Caloric Restriction Promotes Immunogenicity of the Aged Rat Skin

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Original Article

ABSTRACT

Introduction: The positive effect of caloric restriction (CR) in the intervention of age-related skin and hair disorders is a disputed issue, and the mechanisms by which it influences the immune response in old age are still not well understood. This work postulated that CR could prevent age-related cutaneous disorders by enhancing skin immunogenicity and regenerative cells expression with downregulation of inflammatory, oxidative and apoptotic stressors.

Material and Methods: A total of 45 female Wister rats were divided into three groups; adult control, aged, and aged-CR groups. The CR group was subjected to caloric restriction from 13 to19 months of age. Dorsal skin samples were collected for each group and subjected to light and scanning microscopy, morphometric, biochemical, molecular and immunohistochemical studies.

Results: In contrast to the devastating impact of aging on rat skin and hair, CR resulted in upregulations of both oxide synthase (iNOS) and CD4 gene immune cells expressions, p53, cytokeratin (CK) 19, and transforming growth factor-beta 2 (TGF-β2) immune expression levels. Additionally, we observed amelioration of both inflammatory and oxidative stress factors with preservation of most of skin and hair internal and external structures, collagen, and elastic fibers percentage area.

Conclusion: These initial outcomes strongly suggest that in aged rat skin, the CR can protect against skin and hair aging through elevations of both innate and adaptive immunity. These effects were mediated through prohibition of the catabolic effects of apoptotic/oxidative and inflammatory stressors and promotion of the anabolic effects of stem and immune cells and will be explored in further studies.

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Key Words: Aging; caloric restriction; immunogenicity; skin; stressor.

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INTRODUCTION

The effectiveness of caloric restriction (CR) by enhancing organism longevity has been proven, but its effect on skin ageing is still relatively unclear[1]. Recent findings have shed new light on the mechanisms through which caloric restriction affects immunity[2,3]. The immune system has been found to lengthen lifespan through its pivotal protective roles, as it protects the organism against both programmed internal disturbance and/or external agents[4].

Ageing involves the progressive accumulation of deleterious changes in cells and tissues over time[5], including immune and stem cells[6,7]. The loss of collagen and elastic fibres is considered a characteristic histological finding in aged skin[8]. The recognized folds, ridges and creases in aged skin are related to body mass collapse, tissue dehydration, dermal disintegration and junction disunion. Exogenous factors such as sun exposure can induce premature skin ageing or photoaging[9]. Endogenous mechanisms such as glycation, free radicals, cell cycle deviation, and cellular, molecular and mucocutaneous inflammation are involved in skin ageing[10,11]. The wear and tear theory of the ageing process has been attributed to autoimmunity directed mainly by the thymus gland[4].

Studies of the effect of caloric restriction on skin are crucial, as it is the largest mammalian organ that acts as a primary barrier and provides a model for current research on cell adhesion, inflammation and tissue stem cells[12,13].

It has been suggested that caloric restriction attenuates age-related oxidative and inflammatory stress in many animal species without impairing cell-mediated immunity[14-16]. It is supposed that CR might impede ageing and immunosenescence amendments in signal transduction and gene expression mechanisms[17]. In addition, studies have reported that dietary restriction has a beneficial role in preventing immune system disorders, focusing on T lymphocytes and macrophages, through improvement of antioxidant defence mechanisms, diminishing changes in T lymphocytes and impeding DNA mutation frequencies[18]. Moreover, caloric restriction preserves stem cell regenerative function and monitors stem cell senescenc [19], and significant immune-stem cell interactions inversely influence inflammation-associated tissue dysfunction[20].

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Adjustment of therapeutic and preventive strategies in the skin ageing process appears to be strongly linked to anti-inflammatory, antioxidant, anti-apoptotic and stem cell and immunity promotion processes. Therefore, this study was designed to focus on how CR prevents catabolic effects related to ageing and enhances anabolic effects through the promotion of cellular and innate immunity in aged rat skin.

MATERIAL AND METHODS

Animals

This study was carried out on 45 female Wister rats (15 animals 1.5 months of age and 30 animals 12 months of age) which were obtained from the Research Animal Centre of the National Research Centre of Egypt. All of the rats were in good health, and the animals were treated, and used according to the standards for the use of animals in research. All animals had ad libitum access to drinking water and were fed a standard diet (7–10 percent fat, 68–70 percent carbohydrates, 18–20 percent protein, 1–2 percent vitamins and minerals, 210 kcal/100 g/day) for 4weeks of acclimatization. During this period, the adult rat consumed 25 gm/rat on average, while the aged rat consumed 30 gm/rat.

Study design

After the acclimatization period, the animals were divided into3 groups as follows (Figure 1):

Group I (adult control), consisting of 15 animals 2.5 months of age which were received a standard diet ad libitum until 8.5 months of age.

Group II (aged), consisting of 15 animals 13 months of age which were received a standard diet ad libitum until 19 months of age.

Group III (aged-CR), consisting of 15 animals 13 months of age that were individually housed.

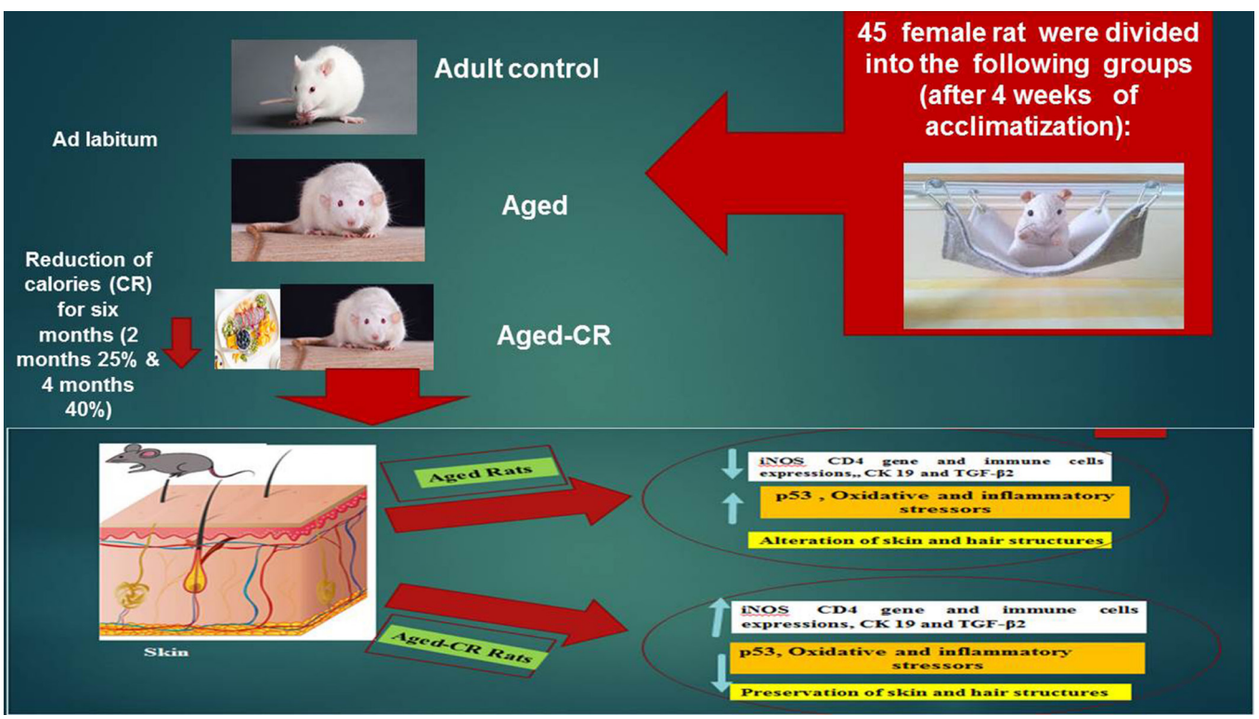

Fig. 1: Graphic abstract

The CR was began at 13 months of age (10 % restriction), increased to 25% restriction at 15 months of age, and then held at 40% restriction for 4 months until each animal's life ended at 19 months of age[21].

