Epidermal Cell Proliferation in Calorie-restricted Aging Rats +

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ABSTRACT:

Calorie restriction (CR) has been known to produce many beneficial health effects, and lowered cell proliferation from CR has been shown to produce anti-cancer effects in some tissues. In this study the rate of epidermal cell proliferation in aging Fischer 344 rats from *ad libitum* fed (AL) and CR colonies was assessed in relation to changes in epidermal thickness with age. Proliferating cell nuclear antigen (PCNA) was detected using immunohistochemical method on paraffin sections in the epidermis of dorsal skin and footpad in these animals obtained from the National Institute on Aging. The proliferating cell index was compared with morphometric measurement of epidermis in young, young adult and old animals (six per group). Data were analyzed by Excel and SPSS 14.0 softwares for statistical evaluation. Two-way analysis of variance (ANOVA) was applied to data to test the effects of age, diet, and age-diet interaction.

The following significant effects were noted: (I) age and age-diet effects in dorsal skin epidermal width, and PCNA; (II) age, and diet effect on footpad epidermal thickness, and PCNA index. There was a trend of increasing epidermal thickness in the dorsal skin in normally feeding aging rats which was depressed with CR in the two younger groups. PCNA index showed a trend of attrition from young to old. The thickness of epidermis in foot pad showed a curvilinear trend in both AL and CR groups with lowest mean values in the old group, and more predominant effect in CR-exposed animals. The proliferation index in the foot pad demonstrated a trend of reduction in old specimens with lower mean values in each corresponding CR age group. This report agrees with CR-inhibited cell proliferation reported in many organs by other investigators, and the observed results might have been caused by physiological or endocrine mechanisms affecting the epithelium in these calorie-restricted animals.

Keywords:

Aging; Calorie restriction; Skin; Rat Skin; Fischer Rat; PCNA; Histology; Morphometry; Epidermis

INTRODUCTION

Calorie restriction (CR) as a metabolic intervention has been shown to incur many health benefits which include attenuation of aging changes, increase in longevity, cardiovascular improvements, and prevention of auto-immune diseases. CR can extend life span as seen from longevity studies with calorie restriction and *ad libitum* (AL) feeding in diverse species including mouse and rat strains, the human, and non-human primates [1,2,3]. Arrested tumor development is another effect of CR in rodents as it can inhibit different kinds of neoplasia in mouse models [4]. In rodents CR may reduce circulating growth factors ultimately inhibiting tumor formation [5]. Anti-aging effects of CR mediated by decreased cell proliferation may render body tissues refractory to age-mediated diseases and intrinsic aging. CR has been reported to alter cell proliferation in rodent tissues and inhibit pace of DNA replication which may make those cells less susceptible to DNA damage by carcinogens. CR has a profound effect on expression of genes relevant to cancer growth [6, 7.8]. Therefore, cell proliferation as analyzed by immunohistochemistry and labeling techniques has been useful in carcinogenic bioassays, skin photo aging studies, histopathology of gastro-intestinal tissue, and as a biomarker of toxicity experiments [9, 10]. Clinical benefits of CR on skin-related disease conditions have also been reported. CR can reduce ulcerative dermatitis in mice, and short-term fasting has alleviated symptoms of contact dermatitis [11, 12].

There is limited information about the effects of CR on morphological parameters during intrinsic aging of skin in laboratory models. Laboratory animals reared under controlled conditions with age documentation are difficult to obtain, and are expensive. Nevertheless, cellular replication in rodent tissues subjected to dietary intervention has been the subject of a number of studies. Calorie restriction produced abnormalities in skin and hair growth in mice [13]. Lok *et al*. [14] observed a reduction in cell proliferation in gastro-intestinal epithelial cells, the mammary gland, and the dermis in young adult mice with dietary restriction. The study was done with mitoses counts following [^{3}H] labeling of tissues. Effects of dietary restriction on proliferating cell nuclear antigen (PCNA) in gastro-intestinal tissues were reported in Fischer 344 rats [6]. Cell proliferation in keratinocytes and mammary epithelial cells in 7-week-old mice was reduced with two weeks of CR imposition [15]. Epidermal cell proliferation was reduced by CR and alternate-day fasting in young mice [16].

In our previous cross-sectional studies of colony-raised Fischer 344 rats, the normal age induced progression of epidermal and dermal changes were found to be modulated by CR [17, 18]. These studies involved morphometric assessment of skin compartments from different body areas in CR rats compared with those from AL groups. As an extension of these studies it was important to know if the rate of epidermal cell proliferation as quantified by immunohistochemistry can be compared to aging changes in epidermal thickness in these colony-raised rats.

MATERIAL & METHODS

Animals: Fischer 344 male rats obtained from the Caloric Restricted Colony of the National Institute on Aging were used in this study. In that colony CR is initiated at 14 weeks of age at 10% restriction, subsequently increased to 25% restriction at 15 weeks, and 40% at 16 weeks, and maintained for the rest of the animal's life. Details can be found in the aged rodent colony handbook of the National Institute on Aging. Animals were shipped to our facilities along with food (AL and CR) and feeding instructions, and were housed in individual cages for one week before sacrifice. The following age groups from *ad libitum* (AL) and calorie-restricted (CR) colonies were used in this study, and each group had six animals: 4 months (young, Y), 12 months (adult, AD), and 24 months (old, O). The CR rats were fed the following amount per day, which is a 40% caloric restriction from their ad lib intake: 4 mo. - 11.5 grams, 12 mo. - 11.0 grams, 24 mo. - 10.5 grams.

Rats were sacrificed between 10AM and 12 noon by carbon dioxide asphyxiation as approved under institutional guidelines of animal care. Skin samples obtained from dorsal interscapular region (DS) and footpad (FP) of these animals were fixed in Bouin-Hollande's fluid for 48 hours, and processed by paraffin microtomy.

