشد فیبروبلاست ها می گردد که این اثر وابسته به دز می باشد. بنابراین هیدروکورتیزون می تواند به منظور جلوگیری از تشکیل بافت گرانوله اضافی و تسریع در التیام زخم اسب به ویژه در قسمت های پائینی اندام های حرکتی مورد استفاده قرار گیرد.

**کلید واژه ها:** هیدروکورتیزون، فیبروبلاست، رشد، اسب