Histomorphometrical and Histopathological Assessment of in Ovo Methionine Injection on the Skin Layers and Its Collagen Bundles of Chicken Embryo

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ABSTRACT

Introduction: Skin resistance is greatly dependent on its collagen amount and nutritional substances such as crude protein that affect the skin collagen can indirectly influence the skin resistance and increase its strength. In this regard, this study aimed to evaluate the effect of in ovo methionine injection on histomorphometry of the skin layers.

Methods: Thirty fertilized eggs of Ross 308 broiler chicks were randomly divided into 3 equal groups of 10 eggs each. On day 4 of incubation, the first and the second group (treatment groups) were injected by 40 mg and 50 mg of methionine (Met) dissolved in 0.5 mL of phosphate buffered saline into the yolk sac, respectively. The third (control) group was only received 0.5 mL of phosphate buffered saline. On the day 18 of incubation, the eggs were removed from the incubator and the embryos were killed humanely. Then, the samples were taken from the skin of thoracic region of each embryo for histomorphometric study under the light microscope.

Results: The results indicated no significant increase in the epidermal thickness in Met (treatment) groups except for Met 40 compared to controls and Met 50 ($P<0.05$). Dermal thickness in the Met groups showed significant increase in Met 50 group compared to the other groups ($P<0.05$). Collagen bundles thickness of both dermis layers decreased in Met groups.

Conclusion: Obtained results revealed that methionine may stimulate immature collagen synthesis.

Key Words:
Skin, Collagen bundle, Methionine, Chicken embryo

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1. Introduction

Skin resistance is greatly dependent on the amount of skin collagen. So any nutritional substance that affects the skin collagen may indirectly influence the skin resistance [1].

In human, collagen comprises one-third of the total protein, accounts for three-quarters of the dry weight of skin, and is the most prevalent component of the extracellular matrix [2]. Collagen serves as a critical structural foundation for soft tissues, related organ systems, and bone, also it accounts for a considerable range of mechanical and biological properties. Thus, collagen-based materials have been candidates for tissue repair and replacement for decades. The collagen family is recognized by its triple helical macromolecular structure and structural roles in the extracellular matrix [3, 4]. Collagen architecture is one of the main determinants of the mechanical properties of tissues [5].

Methionine is an essential amino acid in all animal species. It is also recognized as the first limiting amino acid in poultry [6]. However, excess methionine causes not only growth retardation but also damages to various organs. Since collagen is the most abundant and structurally important protein in the body, its synthesis must be closely related to the growth and development. Furthermore, methionine is important in the formation of collagen [7]. Considering the importance of methionine, we aimed to investigate the in ovo injection of methionine on skin histomorphology and histopathology of chicken embryo as an animal model. Chicken embryo is a good animal model for research because of its availability and short embryonic period [8, 9].

2. Materials and Methods

All experiments were performed in compliance with the Iranian Veterinary Organization ethic rules. According to the law, no specific authorizations needed for work on avian embryos before their hatching. Our experiments were terminated on developmental day 18, i.e. 3 days before hatching, by chilling the eggs on ice for 20 minutes.

In this study, we used crystalline DL-methionine (99% purity, Evonik Degussa, Germany). Methionine solution was prepared by dissolving in phosphate buffered saline (PBS) at 30˚C in a water bath [10], then, injected solution was sterilized by using a syringe filter (0.22 µm). The solution was preserved at 4˚C for further use.

Thirty eggs (weighting 50±0.4 g) of Ross 308 broiler chicks were obtained from a local commercial hatchery from a maternal flock 41 wk in lay. The eggs were incubated (18-day incubation period) under optimal condition (37.7˚C and 60% relative humidity) in an incubator (Model PLC_DQSH, V:4; Belderchin Damavand, Iran). On day 4 of incubation, eggs were candled and infertile or early dead embryos were removed. Then, fertile eggs were randomly divided into 3 equal groups of 10 eggs. The large end of the eggs (injection site) was sterilized prior to incubation with 70% ethanol. At this time, the yolk was identified by candling and injected with 0.5 mL of in ovo feeding solution [11] using a 24G hypodermic needle (25 mm long). Two treatment groups each received 40 mg and 50 mg methionine in 0.5 mL PBS, and control group just received 0.5 mL PBS [12]. Needle punctures in the shell were immediately sealed with paraffin wax [11]. After that, eggs were returned to the incubator.