Histology: Paraffin sections were stained by hematoxylin-eosin-phloxine sequence for histomorphometric study. To assess levels of cell proliferation paraffin sections were subjected to imunohistochemical staining for proliferating cell nuclear antigen (PCNA) as described previously [19, 20]. PCNA staining was performed with a commercial kit from Zymed laboratories which uses a uses a biotinylated PCNA

monoclonal antibody (clone PC10), thus eliminating the need for a species-specific secondary antibody. Streptavidin-peroxidase is used as a signal generator, and DAB as the chromogen to stain PCNA-containing nuclei dark brown. Deparaffinized slides were immunostained after pre-treatment with 0.25% trypsin solution followed by EDTA, or citrate buffer, or L.A.B. solutions (Polysciences Inc., Warrington, PA) for epitope retrieval, and sections were blocked against endogenous peroxidase. Immunostained sections were counterstained lightly with hematoxylin to accentuate the contrast between non-reactive nuclei and brown stained reactive cells.

Histomorphometry was carried out following procedures described earlier [21, 22, and 17]. Epidermal thickness was manually measured with a calibrated ocular micrometer scale introduced into the microscope eyepiece. Linear measurements were taken from the basement membrane up to the end of the granular layer in interfollicular sites. For PCNA-positive cell counts the microscope ocular was fitted with a square lattice grid and counts made at 450X magnification. Epidermal nuclei present within the grid were counted avoiding follicular areas. Nuclei with intense brown staining or sharply stained granules were considered positive for the immunoreactivity, and nuclei with diffuse and faint staining were disregarded. The PCNA labeling index (PCNA-I) is the number of positive nuclei divided by the total number of keratinocyte nuclei X 100. All microscopic measurements were taken in a blinded fashion.

Statistics: Individual means with SD for the 2 parameters i.e., epidermal width and PCNA-I from DS and FP belonging to six groups (AY, AAD, AO, CY, CAD, CO) were generated and analyzed. The effects of CR were analyzed with a 2 X 2 analysis of variance (ANOVA) to determine the effect of age, diet, and age by diet interaction. Data analysis was done with an SPSS 14.0 and Excel softwares. Group means were compared following the method of Bonferroni.

OBSERVATIONS

In AL rats the DS epidermal thickness (depth in μm) readings in three age groups (Y, AD, O: mean± sd) were 11.4±1.1, 12.3±1, and 12.7±1.8. Epidermal width was slightly suppressed in CR rats in two younger groups (10.8±0.9, 9.8+1.1, and 12.41+1.5).The age, and age X diet effects were significant (age: F_{CAL} [2,30]=8.0, $p < 0.002$; age-diet interaction: F_{CAL} [2,30]=3.9, $p< 0.03$).

The means for proliferation rate (PCNA-I) in DS epidermis were 10.5 ± 5.7, 18.6 ± 5.4 and 6.9 ± 4.4 in young, adult, and old animals from AL groups. In CR rats the index was 11.0 ± 8.2, 8.7 ± 5.4, and 6.8 ± 2.2 in the three age groups. Age and age X diet effects were significant (age: F_{CAL} [2, 30] =4.57, $p < 0.01$; age-diet interaction: F_{CAL} [2,30]= 3.34, $p < 0.04$).

In the footpad epidermis the thickness mean values in AL rats were 128.5 ±23.1, 138.9 ± 28.2, and 99.9 ±11.4 in Y, Ad, and O groups. Lower values were noted in CR animals i.e., 104.2 ±26.7, 118.9 ±8.7 and 75.3 ±6.6 in the three age groups. Age and diet effects were significant (age F_{CAL} [2, 30]=14.04, $p < 0.001$; diet F_{CAL} [1,30]= 12.36, $p< 0.001$).

FP PCNA-I values showed diminished values in CR rats compared over AL animals (AL: 29.6 ±2.7, 28.4 ± 5.2, 21.6±3.9; CR: 22.4 ±4.7, 19.4±3.0, and 20.3 ±3.6). Age and diet effects were significant (age: F_{CAL} [2, 30] = 4.87, $p < 0.01$; diet F_{CAL} [1,30]= 19.28, $p< 0.001$).

The accompanying photomicrographs show representative sections from footpad immunostained for PCNA (Fig.1A-D). Figures 2(A-D) represent group means with error bar (1 SD), and figures 3(A-D) show overlaid scatter graphs with individual data points and a fit line running through the mean values indicating the trends.

DISCUSSION

For studying intrinsic aging of skin researchers have used various rodent species as potential models due to their short life span and fast aging. However, due to prohibitive cost and the difficulty in obtaining these models from a germ-free colony with accurate age documentation access to aging animals has been restricted to aging biologists. The Fischer 344 rat has a medial life span of 24 months [23], and is a well established model in biomedical and aging research [24, 25, 26]. The present cytological and immunohistochemical observation of the aging epidermis is a cross sectional study representing major phases of life cycle in this species. The data indicate certain trends although uniformly linear changes across the three studied stages were seldom detectable in these two parameters (epidermal width, and PCNA) from the two skin samples (DS, and FP). This is reminiscent of reported fluctuation of epidermal parameters like cell populations or cell kinetics over the entire life cycle in rodent skin [27, 28].

In respect of the effect of chronological age on epidermal width we observed a dichotomy in samples from the DS and FP. A linear increase from young to old was notable in DS, and a similar trend in the FP epidermis was noted only up to the adult stage. We have reported linearly increasing epidermal thickness in the ventral skin of growing Fischer 344 rats [17]. CR seemed to inhibit this parameter in DS only in younger animals the effect being lost in the oldest group. In FP samples epidermal width followed a curvilinear trend in AL animals, and a similar trend with lower mean values resulted in food restricted specimens. Published studies on dorsal skin thickness in the normal aging rats have reported slightly different trends. In Fischer 344 rats epidermal thickness remained nearly constant from 3 to 22 months of age after an initial increase [29]. Average epidermal thickness in Wistar rats decreased progressively up to the 4^{th} week, and then remained constant up to the age of 34 months [30]. In a study of age-associated epidermal size and proliferation rate in mice no single age-associated change in epithelial structure was observed; epidermis from the ear and foot pad showed increase in thickness while palate epithelium became thinner in old animals [31]. Aging as a biological phenomenon may be presumed to precipitate similar alterations in similar tissues, but regional differences in same animals show dissimilar patters in different epithelial types [31].