All of the animals weights were registered. All procedures were authorised by the Faculty of Medicine's Ethical Committee at Menoufia University in Egypt. Note: Since two rats died in the aged group during the experiment, ten rats from each group were used for final statistics data. An intraperitoneal injection of ketamine (5 mg/100 g body weight) and xylazine (2 mg/100 g bodyweight) was used to anesthetize the animals. They were weighed, and the results were logged. The rats were then sacrificed with an intraperitoneal anaesthetic overdose.

Microscopic study

Full-thickness dorsal skin specimens (5 mm) were fixed in 10% formalin saline for 24 hours for light microscope examination. After that, the specimens were processed to make paraffin block. The paraffin sections (5 µm) were cut to be stained with hematoxylin and eosin (H&E), Masson's trichrome stain (for collagen fibers), and Orcein stain (for elastic fibers). Semithin sections (0.5–1 µm) were cut and stained with 1% toluidine blue[22].

Hair shafts from both the control and experimental groups were incised near to the skin surface of the dorsal abdominal surface for scanning electron microscopic (SEM) analysis. For 24 hours, the specimens were fixed in 2.5 % glutaraldehyde in 0.1 mol/l PBS. The tissue was washed twice in PBS, fixed with 1 % osmium tetroxide (OsO4), rinsed with buffer, and dehydrated in ethyl alcohol in ascending grades.

The specimens were dried in a carbon dioxide critical point apparatus, mounted in stubs, and coated with gold using low voltage DC sputtering and the BAL-TEC 030 critical point apparatus before being examined with a scanning electron microscope JOEL5300 JSM (musashino 3-chome akishima Tokyo 196-8558, Japan)[23].

Immunohistochemical study (IHC)

Skin sections (n = 10) were deparaffinized, endogenous peroxidase activity was blocked with H_2O_2 in methanol, and the sections were heated in a microwave pressure cooker for 20 minutes in 0.01 mol/l citrate buffer. After allowing the slides to cool to room temperature, nonspecific binding was blocked for 20 minutes at room temperature with normal horse serum. The following antibodies were used in the IHC analysis: rabbit anti-CK19 (BIOSS Cat#, bs-1028R), rabbit monoclonal anti-CD4 (Cat#, ab183685, 1:1000), polyclonal anti-TGF beta 1 (Cat#, ab25121, 1: 2000), polyclonal anti-iNOS (Cat#:ab3523, 1:500), and mouse monoclonal anti-P53, tumor marker (Cat#, ab1431, All of the primary antibodies were validated before being used. Mayer's hematoxylin (Bio-Genex, Menarini Diagnostics, Antony, France Cat #, 94585) was used for counterstaining[24].

Biochemical study

Skin tissue samples from 10 animals from each group were homogenized with 10 volumes of ice-cold 0.25-M sucrose and then centrifuged at 10,000 g to measure biochemical parameters in the resulting supernatant for detection of cytokine and antioxidant levels. TNF- and IL-6 levels in rat skin were measured using commercially available enzyme immunoassay kits [enzyme-linked immunosorbent assay (ELISA)]. Rat ELISA TNF- (R&D Systems, Europe, Abingdon, England, Cat#, HSTA00D), and Rat IL-6 ELISA (Diaclone SAS, Besancon, France, Cat#, 670.010.096) were used in this study. All of the samples, standards and controls were analyzed twice. The activity of cytokines was measured in pg/mg tissue protein. We also used the tissue supernatant to detect Malondialdehyde (MDA) as a lipid peroxidation marker using the Draper *et al*.[25] method, while CAT activity was calculated using the Hadwam[26] method. Rat ELISA CAT and Rat Reduced Glutathione ELISA kits (MyBioSource, SanDiego, CA, USA, Catalog #, MBS726781 and MBS046356, respectively) were used. The oxidant/ antioxidant activities were measured in micrograms per milligram of tissue protein.

iNOS and CD4 mRNA expression in the skin

The extracted RNA (5 ml) was added to a master mix (20 ml) to give a total reaction volume of 25 ml per sample for both CD4 and iNOS gene expression. 12.5 mL Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California), 5.5 mL DEPC treated nuclease free sterile water (Fisher Scientific, Pittsburgh, Pennsylvania), and 1.0 mL DEPC treated nuclease free sterile water (Fisher Scientific, Pittsburgh, Pennsylvania) were combined to make the master mix (6.2 mM) [(CD4 F: GAGAGTCAGCGGAGTTCTC; CD4 R: CTCACAGGTCAAAGTATTGTTG), (iNOS F; TCCTGGACATTACGACCCCT; iNOS R: AGGCCTCCAATCTCTGCCTA)] primers (In*vitro*gen, Carlsbad, California). The run did in the presence of β-actin gene (F; AATTTCTGAATGGCCCAGGT- R; TTTGTGTAAGGTAAGGTGTGC). The run was carried out in the presence of the -actin gene (F; AATTTCTGAATGGCCCAGGT- R; TTTGTGTAAGGTAAGGTGTGC). Before loading the samples into the rotor's wells, they were spun. The following cycling conditions were used: initial denaturation at 95°C for 10 minutes; 40 cycles of 64°C for 15 seconds; and extension at 72°C for 30 seconds. During the extension process, data was collected. Each RT-PCR was performed three times with the same results each time. The Rotor-Gene6000system was used to carry out the reaction (QIAGEN, USA). The following equation was used to conduct comparative quantitation data analysis:

Ratio target gene expression = Folded change in target gene expression (sample/control)

Folded change in reference gene expression (housekeeping gene/control).

Quantitative morphometric study and statistical analysis

An automated image analyser (ImageJ 1.47v, National Institute of Health, and Bethesda, MD, USA) was used to perform quantitative morphometric skin sections. The following were included in the Quantitative Morphometric Study: (1) Morphometric analysis of epidermal and dermal thickness (m) in Hx & E stained sections (2) The percentage area of both collagen and elastic fibres by Masson's trichrome and Orcien stained sections respectively as well as CK19, P53, TGF-β2, iNOS and CD4 immunohisto chemistry(cytoplasmic and nuclear) marker reactivity. For each animal (n = 10) five representative fields scattered in the preparation (400, Olympus light microscope BX51TF; Olympus, Tokyo, Japan) from three non-adjacent parts were analyzed. The data was presented as mean ± SD. We used the Kruskal–Wallis (ANOVA on rank) and Dunn's Multiple Comparisons Test to evaluate non-parametric data from multiple groups. We considered results to be significant at $p < 0.05$. SPSS software version 14 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis.

RESULTS

The validity of our CR paradigm was defined in this work through the insignificant decrease in body weights in aged-CR animals compared with aged rats (Table 1).

Light microscopy analysis

H&E-stained sections of the control rat skin showed a thin keratinized multi-layered epidermis (mean thickness: 137.10 µm), connective tissue dermis with regularly distributed hair follicles and sebaceous glands (mean thickness: 1259.3 µm). In addition, the epidermal dermal junctions (EDJs) showed a papillary configuration (Figure 2A). Compared with the control group, the aged rat skin showed marked decreases in both epidermal and dermal thickness (p<0.0001). The epidermis showed discontinuity and the EDJ showed flatness with adjacent necrotic cells. The dermis showed cellular and connective tissue loss. Additionally, it showed clumped fragmented collagen bundles and distorted degenerated hair follicles with inflammatory cell (Figure 2B). In comparison with aged rats, aged-CR rats revealed a significant (p<0.0001) increase in both epidermal and dermal thickness. Except for focal areas of dermal inflammation and vacuolation, all skin structures were markedly preserved (Figure 2C).

By Masson's trichrome staining, the adult control skin collagen fibres appeared as fine interlacing green bundles in the papillary dermis and thick, wavy irregular green bundles in the reticular dermis (Figure 3A). In comparison with the adult control, the collagen fibres of the aged group appeared thin, fragmented, less dense, unevenly arranged and significantly decreased (p<0.0001) (Figure 3B). After caloric restriction in the aged-CR groups, the collagen fibres were significantly (p<0.0001) preserved and appeared dense and regular (Figure 3C).