At the end of the experiment, on day 18 of incubation, the eggs were removed from the incubator and killed by placing on the ice. Then, the eggs were opened at the wider end [13]. The skin samples of approximately 1.5 cm^2 area were excised from the left of the thoracic region next to the latest rib near the mid line and stored in 10% buffered formalin for further histological processing. Following routine preparation of tissues, serial sections of paraffin embedded tissues of 5 µm thicknesses were cut using a microtome (Slee-Germany) and stained by Masson's trichrome (to assess the orientation and thickness of collagen bundles) and hematoxylin-eosin (H&E for histopathologic evaluation) methods and examined under the light microscope.

The study samples contained both epidermis and dermis layers. By Masson's trichrome staining, the average diameter of 10 collagen bundles in each of 10 fields were measured by using digital lens (Dino-Eye, AM7023, 5Mp, Taiwan) in the papillary and reticular layers. Also by H&E staining, the thickness of epidermis and dermis were measured with magnification of x400 and x100, respectively. Finally, the probable presence of inflammatory cells was evaluated in the dermis by this method.

Obtained data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 16, Chicago, USA). Differences between groups were compared by Tukey test following ANOVA, and a $P<0.05$ was considered as statistically significant. Results are reported as least squares means with standard errors.

Table 1. Histometric measurements (Mean±SE) of skin and its collagen bundles*.

	Epidermal thickness (µm)	Dermal thickness (µm)	Reticular bundles thickness (µm)	Papillary bundles thickness (µm)
Control	34.63±0.77[b]	808.35±44.73[a]	3.18±0.11[a]	3.41±0.1[a]
Met 40	69.75±4.99[a]	2048.39±95.14[b]	3.09±0.1[a]	3.42±0.16[a]
Met 50	48.09±1.97[b]	969.23±70.66[a]	2.66±0.15[b]	3.11±0.06[b]

* Means bearing different superscripts in a column differ significantly (P<0.05).

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3. Results

Histological and histometrical findings

The epidermis mainly consists of a stratified squamous keratinized epithelium. Administration of methionine to chicken embryo resulted in an insignificant increase in the epidermal thickness in Met 50 group compare to control group (P>0.05), while Met 40 group showed a significant increase compared to 2 other groups (P<0.05) (Table 1).

Dermal thickness in Met 40 group revealed significant increase compared to other groups (P<0.05). There was no significant difference between Met 40 group and control group (P>0.05) (Table 1).

Histometrical assessment of dermal collagen bundles thickness in the papillary layer showed no significant difference between the Met 40 group and control group (P>0.05). However, papillary collagen bundles in the control group were significantly thicker than Met 50 group (P<0.05) (Table 1).

Although the average collagen bundles thickness in reticular layer of Met 40 group was lower than that in the control group, it was not significant (P>0.05). On the other hand, obtained results displayed significant decrease in Met 50 group compared to the Met 40 and control groups (P<0.05) (Table 1).

Histomorphological findings

Dermis collagen bundles architecture was randomly organized in all control and treatment groups. Papillary collagen bundles in Met groups were organized sporadically compared to control group (Figure 1). Observations of reticular layer were similar to papillary layer.

Histopathological findings

Microscopic findings did not show any signs of inflammation in the tissues. On the contrary, the distribution of immune cells was similar in all groups (Figure 1).

4. Discussion

The skin is composed of the epidermis and dermis [14]. Increasing the epidermal thickness in Met groups is the first body defense barrier. It seems that sulfhydryl base of methionine can stimulate epidermis formation through reinforcing its matrix [14].