In this context it may be pertinent to compare studies on age-associated changes in epidermal thickness in man. Recently *in vivo* measurement of epidermal thickness with optical coherence tomography and confocal laser microscopy in human volunteers from 21-82 years has been reported, and no differences between age groups were observed [32]. Epidermal thickness was found to be constant in sun-exposed and protected skin in healthy volunteers representing 1^{st} to 9^{th} decades [33].

The same parameter was unaffected by aging as observed in other human studies [34, 35]. However, Gilhar et al. [36] noted that aged human skin was associated with thinning of the epidermis and decreased epidermal proliferation. On the contrary, increase in epidermal thickness with aging in human volunteers was revealed with confocal laser scanning electron microscopy and ultrasound imaging [37, 38]. The variations in human data may be explained possibly by the fact that different kinds of populations were surveyed by the investigators, and the subject remains equivocal.

A trend of diminution of cell multiplication rate with aging was conspicuous in FP samples which was further accentuated by CR imposition. The effect of age on PCNA in the DS of AL rats showed a spike in 1-year-old animals before a downturn in the old group; CR produced a more consistent age-related linear decrease in mean values. In rodents age-associated epidermal cell multiplication or cell proliferation rate shows a variety of trends in different species which makes it difficult to compare with our results. While a negative effect of age on epithelial proliferation was recorded in some early studies [31], other investigators have documented increased rate of cell proliferation, or a lack of change with age. In C57B1/6NN mice studied with autoradiography no significant differences in labeling index between young and adult animals was seen [31] , and in the hairless mouse epidermis, the average number of drug-arrested mitoses remained steady until old age [28]. In Sprague-Dawley rats Bertalanffy *et al.* [27] observed epidermal mitotic rates increased with age, particularly with senility. This was observed in plantar and ear epidermis from young, adult, and senile rats. In the same species a fall in the rate of epidermal proliferation in the dorsum, foot, and tail in varying degrees related to ages of 7, 14, and 52 weeks of age was observed [39]. The present results in Fischer344 rats resemble the age-associated lowering trend reported in Sprague-Dawley rats in terms of cell proliferation. It is of interest to note that the rate of turnover of human epidermis was found to be decreased with age in many early reports [28] but a meticulous study by Thuringer & Katzberg [40] showed increased mitotic index in senile individuals.

The present cross-sectional study indicating effects of normal aging and CR on cell proliferation differs from a report on cell proliferation rate in diverse organs in F344 rats and B6C3F1 mice [41]. In that experiment F344 rats at 7, 10, 13, and 20 weeks of age were studied with PCNA, and bromodeoxyuridine. Age-related differences in the skin and stomach were not evident although labeling indices varied according to age in liver and kidney. The effect of CR in suppressing keratinocyte proliferative potential presently observed is somewhat opposite to results reported with *in vitro* studies conducted with skin fibroblasts. There was a progressive loss in proliferative capacity of these cells from AL mice [25, 42],

and CR preserved the proliferative capacity [42]. However, in monkey skin cells (fibroblasts) cellular proliferation which declined with age was not altered by CR [43].

The mechanism behind CR-induced depression of epidermal size and cell multiplication in both tissues of aging rats remains speculative without further measurement of physiological parameters. It is possible that diet-induced weight loss might contribute to attrition of these parameters. The action of CR however, appears to be independent of body weight reduction in rodents as suggested by some studies. In CBA mice epidermal mitotic activity was related to food intake rather than to body weight directly [44]. Fasted mice did not reduce their total food intake or body weight, yet still experienced the beneficial effects on physiological parameters as seen in standard CR protocols [45, 23]. In C57BL/6 mice lower cell proliferation was not observed with reductions in body weight [46].

Within recent years many biochemical studies in different species have been reported on the mechanism of CR effect on various organ systems. CR-mediated inhibition of age-related growth in epidermal thickness and a concomitant modulation in cell proliferation in rat epidermis are conceivably elicited by metabolic adaptations like altered anti-oxidant system, endocrine secretions, or growth factors as summarized by Fontana & Klein [2]. Physiological processes related to energy metabolism are regulated by endocrine factors, and CR effects may be mediated through changes in cell signaling by epidermal growth factor, and insulin-like growth factors [47, 48]. Cell proliferation and DNA replication has a central role in carcinogenesis, and insulin-like growth factor I (IGF-I) has been thought to play a role in hypo proliferative response to CR [16, 49]. The possible physiological alterations involved in causing morphological manifestations in the epithelium in these age-graded calorie-restricted rats remain to be elucidated.

Our observations in the rat skin are comparable to rodent and some human studies where dietary restriction has been shown to reduce cell proliferation. In most studies on rat liver, or gall bladder, this depressive effect has been described except the rat jejunum where no effect was reported (reviewed by Klebenov [50]). CR reduced proliferative response of rat hepatocytes [6, 51], but long-term CR was also associated with enhanced proliferation in aged cells [51]. In young mice CR as well as alternate-day fasting reduced cell proliferation in epidermal cells, splenic T cells, and mammary epithelial cells [49, 16]. Fasting in obese human volunteers led to a reduction in mitotic index and a thinning of epidermis [52].

In abdominal skin and liver from Sprague-Dawley rats depressive effect of CR on *in vivo* DNA synthesis was noted only in the early stages of life [53] comparable to the present finding of epidermal width estimation in DS samples. In that study the authors applied a two-way factorial analysis to determine effects of age, and diet, and their interactions, and variable age, or interaction effects were detected on the different parameters of the study i.e., DNA synthesis, RNA/DNA ratio, protein/RNA ratio etc. By applying the same statistical measure in our study significant effects of age on the two cytological parameters were noted predominantly, while age-diet interaction effect was seen only in some occasions.