By Orcein staining, the control skin elastic fibres appeared as thin red branched network fibres in the papillary dermis and a thick condensed network in the reticular dermis (Figure 4A). In the aged group, the elastic fibres were few in number and appeared thin, short, fragmented, dispersed and markedly decreased (p<0.0001) (Figure 4B). This decrease was significantly (p<0.001) prevented after caloric restriction in the aged-CR groups, and the fibres appeared nearly as the control (Figure 4C).

By toluidine blue staining, the control adult rat skin showed a stratified squamous epidermis that was mainly composed of keratinocytes. The dermis showed sebaceous glands with basal flattened cells surrounding large polyhedral cells with rounded nuclei and foamy cytoplasm. Additionally, there were hair follicles and mast cells around the blood capillary (Figures 5A-C). Conversely, the skin of aged rats appeared thin and hypocellular, with scarce melanocytes, mast cells, and Langerhans cells and vacuolated keratinocytes with desquamated stratum corneum. Most of sebaceous glands appeared swollen with accumulated secretions, and their cells became shrunken with dark, irregular nuclei associated with degenerated hair follicles (Figures 6A-C). Again, the aged-CR rats showed preserved epidermal and dermal architecture (Figures 7A-C).

Scanning electron microscopy (SEM) results

By SEM, the epidermis of the adult control rat appeared slightly corrugated and covered with a homogenous stratum corneum lamellar structure. The dermis is formed of compact, well-organized collagen fibres. Likewise, the hairs were straight and grew densely in the presence of outer organized cornified leaflet sheets (Figures 8A-C). Conversely, the epidermal surface of the aged group showed deep furrows and sparse thinning hairs. Some hairs appeared deformed and broken, while others were lost. The outer cornified hair shaft leaflets were either lost or less organized. The majority of the hair appeared variable in size and length (Figure 9A-C). In contrast, the skin sheet of aged-CR rats was restored in its ordinary structures, though at levels that were still below the control. The collagen fibres under the epidermis were evident with correct arrangement, and the hair showed a new normal organized leaflet sheet with normal leaflet sheet thickness (Figures 10A-C).

Immunohistochemical results

CK19 expression

In adult control rat skin, CK19+ stem cells marker were identified as light brown cytoplasmic colour in the epidermis, the bulge region and outer root sheath of the hair follicle and the sebaceous gland (Figure 11A). In aged rat skin, the level of CK19 reactivity was significantly (p<0.0001) reduced (Figure 11B), whereas the reactivity was significantly (p<0.0001) recovered in aged-CR skin (Figure 11C).

TGF-β2 expression

In adult control rat skin, TGF-β2 reactivity was detected in the cytoplasm of epidermal cells, hair cells (Figure 11D). Conversely, aside from positive dark brown clumped granulation tissue in the papillary dermis, the reaction was significantly (p<0.001) decreased in aged rat skin (Figure 11E). The decrease was significantly(p<0.001) increased in the CR-fed groups in the epidermis hair follicle, with many new blood vessels and fibroblast reticular dermis detected (Figure 11F).

P53 expression

With respect to adult control rats, which showed weak expression of p53 (Figure 11G), aged rat skin showed significant epidermal and dermal (p<0.0001) dark brown nuclear reactions (Figure 11H). Caloric restriction in aged rats resulted in a significant (p<0.0001 reduction in the number of cells positive for p53 (Figure 11I).

iNOS expression

In the skin of adult control rats, iNOS immune cells were identified as light brown cytoplasmic reactions in epidermal cells and hair follicles (Figure 12A). However,

aged rat skin showed a significant (p<0.0001) increase in the nuclear dark brown reaction infiltrating the dermis related to inflammatory cells (Figure 12B). Caloric restriction in aged rats resulted in significant (p<0.001) increases in both epidermal and dermal iNOS reactions (Figure 12C).

CD4 expression

In adult control rat skin, CD4+ T helper cells marker were dark brown in the dermis (Figure 12D), and their numbers were significantly (p<0.0001) reduced in aged rat skin (Figure 12E). This is decrease was significantly (p<0.0001) ameliorated in the CR rats (Figure 12F).

Biochemical results

As both oxidative and inflammatory stresses are involved in skin pathogenesis, our study compared the levels of oxidant and antioxidant indicators in the skin among the studied groups. In the aged group, MDA, a marker of lipid peroxidation and oxidative stress, was significantly (p<0.0001) elevated relative to both the adult control and aged-CR groups. In contrast, CAT levels, which are indicators of antioxidant capacity, were significantly (p<0.0001) decreased in the aged group relative to the adult control and aged-CR groups. In addition, the levels of the inflammatory cytokines IL-6 and TNFα in the skin were significantly higher in the aged group than in the adult control group. Compared with aged rats, aged-CR rats showed significant (p<0.0001) improvements in both cytokine levels, reaching nearly the level of the adult control rats (Table 1).

mRNA expression results

Compared with adult control rats, aged rat skin showed a significant (p<0.0001) increase in iNOS mRNA gene expression. However, CR in aged rats induced a significant (p<0.0302) increase in iNOS mRNA expression (Figure 13A). Compared with adult control rats, aged rat skin showed a marked (p<0.0001) decrease in CD4 mRNA gene expression. Similarly, CR in aged rats induced a significant (p<0.0001) increase in skin CD4 gene expression (Figure 13B).

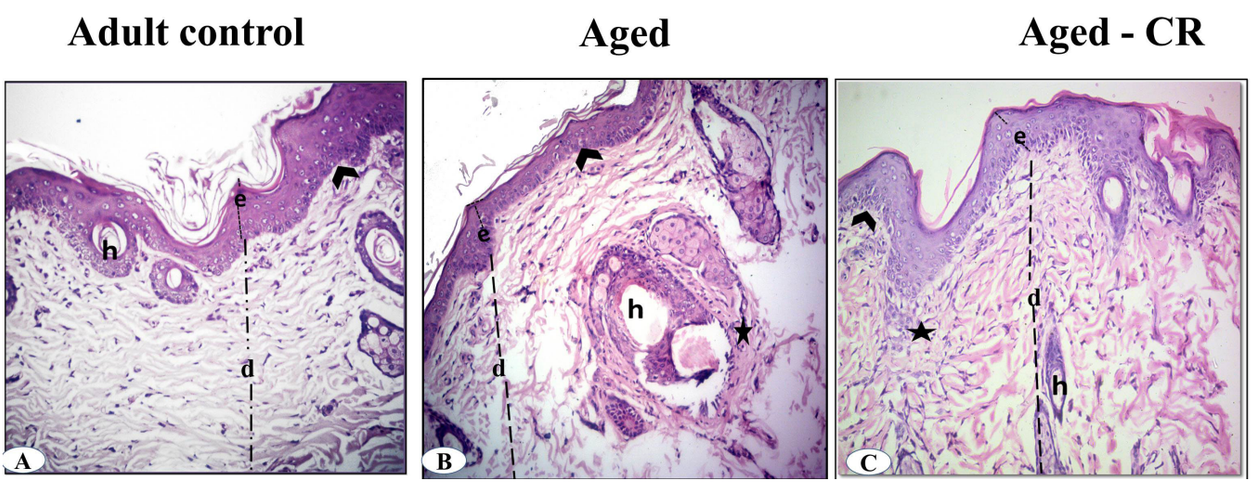

Fig. 2: H& E- stained skin rat sections (×400) of different experimental groups show: **(A)** adult control skin with normal epidermis (e), dermis (d), hair follicles (h) and epidermal/dermal junction, DEJ (arrowhead) . **(B)** Aged skin shows interrupted (arrow) thin epidermis and disintegrated dermis with degenerated hair follicles (h) that surrounded by inflammatory cells infiltration (star). Note. flat DEJ (arrows head).**(C)** Aged-CR skin and hair follicles (h) appears similar to control except for the presence of dermal inflammatory cells (star).