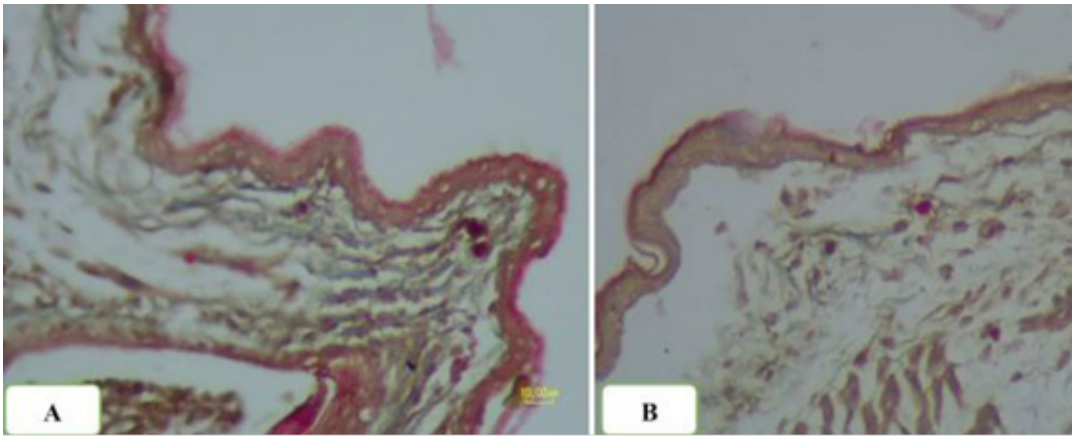

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Figure 1. Histomorphology and histopathology evaluation of dermis papillary layer. Collagen bundles architectures were organized randomly in both control (A) and methionine groups (B: Met 50). There is no sign of inflammatory cells in Met group compared to control (Masson's trichrome, ×400).

In load-bearing tissues, collagen is abundantly present and its mechanical properties depend on the collagen fiber architecture, e.g. collagen fiber orientation, content, and crosslinking [15, 16, 17]. Fibrillar structure is reinforced and its disassembly is prevented by the formation of covalent crosslinks between the collagen molecules [14, 18].

Mechanical properties of skin are highly dependent on collagen density and crosslinks between their fibrils [19]. The assessment of collagen bundles thickness and orientation is the most common method for the analysis of these bundles [20]. Histological examination by 1 or 2 observers using polarized light is the most common method to determine collagen orientation [21].

Different studies have described the thickness changes of collagen bundles in the face of various factors. Carneiro et al. (2007) performed a light and electron microscopic study on skin of mice, which were exposed to UVB radiation [22]. Also, morphometry of dermal collagen orientation has studied by Fourier analysis [21]. De Vries et al. (2000) described 2 methods, the laser scatter method and the fast Fourier transform, to measure skin collagen bundles orientation and spacing [23]. Ferdman and Yannas (1993) developed a light scattering technique to analyze the orientation and diameter of collagen fibers in histologic sections of connective tissue [24].

Considering the importance of healthy skin in birds, many studies have been done in this regard. Study results by Cahaner and Gutman (1993) revealed that chicks fed with diets containing relatively wide calorie to protein ratios had weaker skin than those fed with diets with narrower calorie to protein ratios. Regardless of diet, males had stronger skin than females. Although the magnitude of the differences varies with age, breast skin is stronger than thigh skin, with skin from the back stands in the middle [25].

Histomorphometric results in our study showed no significant decrease in reticular collagen bundles in Met 40 group compared to control ($P>0.05$). However, the difference between the control group and Met 50 regarding reticular collagen bundles was significant ($P<0.05$), i.e. methionine has negative effect on collagen bundle synthesis. The results in papillary layer were similar to those in reticular one (Table 1). This result is contrary to some studies that showed increase in dermal collagen fibril diameter, number, and function of fibroblasts as a result of UV radiation [22, 26, 27, 28]. On the other hand, Taniguchi et al. (1987) reported that collagen extractability in the neutral salt extract from the skin of newborn rat was greater in the excess methionine group than that in the control group. Collagen extractability in the neutral salt indicates a lesser degree of crosslink formation in pup's skin treated with excessive methionine.