The present report in Fischer344 rats has some clinical implications regarding prevention of skin cancer with calorie deprivation. A link between increasing age and development of skin cancer has been discussed [54]. It has also been shown that dietary energy restriction with 40% calorie reduction from fat and carbohydrate can inhibit induced forms of cancer in the mouse [55]. Similar studies on the induction of skin cancer in this model will be helpful to elucidate this fascinating aspect of interrelationship of diet and carcinogenesis.

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Explanation of Figures

Figs. 1A-D (Photomicrographs)

Light microscopic photographs of the rat foot pad skin showing immunohistochemical staining of PCNA positive epidermal cells. The brown positive cells are localized to the basement layer (arrows in Figs. 1A and C). The cells are sparse in the CR animals. (original magnification X 200; scale marker 50 mμ). The sections have been lightly counterstained with hematoxylin. A : AY; B: CY; C: AAD; D : CAD. EPI: epidermis; SC:corneal layer.

Figs.2 A-D. Means with error bar of one standard deviation in the four groups (DS Epi, DS pcna, FP Epi, FP pcna) across the three age groups in rats.

Figs. 3 A-D. Overlay scatter plots of actual datapoints showing the distribution in four groups of this study. A linear fit line drawn through the means shoows the trend of changes in aging rats from *ad lib* and CR groups.