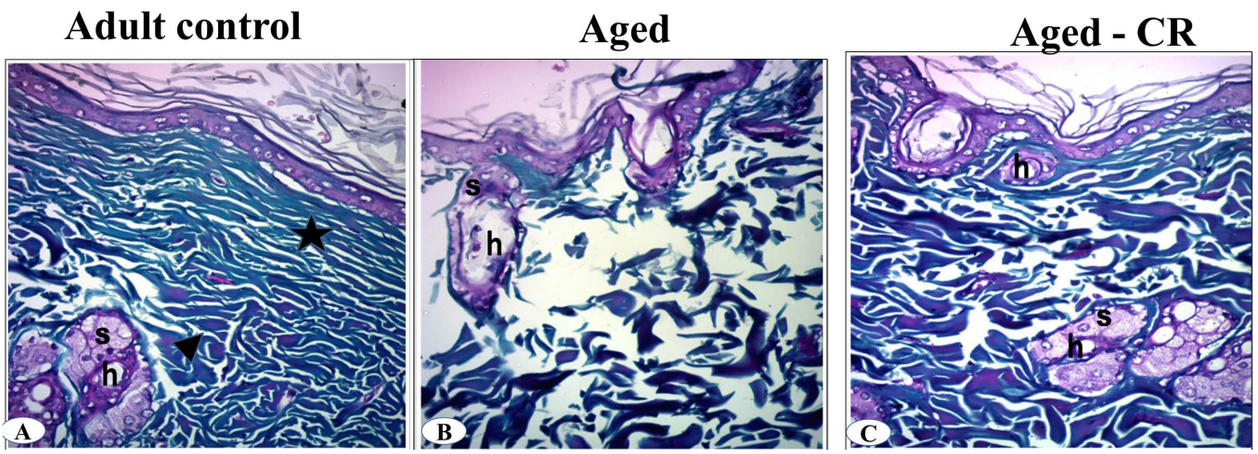

Fig. 3: Masson's trichrome stained skin rat sections (×400) of different experimental groups show: **(A)** Adult control skin with thin interlacing compact bundles of collagen fibers (star) in the dermal papillary layer and wavy bundles with different directions in the dermal reticular layer (triangle). Note. The normal structure of hair follicles (h) and its associated sebaceous glands (s) **(B)** Aged skin with few disrupted and disintegrated collagen fibers and reduced fibroblast population.Note. degenerated hair follicles (h) and sebaceous glands (s) **(C)** Aged-CR rat skin restored its normal components. Note. The normal structure of hair follicles (h) and its associated sebaceous glands (s)

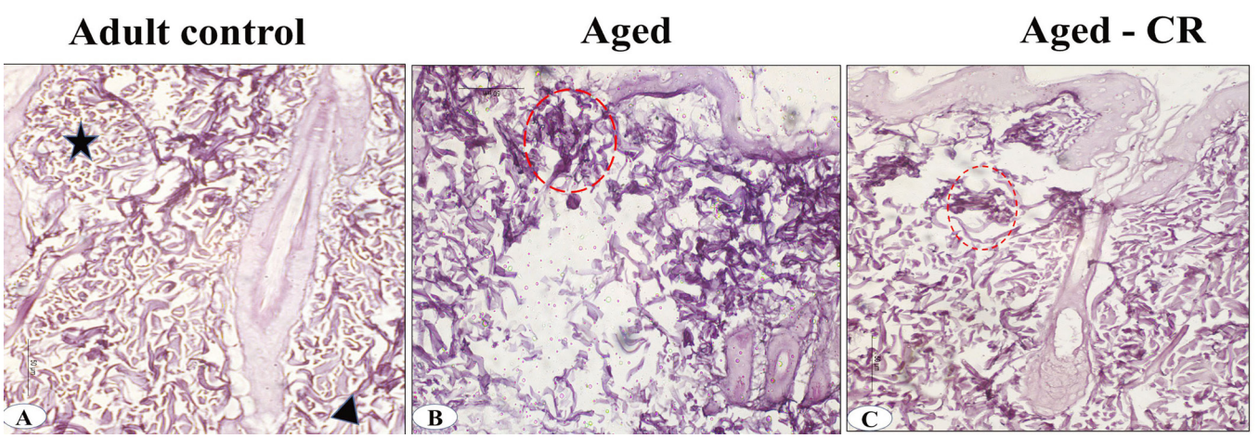

Fig. 4: Orcien stained skin rat sections (×400) of different experimental groups show: **(A)** Adult control skin with mainly thin branched elastic fibers in the papillary dermis (star) and mainly thick elastic fibers in the reticular dermis (triangle). **(B)** Aged rat skin, the elastic fibers are fragmented, dispersed and markedly decreased with presence of diffuse papillary clumped elastic fibers (circle). **(C)** Aged-CR rat skin restored its normal components except for the presence of focal areas of clumped fibers (circle).

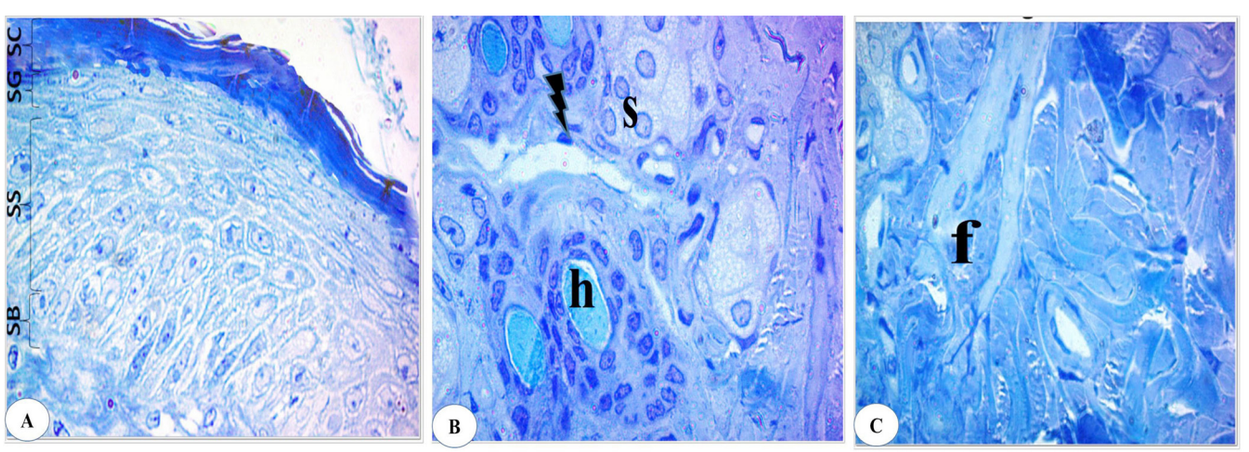

Fig. 5: Toluidine blue- stained semithin sections of adult control rat skin (×1,000) show: **(A)** Epidermal stratification formed from below to above as follows; stratum Basale (sb), stratum spinosum (ss), stratum granulosum (sg) and lamellar stratum corneum (sc) that formed mainly by keratinocytes (k). **(B)** The dermis shows hair shaft (h), mast cell (corrugated arrow) around the blood capillary. The sebaceous gland (s) is formed of basal flattened cells and large polyhedral cells with rounded nuclei and vacuolated cytoplasm. **(C)** Normal structure of the hair follicle (f).

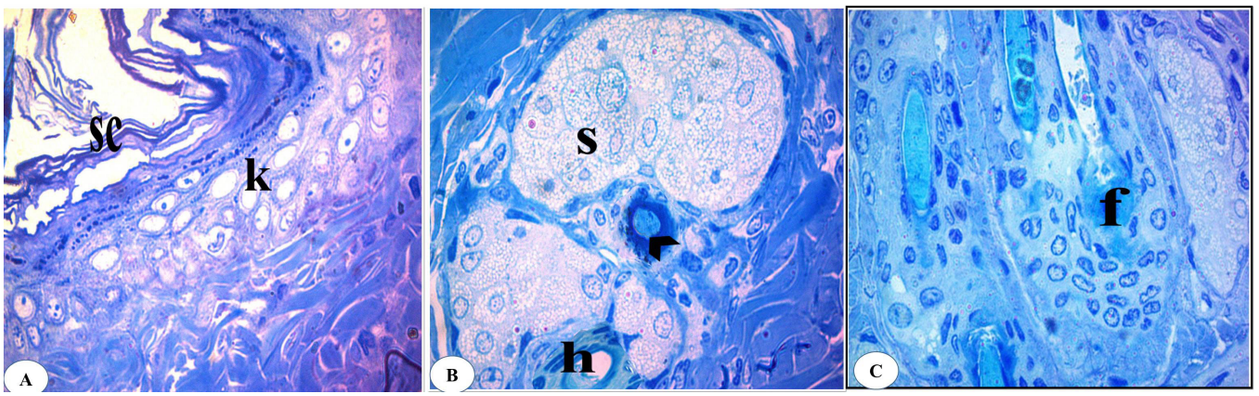

Fig. 6: Toluidine blue- stained semithin sections of aged rat skin(×1,000) show: **(A)** Thin epidermis , hypo cellular with vacuolated keratinocytes (k) and desquamated stratum corneum (sc). **(B)** Sebaceous gland shows swollen polyhedral cells with accumulated secretion and degenerated hair shaft (h) and sheath (arrowhead). **(C)** Distorted structure (f).