So according to our findings, we hypothesize that methionine administration in chicken embryo decreases collagen bundles due to reduction in crosslinks. This hypothesis is more highlighted when histomorphology of dermis layers is noticed. According to Figure 1, densely packed bundles in reticular and papillary layers of dermis of the control group were more than those of treatment groups. Furthermore, there were more crosslinks between collagen bundles in the control group compared to the Met ones. On the other hand, dermal diameter of Met groups increased in comparison with the control group (Table 1) because of the effect of methionine on collagen production. It suggests that methionine has a positive effect on the fibroblasts number and or their activity but the produced procollagen cannot be converted to collagen because of not enough crosslinks. This finding is consistent with the results of some other studies [7]. We also propose that lack of an antioxidant such as vitamin C, because of its role in the hydroxylation of collagen fibrils [14], has led to formation of the immature collagen bundles that could not be seen by light microscope.

Kligman et al. (1989) and Carneiro et al. (2007) confirmed that decrease in collagen bundles diameter and return to control levels after stopping the UV-irradiation could be the result of enzymatic digestion by collagenase secreted via infiltrated inflammatory cells [22, 29]. This process cannot be the cause of decreasing the collagen bundles diameter in our study. According to histopathological findings, there was no significant change in the inflammatory cells in the skin of treatment groups compared to the controls (Figure 1), i.e. inflammatory cells did not participate in decreasing the collagen bundles diameter in Met groups. According to results by Taniguchi et al. (1987), water and hexosamine contents of the skin did not differ between excess methionine and control groups. Excess methionine possibly increases production of glycosaminoglycans and probably proteoglycans, as it is shown by the generally higher values of hexosamine contents. These researchers showed that the noncollagenous proteins and type III collagen in the excess methionine group was greater than those in the controls. Thus, increase in space between collagen bundles in methionine treatment groups does not associate with edema.

Skin extracellular matrix (ECM) is composed of some sulfated materials called glycosaminoglycans. Methionine has a sulfhydryl group, which can be used in the ECM production. So, this amino acid can reinforce the ECM [7] and improve wound healing too [21]. These

findings indicate that methionine decreases the collagen bundles diameter in Met groups due to reduction in the crosslink formation between collagen fibrils.

Connective tissue growth factor (CTGF) is a cysteine-rich peptide synthesized and secreted by fibroblastic cells after activation with transforming growth factor beta (TGF-β) that acts as a downstream mediator of TGF-β-induced fibroblast proliferation [30]. Therefore, injection of TGF-β into the subcutis of neonatal mice induces the formation of granulation tissue [31]. Duncan et al. (1999) conducted in vitro and in vivo studies to determine whether CTGF is essential for TGF-β-induced fibroblast collagen synthesis [32]. In vitro studies with normal rat kidney (NRK) fibroblasts demonstrated that CTGF potently induces collagen synthesis and transfection with an antisense CTGF gene blocked TGF-b stimulated collagen synthesis [33, 34]. CTGF and TGF-β are potent stimulators of collagen biosynthesis, ECM accumulation, and mesenchymal cell proliferation and activation [35, 36, 37]. Thus, it is important to be aware of any pathological effect on collagen metabolism that excess methionine may have on the skin.

The collagen is produced by 2 genes: COL1A1 and COL1A2 which are on the 17th and 7th chromosomes, respectively. Some transcription factors such as CBF, Sp1, and SMAD stimulate the collage gens expression [36]. We propose that methionine can increase the collagen synthesis due to increase in the collagen genes transcription factors and some related cytokines.

According to our results by light microscope examinations, there was no difference in collagen fibril orientation between the study groups. In other words, collagen bundles architecture appeared to be randomly organized (Figure 1). This finding was similar to some previous studies on human [21]. It shows that supplemented methionine does not affect collagen architecture of the skin.

Based on some previous studies [7], we suggest that decrease in crosslink formation of skin with excessive methionine is the cause of collagen bundle diameter reduction. This idea can be used to study how excess methionine affects the metabolism of the rat skin. It also indicates that decrease in the collagen bundles diameter in methionine administrated groups does not related to the collagenase enzyme.

Our study demonstrates that excess methionine intake results in the immaturity of skin. The results showed that methionine decreases collagens crosslink formation, while the higher content of collagen will produce when it accompanies with vitamin C [38] or some various nutritional factors such as vitamin B6 [7]. So excess methionine affects the content of immature collagen in the rat skin and causes a significant change in the nature of collagen.

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