FIG 1A

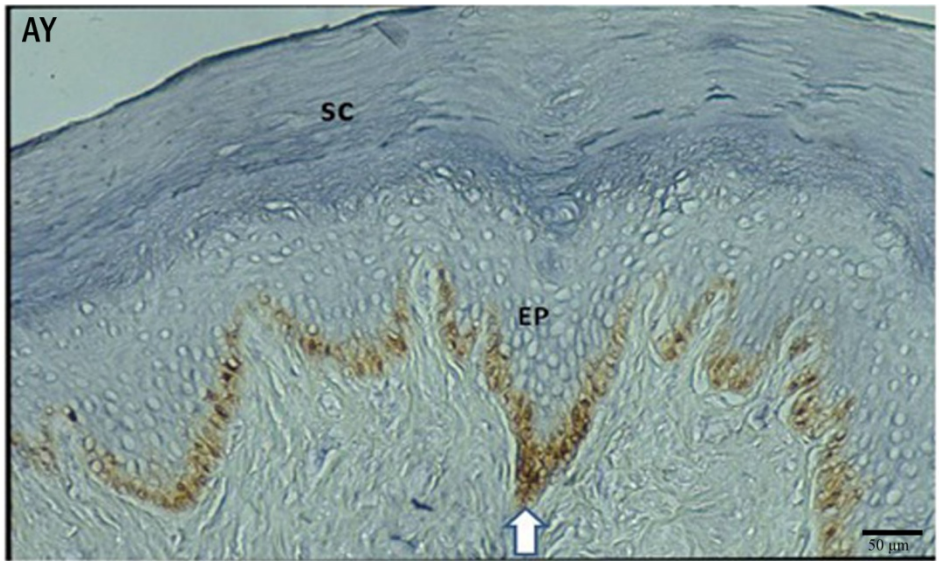


FIG 1B

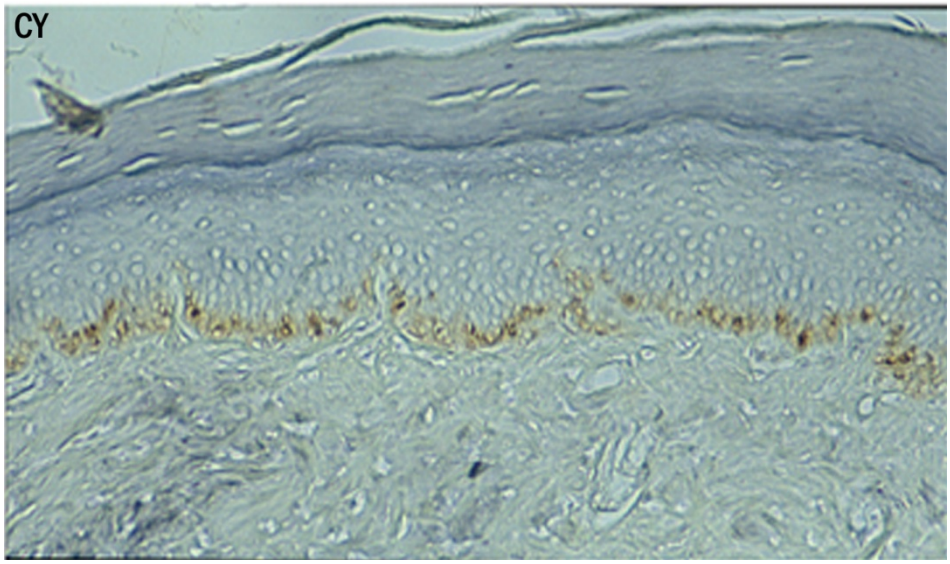


FIG 1C

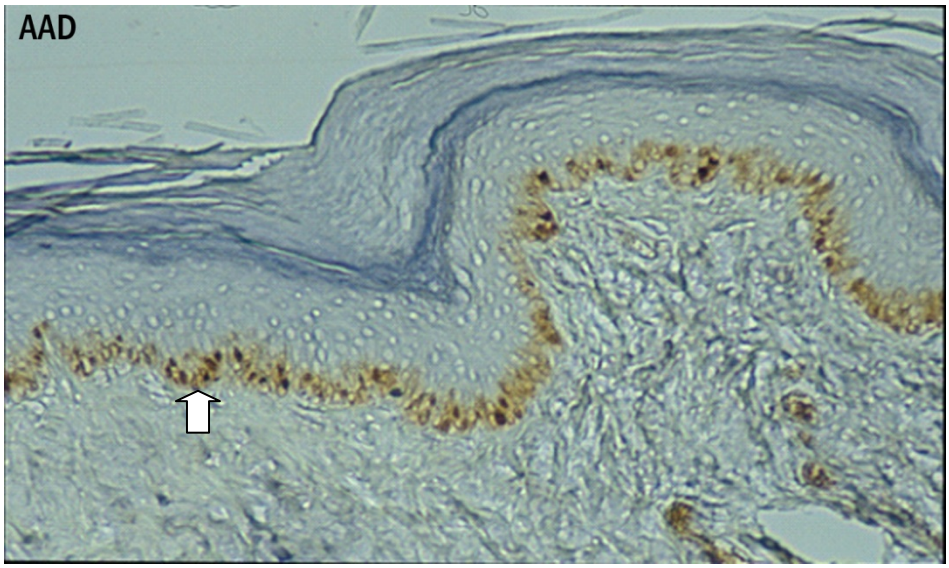
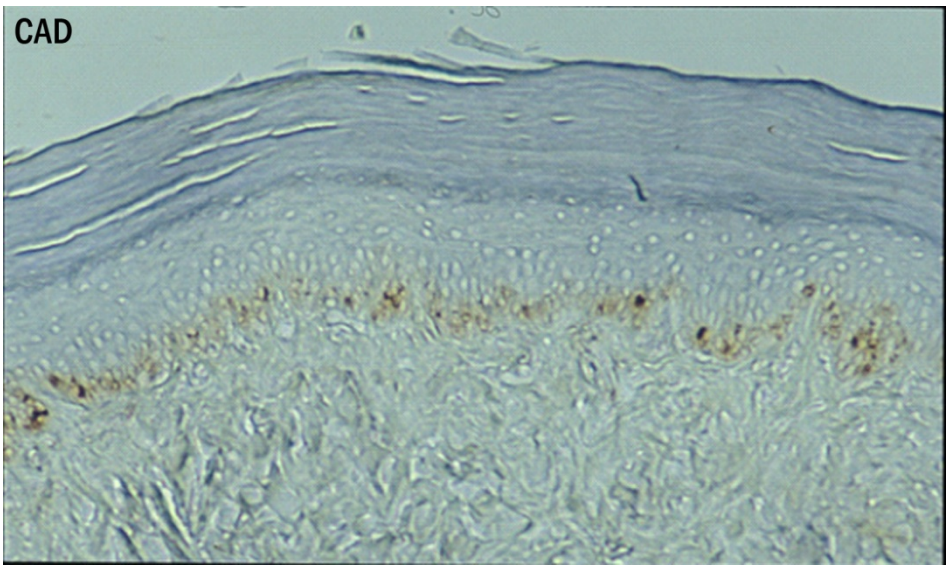


FIG 1D



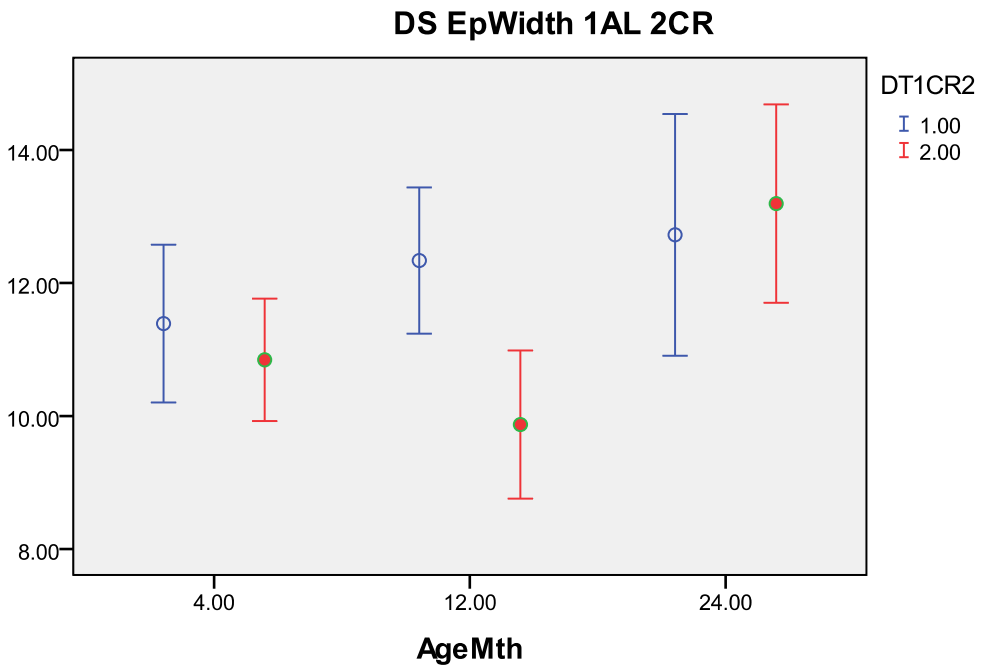

DS EpWidth 1AL 2CR
DT1CR2
1.00
2.00
14.00
12.00
10.00
8.00
4.00
12.00
24.00
AgeMth