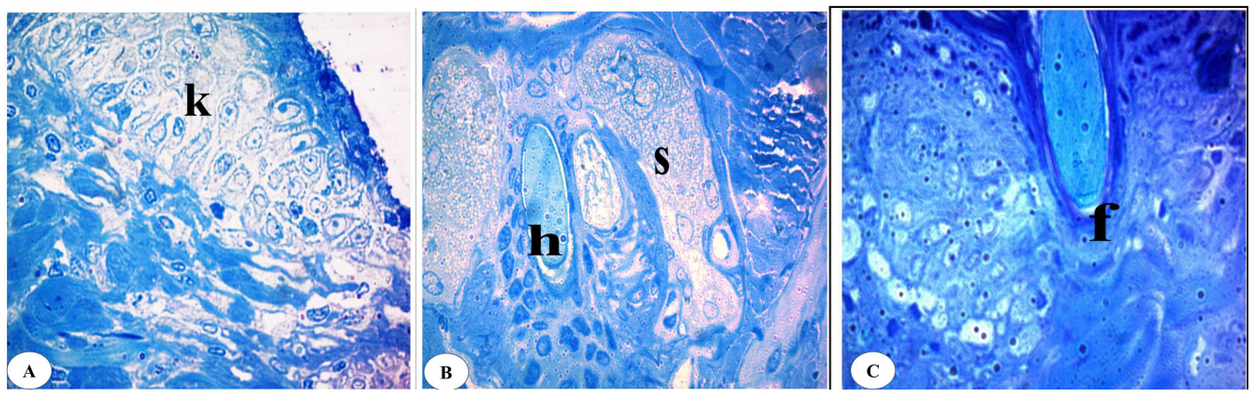

Fig. 7: Toluidine blue- stained semithin sections of skin of aged-CR rat (×1,000) show: **(A-C)** Nearly normal epidermal and dermal structures apart from mild swollen sebaceous glands (s). K: keratinocytes (k); h; hair shaft ; f: hair follicle

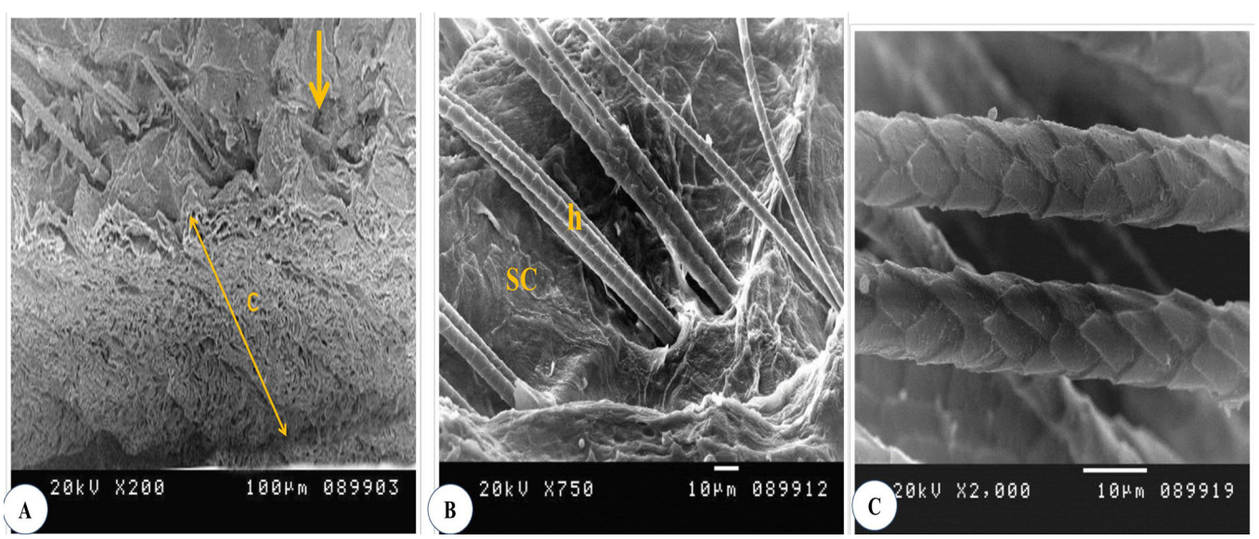

Fig. 8: Scanning electron microscope (SEM) images of adult control rat skin show: **(A)** Thick Skin sheet with shallow furrows (arrow) in its surface. The dermal layer has regular compact collagen layers (c). **(B)** The surface squamous cells are covered with homogenous stratum corneum (sc). The hairs (h) are straight and bushy. **(C)** The hair shaft with normal cornified leaflet sheet arrangement.

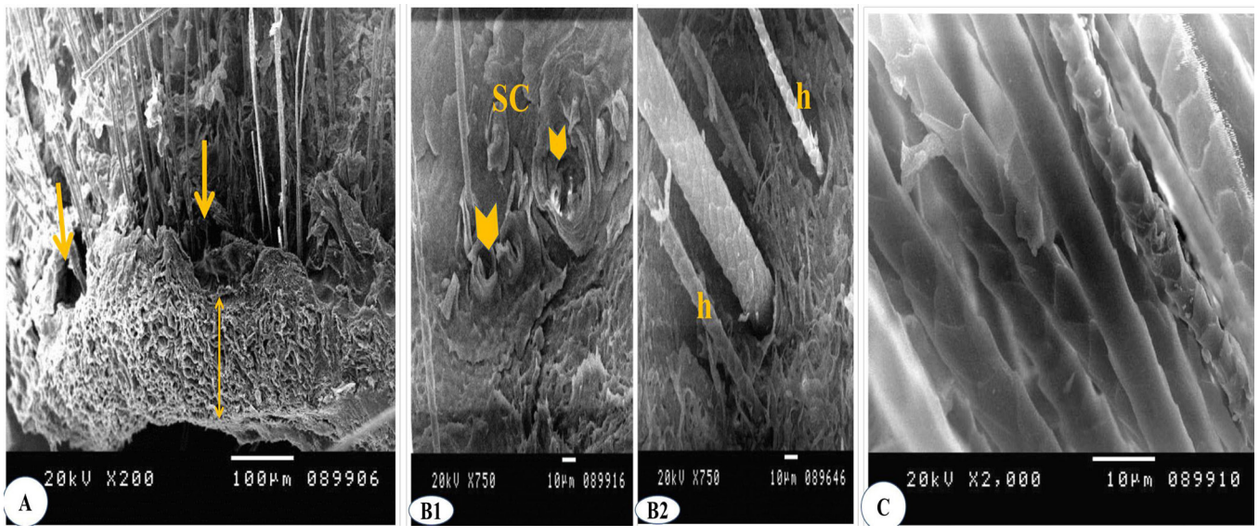

Fig. 9: SEM images of aged rat skin show: **(A)** Thin skin sheet with a corrugated surface, deep furrows (arrows) and less compact collagen layers. **(B1-2)** The stratum corneum (sc) is disturbed with many clumped scaly disorders with avulsed hairs (arrow heads). Some hairs (h) are week, broken and different in size (mostly thin). **(C)** The hair shaft leaflet sheets are either lost or less organized.