FIG 2A

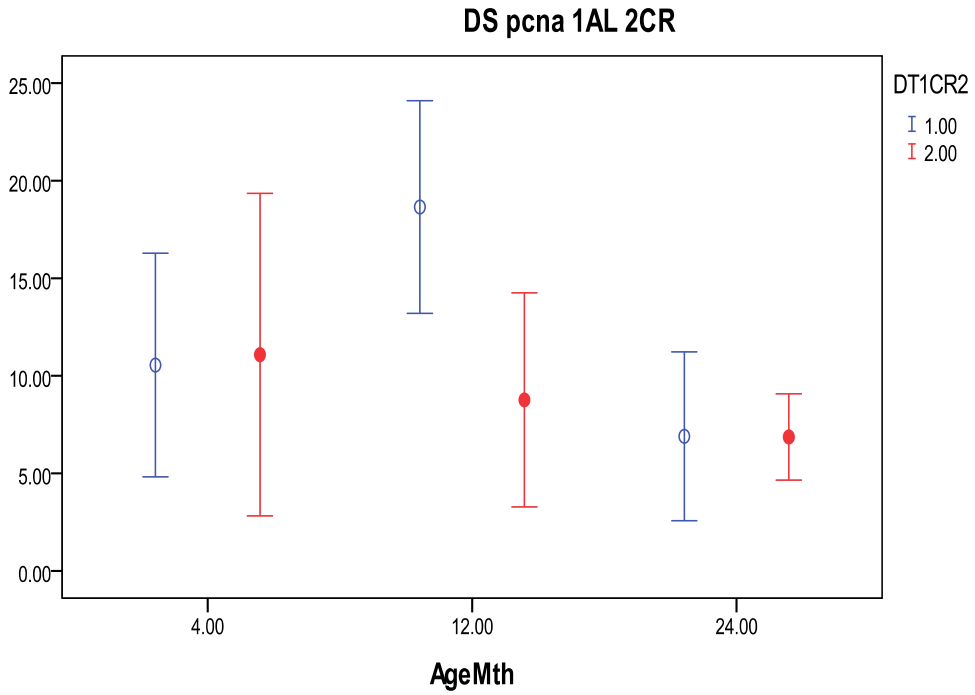

DS pcna 1AL 2CR
DT1CR2
1.00
2.00
25.00
20.00
15.00
10.00
5.00
0.00
4.00
12.00
24.00
AgeMth

FIG.2B

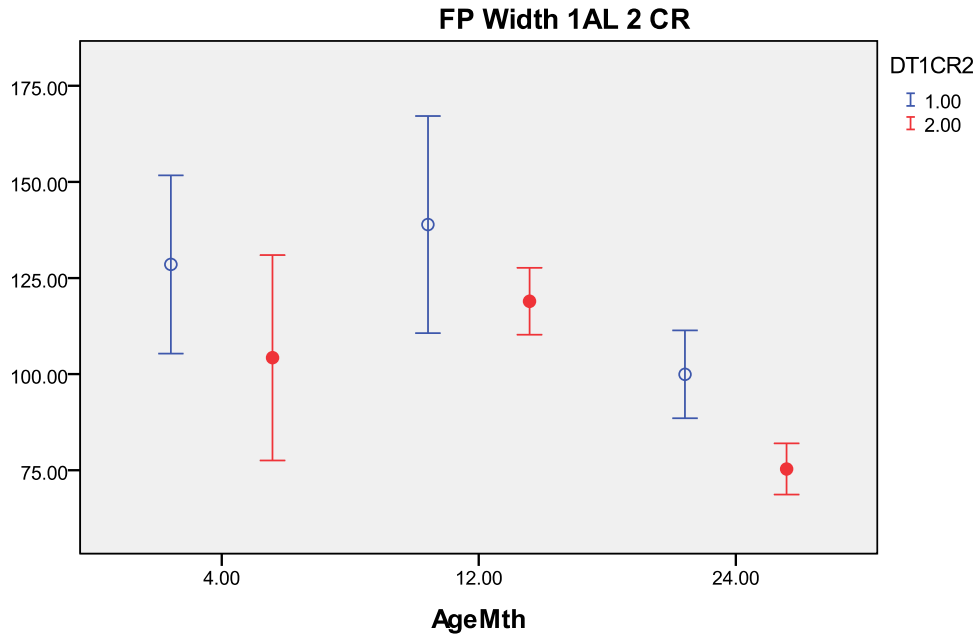

FP Width 1AL 2 CR
175.00
150.00
125.00
100.00
75.00
4.00
12.00
24.00
AgeMth
DT1CR2
1.00
2.00

FIG 2C

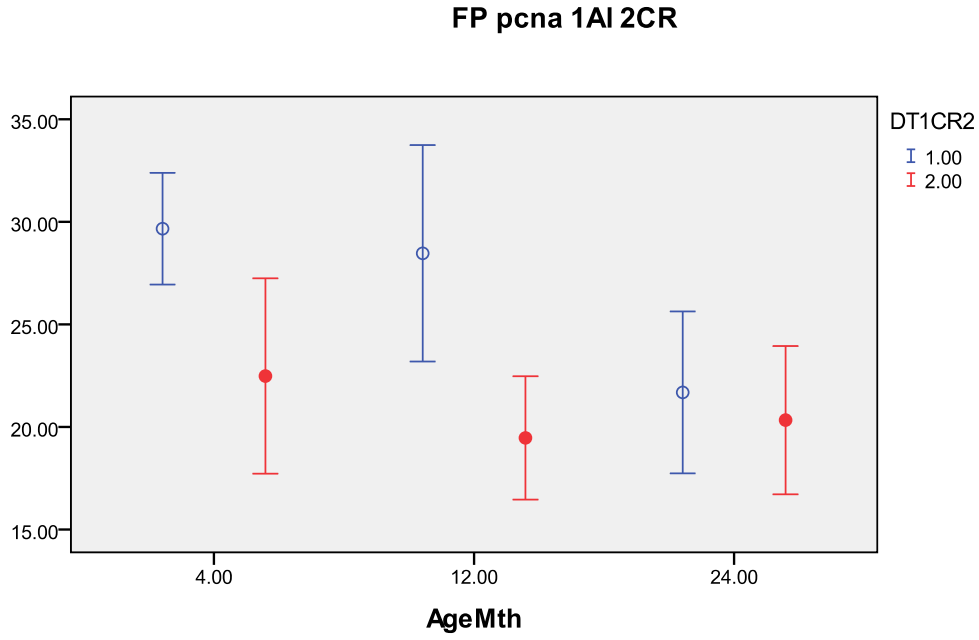

FP pcna 1Al 2CR
35.00
30.00
25.00
20.00
15.00
4.00
12.00
24.00
AgeMth
DT1CR2
1.00
2.00

FIG 2D

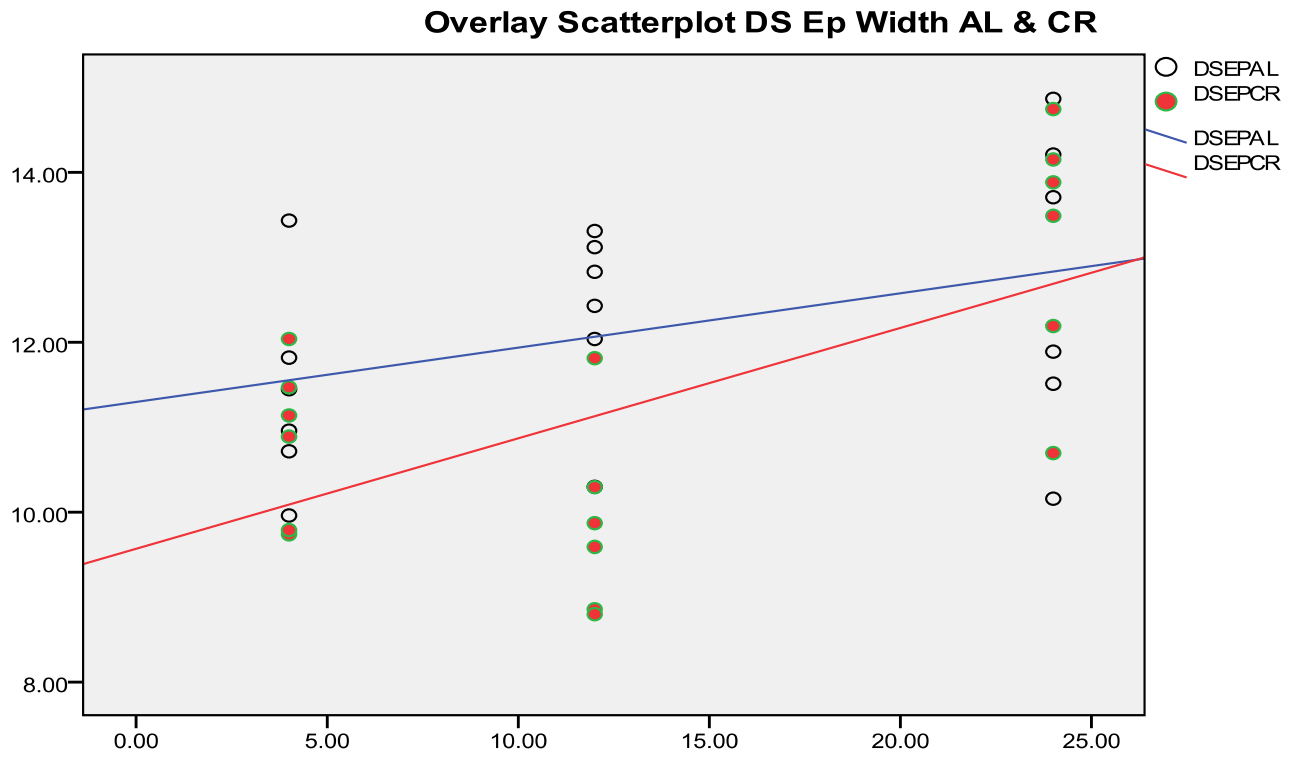


FIG 3A AGE GROUPS >

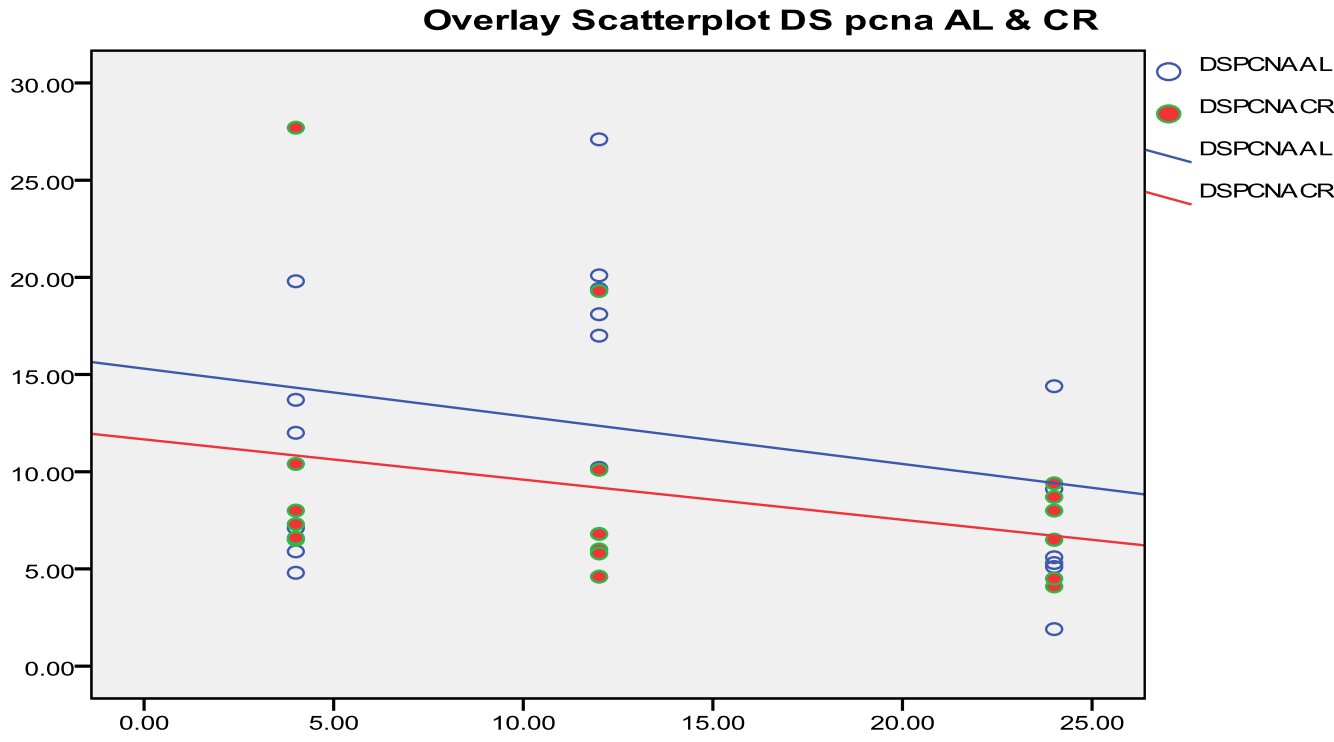


FIG 3B AGE GROUPS >

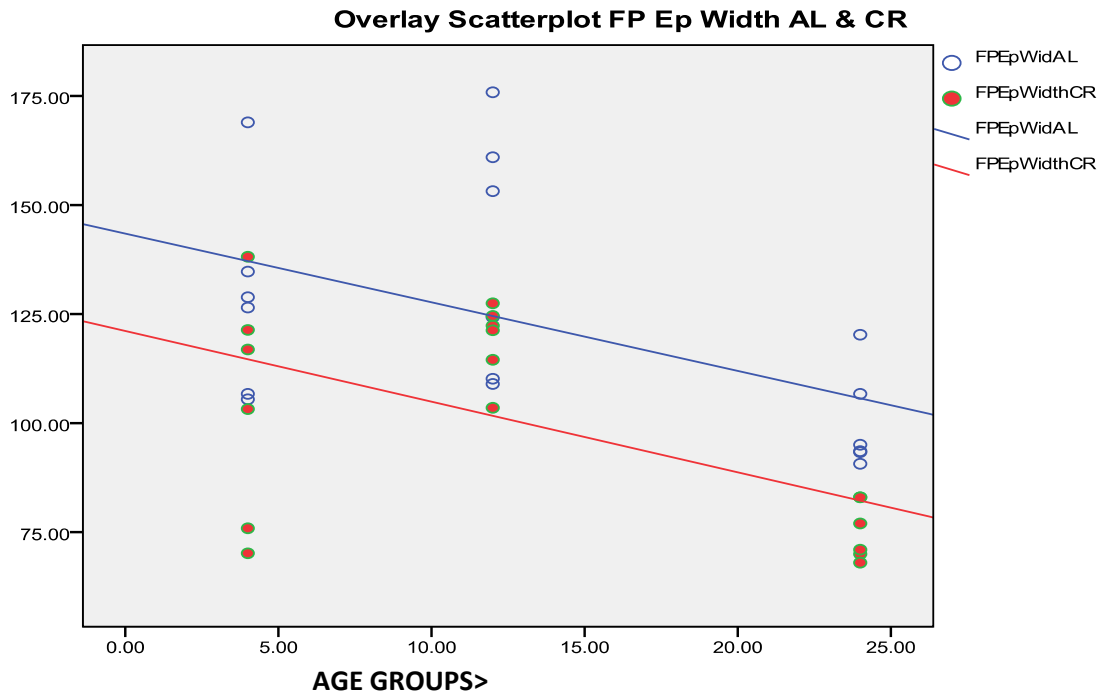

Overlay Scatterplot FP Ep Width AL & CR
FPEpWidAL
FPEpWidthCR
FPEpWidAL
FPEpWidthCR
175.00
150.00