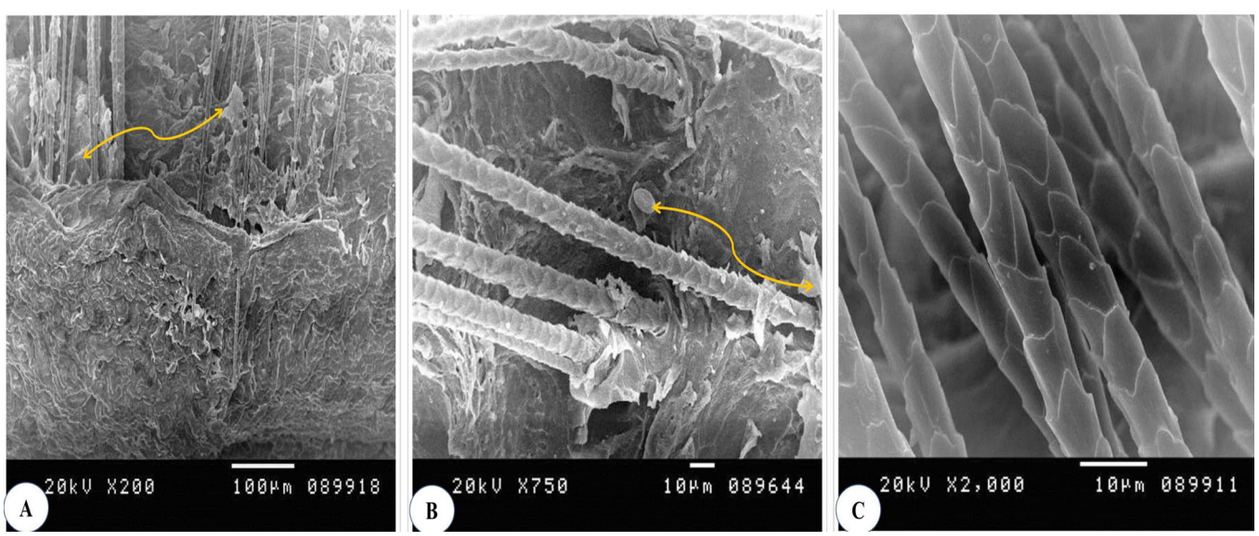

Fig. 10: SEM images of aged –CR rat skin show **(A-B)** Preserved skin sheet structures except for some scaly disorders (double curved arrow) in the stratum corneum. Note. straight hair. **(C)**The hair shaft appears similar to the control with a delicate well organized hair leaflet sheet.

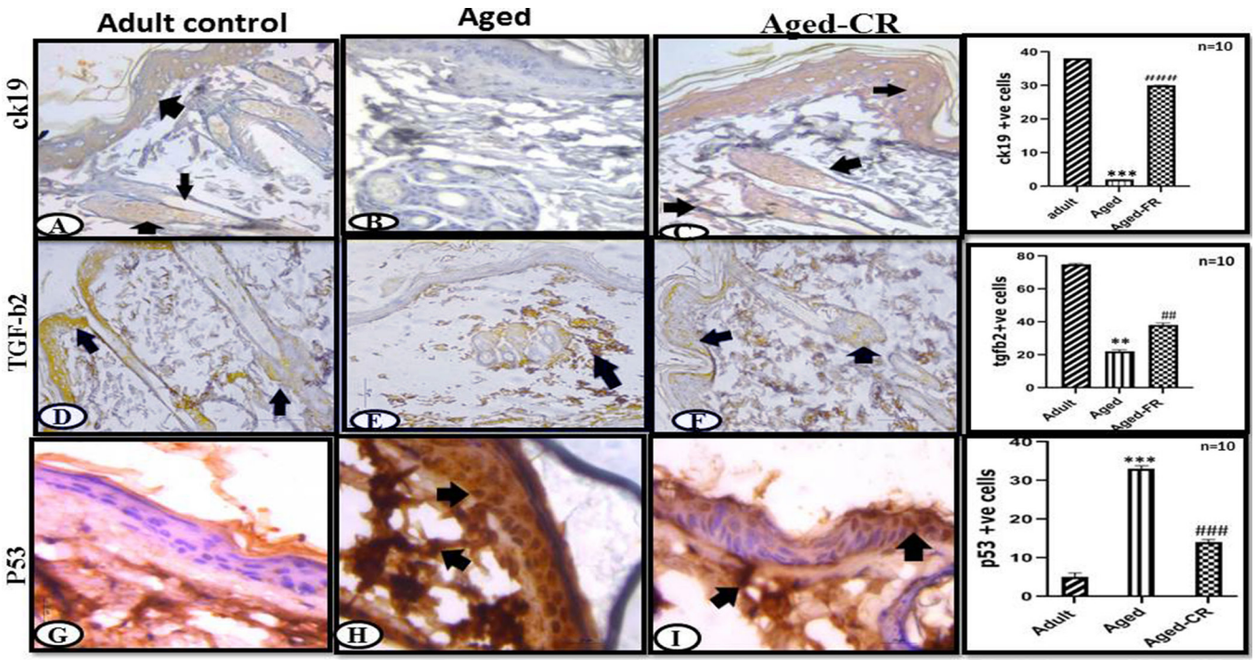

Fig. 11: Representative ck19, TGF- B2, p53 immunostaining in rat skin of different groups. The immunoreactivity is denoted by short arrows. Right bars: **P*< 0. 01 , ***P*< 0.001,****P*< 0.0001 compared with control; #*P*< 0. 01, ##*p* <0.001, ### *p* <0.0001 compared with Aged group. (ck19& p53x1000; TGF-B2x400)

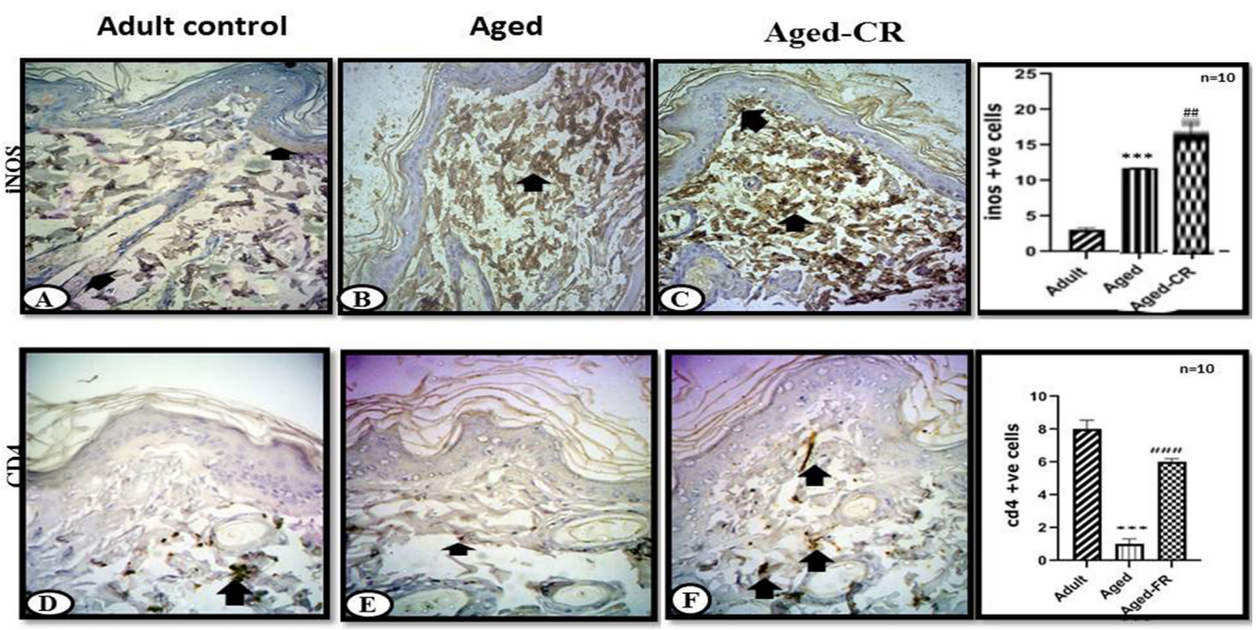

Fig. 12: Representative iNOS, CD4 (x1000) immunostaining in rat skin of different groups. The immunoreactivity is denoted by short arrows. Right bars: **P*< 0. 01 , ***P*< 0.001,****P*< 0.0001 compared with control; #*P*< 0. 01, ## *p* <0.001, ### *p* <0.0001 compared with Aged group.