125.00
100.00
75.00
0.00
5.00
10.00
15.00
20.00
25.00
AGE GROUPS>

FIG 3C

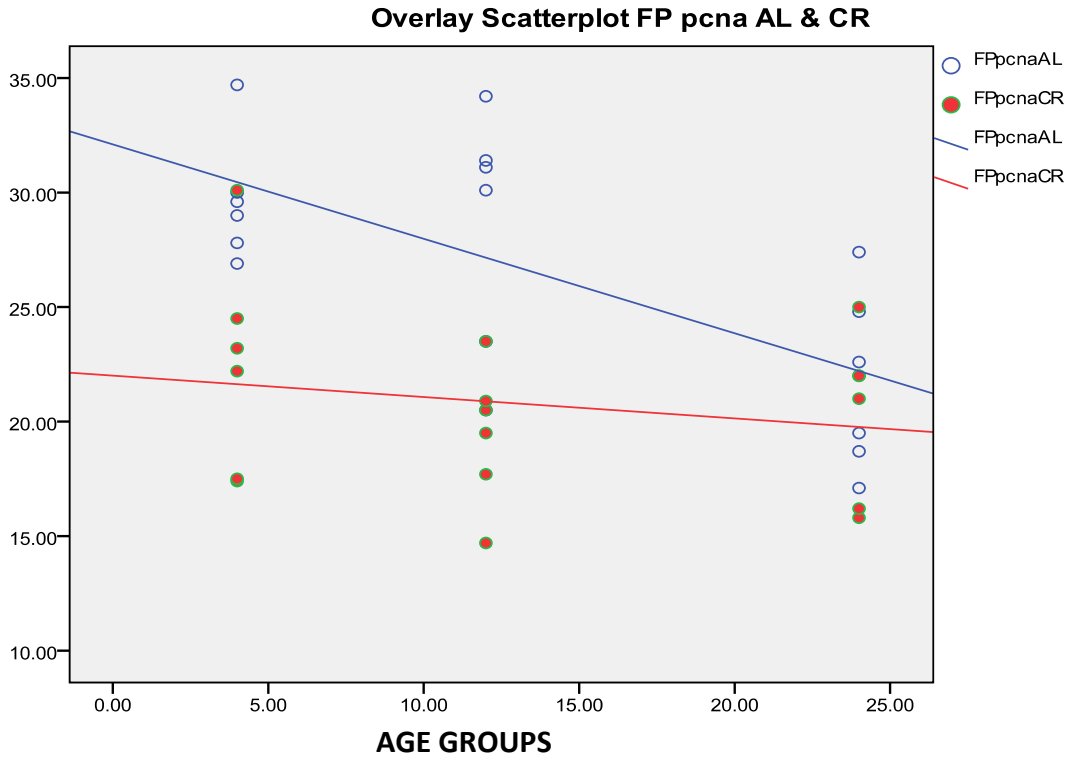

Overlay Scatterplot FP pcna AL & CR
FPpcnaAL
FPpcnaCR
FPpcnaAL
FPpcnaCR
35.00
30.00
25.00
20.00
15.00
10.00
0.00
5.00
10.00
15.00
20.00
25.00
AGE GROUPS

FIG3D