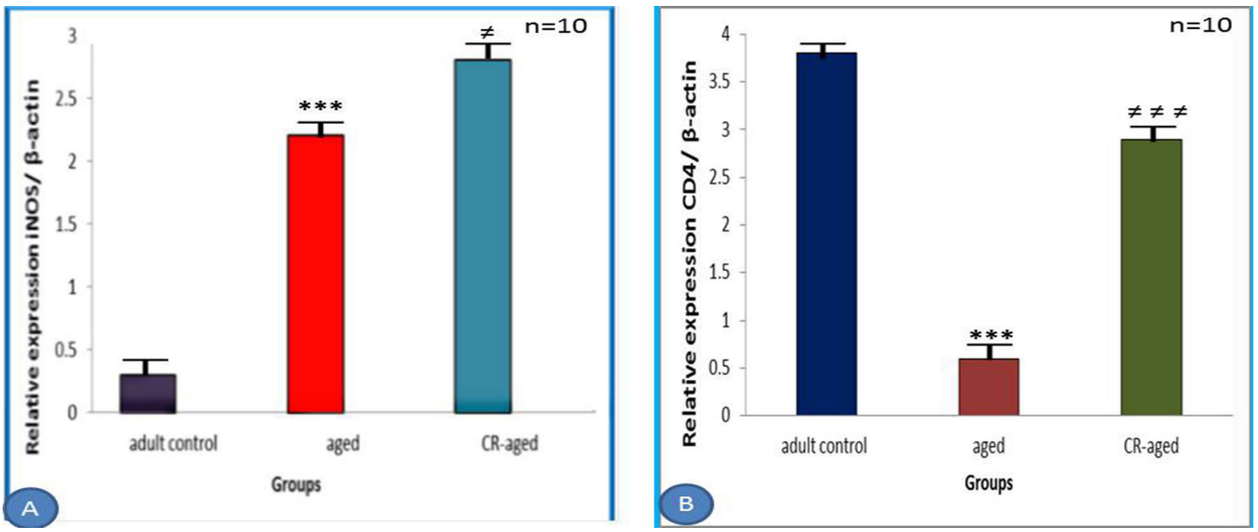

Fig. 13: Quantitative real-time PCR assays from experimental rat for the following skin mRNA transcripts: (A) Inducible nitric oxide synthase (iNOS) and (B) cluster of differentiation (CD4). The results are shown as mean ±EM and expressed after β actin normalization. **p*<0.01, **p*<0.001 ****p*<0.0001, compared with adult control group; ≠*p*<0.01, ≠ ≠*p*<0.001 ≠ ≠ ≠*p*>0.0001, compared with aged group.

Table 1: Body weight, skin measurements and biochemical studies in different experimental groups

Groups / parameter (mean +SD)		Aged-CR	Aged	Adult control
Body Weight (g)		250± 10.5	411.0 ± 8.3***	312.0 ± 2.5≠≠≠
Epidermal Thickness (um)		137.10 ± 0.379	38.56 ± 02***	99.86 ± 0.121≠≠≠
dermal Thickness (um)		1259.3 ± 100.4	708.03 ± 62.03***	902 ± 87.00≠≠≠
Area % of collagen fibers		49.42± 1.020	24.27 ± 4.902***	39.5.37 ± 6.153≠≠≠
Area % of elastic fibers		41.30 ±2.101	28.03 ± 3.411***	36.53 ± 6.384≠≠
Oxidative stress factors (µM/g tissue)	MDA	9.76±3.79	44.90±7.40***	15.82±2.82≠≠≠
	CAT	135.7+2.53	74.64+9.82***	105.31 +5.56≠≠≠
Cytokines pg/mg tissue))	TNF-α	23. 98±1.27	96.9±1.1***	58.05±0.2≠≠≠
	IL-6	25.46±1.54	109±1.2***	37.02±1.8≠≠≠

Footnote: MDA= Malondialdehyde; CAT = catalyze. **p*<0.01, **p*<0.001 ****p*>0.0001, compared with adult control group; ≠*p*<0.01, ≠ ≠*p*<0.001 ≠ ≠ ≠*p*>0.0001, compared with aged group.

DISCUSSION

Immune senescence is naturally associated with the ageing process. Caloric restriction increases healthy life via many mechanisms. However, its role in immune savings during the ageing process is still unclear[27]. Ageing provokes morphological and functional alterations in the skin through intrinsic and extrinsic factors[28]. Oxidative stress and inflammation are recognized as driving forces where they play roles in skin ageing and ageing-related skin disease[29,30]. Recently, oxidative/inflammatory stress related or not related to the ageing process has led to significant declines in both innate and adaptive immunity (immunosenescence)[31,32]. The skin actively participates in immune responses, as it is considered an external barrier to environmental hazards, and it has an internal immune response. In this work, we raise the question of whether CR can induce improvements in cutaneous innate and adaptive immunity in aged rats through elevations of both inflammatory and oxidative stress and follow its pattern through structural changes. Similar to other studies[33-35], we proved that CR could modify the significant effects of age on skin components through significant downregulation of catabolic stress factors, i.e., oxidative markers (MDA and CAT), the inflammatory cytokines IL-6 and TNF-α, and apoptosis markers (P53); and upregulation of anabolic proliferative factors, including ck19 and TGF-β2. These factors were associated with marked protection of skin structural attrition of the epidermis, dermis layers, hair shaft and follicle, collagen and elastic fibres. All of these changes were accompanied by marked upregulations of CD4 and iNOS immunostaining and gene expression.

Vitlic *et al.*[35] studied the stress-immune relationship in aged persons and suggested that stress aborted cortisol production, but chronic elevations can lead to immune system resistance and increased production of inflammatory cytokines that further compromise the immune response. In CR rats, the downregulations of the inflammatory cytokines IL6 and TNF-α and the oxidative stress factors MDA and CAT are in accordance with other studies that proved that long-term moderate CR without

malnutrition induces significant inhibitions of oxidative[16] and inflammatory stress without a significant adverse effect on key in *vivo* cell-mediated immunity[15].

Suárez-Souto *et al*.[36] proved that CR increased the gene expression of most cytokines, both pro- and anti-inflammatory, and hence improved the expression of innate and adaptive immunity mediators in organs. In this work, the marked upregulation of both the iNOS (proinflammatory mediator NO) and CD4 genes and immune expression in the aged-CR group suggested the role of CR in the protection of both innate[37] and adaptive immunity[38]. The positive iNOS immunoreactivity in the aged-CR group should be due to the presence of NO in epidermal macrophages[37].

In the aged-CR group in the current study, our suggestion that the increase in adaptive immunity could be related to the marked upregulation of CD4 gene and immune expression contradicts White *et al*.[2]. They studied the effect of caloric restriction on lymphoid cell populations in two species of mice and correlated delayed lymphocyte maturation only with extended lifespan. In addition, they correlated the reduced functional capacity of T cells to the effect of caloric restriction on the preservation of differentiated CD8+ T cells at the expense of CD4+ T cells. This discrepancy could be due to the different animal species used (mice vs rat); moreover, the ratio of CD4 to CD8 in rats will be considered in future studies. The increase in CD4 could be due to gut microbiota stimulation by CR-induced basal metabolic index loss [38]. This association between and hence prevention of immunosenescence in CD4+ T cells, gut microbiota and immune activation were observed by Lu *et al*.[39] in HIV-infected subjects. We also explained the increase in CD4+ T cells due to the regulatory effect of CR on thymic ageing, as it reduced pro-adipogenic signalling in the thymus and promoted thymopoiesis during ageing[40].

We can correlate the protective effect of CR to both innate and adaptive immunity to the upregulation of the cytokine transforming growth factor-beta 2 (TGF-β2) by a CR diet. TGF-β2 is considered a potent chemoattractant for innate immune cells, neutrophils, monocytes, macrophages, endothelial cells and fibroblasts[41]. Additionally, it attracts adaptive immune cells and Th17 cells and induces their activation and differentiation[42] by delaying naïve T cell activation and differentiation via the inhibition of IL-2 production[43].

As reported by Bhattacharyya *et al*.[34], this work revealed that aged-CR rats showed preservation of mechanical immunity and skin function as an external barrier, as the CR rescued the thickness of the dermis and hypodermis and restored hair follicle growth. This was in contrast to our other data, which showed thinner skin and thin, weak, sparse degenerated hair in aged rats. We can attribute the protective process to the roles of TGF-β2 in hair folliculogenesis and cornification[44]. Furthermore, adult stem cells in tissues are essential for organ homeostasis and repair, and alteration of stem cells has been implicated in skin ageing[45]. We also suggested that CR prevented age-related atrophic changes through increases in interfollicular epidermal (IFE) stem cells (basal layer of the epidermis), hair follicle stem cells (HFSCs) and MSCs (in the bulge), as we detected a significant increase in CK19 expression, a marker of stem cell proliferation and differentiation in both epidermal cells and the hair follicle outer sheath of aged-CR rats[46-48]. The anti-ageing capability of CR is related to its ability to reprogram stemness and enhance the regenerative capacity of stem cells. Schultz and Sinclair[49] revealed that CR improved the functioning of various stem cell populations, including haematopoietic and intestinal stem cells in mice and germline stem cells in flies. Moreover, the anti-ageing capability of CR could be due to the prevention of keratinocyte apoptosis induced in the aged skin group, which was detected histologically through the preservation of basal keratocytes and hair follicle cells associated with the significant downregulation of p53 expression in CR rats, as the apoptotic marker p53 was considered by Gritsenko *et al*.[50] to be a marker for skin cell anti-ageing. Furthermore, the detected preservation of the dermo-epidermal junction (indicated by the presence of rete ridges and dermal papillae) in CR rats, in contrast to the significant flattening of the former in aged rat skin, should be responsible for preserving epidermal resistance to shearing stress and preventing its breakage, which is detected as deep furrows in scanning images of the aged group[51]. We can postulate that this preservation is due to an increase in fibroblasts in the dermis of CR rats anchoring to the basement by their fibrils, aided by the anchoring of intact protected basal keratinocytes to the epidermal side of the basement membrane[52]. Moreover, iNOS may prevent skin furrows by reducing internal leukocyte infiltration[53], which was confirmed by downregulation of inflammatory cytokines.

An impressive integration between the apoptotic/stem cell reciprocal cycles in aged skin was discussed by Matsumura *et al*.[54]. They described that the age-related decline in the renewal capacity of the hair cycle fully involves hair follicle stem cell (HFSC) senescence. DNA damage accumulates in HFSCs during repetitive hair cycling, which leads to COL17A1 proteolysis, an important component of the follicle stem cell niche. Deficiency in COL17A1 results in HFSC loss of stemless and differentiation into an epidermal lineage. This could explain the association of a marked decrease in p53 and increases in both CK19 and TGF-β2 in the CR-aged rat in association with the detection of straight and densely growing hair with intact outer-organized cornified leaflet sheets, in contrast to the sparse, thinned, broken, deformed hairs with loss of cornified leaflet sheets of the hair shaft in aged rat.

In the aged-CR rats described in this work, the increase in fibroblasts should be responsible for the intact dermis, as detected histologically, which is responsible for the production of collagen and elastin fibers that

were significantly detected histochemically and appeared compact and well-organized by SEM[55]. As Rittie and Fisher[51] described, fibroblast cells constitute the majority of dermal cells that secrete extracellular matrix (ECM) proteins. They added that the ECM is formed mainly by collagen and partly by elastin fibers, proteoglycans, and hyaluronic acid, which provide strength, support, and flexibility. Salzer *et al*.[41] detected senescent fibroblasts in the upper dermis of an aged group that gradually acquired the characteristics of the lower dermis, with reduced expression of ECM proteins and increased adipogenic traits. The explained decrease in ECM proteins could be related to their breakdown by matrix metalloproteinases (MMPs, proteolytic enzymes)[56] and degradation by cathepsin K, a lysosomal protease that clears unwanted clumped old elastin[57].

Additionally, we detected intact sebaceous gland cells with profuse secretion in aged-CR rats compared with gland degeneration in aged rats. These factors added a protective immunogenic role to both the skin and hair. The oily and waxy sebum (formed of triglycerides, wax esters, cholesterol esters, squalene, and free fatty acids) secreted by preserved sebaceous glands in aged-CR rats should act as a waterproof barrier and lubricant that protects them from mechanical injury[58]. Likewise, Kabashima *et al*.[59] proved that sebum participates in the immunity of mammals through the production of antimicrobial peptides, cytokines, and chemokines, such as interleukin (IL)-1β, IL-6, IL-8/CXCL-8, and tumour necrosis factors (TNFs).

Ultimately and unfortunately, due to economic issues, this work has some limitations, as, according to the nature of animal studies, it is unknown whether our findings will be accurate to human or other species or environmental conditions. Additionally, only selected CD4 and iNOS cell populations were examined without the detection of changes that would occur at different time points in the ageing process, especially for advanced ageing.

CONCLUSION

Our current study suggests that the initial interaction between the CR and both the host innate immune response through iNOS and the adaptive immune response through CD4 that is generated against skin senescence in rats should be further studied by performing other measurements of the immune response.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تقييد السعرات الحرارية يعزز مناعة الجلد الجرذ المسنين

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الخلفية: التأثير الإيجابي لتقييد السعرات الحرارية في اعتلال الجلد المرتبطة بالعمر هو موضوع متنازع عليه كما ان الآليات التي يؤثر بها على الاستجابة المناعية في الشيخوخة لا تزال غير مفهومة جيدًا. افترض هذا العمل أن تقييد السعرات الحرارية يمكن أن يمنع الاعتلالات الجلدية المرتبطة بالعمر من خلال تعزيز مناعة الجلد وتعبير الخلايا المتجددة مع تقليل تنظيم الضغوطات الالتهابية والأكسدة وموت الخلايا المبرمج.
الطرق: تم تقسيم ٤٥ من اناث االجرذان إلى ثلاث مجموعات: مجموعة التحكم البالغة، و مجموعة الجرذان المسنة، ومجموعة تقييد السعرات الحرارية المسنة. تعرضت مجموعة تقييد السعرات الحرارية المسنة لتقييد السعرات الحرارية من ١٣ إلى ١٩ شهرًا من العمر. جمعت عينات الجلد الظهرية لكل مجموعة وخضعت للفحص المجهري الضوئي والمسح الضوئي، والدراسات البيوكيميائية والجزيئية والكيميائية المناعية.
النتائج: على عكس التأثير المدمر للشيخوخة على جلد الفئران، أدى تقييد السعرات الحرارية إلى زيادة تنظيم كل من جينات الأكسيد (iNOS) و CD4 وتعبيرات الخلايا المناعية، p53، سيتوكراتين CK 19، وتحويل عامل النمو بيتا 2 (2- (TGF) مستويات التعبير المناعي. بالإضافة إلى ذلك، لاحظنا تحسن عوامل الإجهاد الالتهابي والتأكسدي مع الحفاظ على بنية الجلد والشعر والكولاجين والنسبة المئوية لمنطقة للألياف المرنة.
الخلاصة: تشير هذه النتائج الأولية بقوة إلى أنه في جلد الفئران المسنة، يمكن أن يحمي تقييد السعرات الحرارية من شيخوخة الجلد من خلال الارتفاعات في كل من المناعة الفطرية والتكيفية. تمت هذه التأثيرات من خلال حظر التأثيرات التقويضية لضغوط الاستماتة / المؤكسدة والالتهابية وتعزيز التأثيرات الابتنائية للخلايا الجذعية والمناعة والتى سيتم استكشافها في مزيد من الدراسات.