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Original article

Modulation of angiogenesis by topical application of leptin and high and low molecular heparin using the Japanese quail chorioallantoic membrane model

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ABSTRACT

Pathological angiogenesis characterized by uncontrollable vessel growth is an accompanying feature of many diseases. The avian embryo chorioallantoic membrane (CAM) is an excellent model for angiogenesis research. In our study we used a less common Japanese quail CAM model for the testing of angiogenic potential of leptin, high-molecular (heparin sodium) andlow-molecular (nadroparin calcium) heparins. Heparins play a significant role in vascular endothelial cell function, and they are able to modulate the activities of angiogenic growth factors. On embryonic day 7 leptin (5 μg per CAM), heparin sodium (75 IU per CAM) and nadroparin calcium (47.5 IU per CAM) in 500 μl PBS were applied on the CAM surface. After 24 h the fractal dimension (Df) of the vasculature was evaluated. Samples from each group were histologically analyzed and *VEGF-A* and *Quek1* expression were detected by qPCR. Df was significantly increased in the leptin group. A moderate stimulatory effect of heparin sodium and an inhibitory effect of nadroparin calcium were observed. Both leptin and heparin sodium caused a noticeable increase in the CAM thickness compared to the control and nadroparin calcium groups. We observed an increased number of blood vessels and accumulation of fibroblasts. There was no significant impact on gene expression of *VEGF-A* and *Quek1* 24 h after treatment, however, trends similar to the changes in Df and CAM thickness were present. The resulting effect of nadroparin administration on *Quek1* levels was exactly the opposite to that of leptin ($p < 0.05$).

1. Introduction

Specific properties of avian chorioallantoic membrane (CAM) make it a suitable experimental model for various research areas, such as the study of normal and pathological angiogenesis. The advantages of this model are rich vascularization and simple experimental handling. Results obtained using the CAM model reliably correlate with the conclusions obtained in mammalian models (Parsons-Wingerter et al., 1998). Although the chicken CAM model is used more often, the Japanese quail CAM can be used with similar results, while using the advantage of shorter embryonic development, lower cost of breeding and easy manipulation.

Angiogenesis is a process in which new blood vessels form from an existing vasculature. When the balance between the factors affecting the activation and the inhibition of angiogenesis is disturbed, a deregulation occurs, resulting in the development of serious inflammatory or tumor diseases (Folkman, 1995).

The vascular endothelial growth factor (VEGF) plays a major role in the formation of new vessels. VEGFR-1 functions as a negative regulator of vasculogenesis and angiogenesis during early embryogenesis (Fong et al., 1999), while in adulthood it positively regulates inflammatory responses (Luttun et al., 2002). VEGFR-2 provides most of the cellular responses to VEGF, supports angiogenesis and stimulates the growth of vascular endothelial cells (Roskoski, 2007). Pathological angiogenesis is associated with an over-production of VEGF, as is in the case of cancer, arthritis, or asthma (Carmeliet, 2003).

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In Japanese quail, tyrosine kinase homologues of VEGFR2 and VEGFR3 receptors, referred to as Quek 1 and 2, were identified. Both of these receptors are expressed in endothelial cells of birds during early development (Eichmann et al., 1996).

Growth of fat tissue requires a sufficient blood supply and the creation of the new capillaries, therefore the adipose tissue is characterized by rich vascularization. The effect of leptin (tissue hormone produced by adipocytes) on angiogenesis has been investigated with contradictory results. Leptin proangiogenic activity stimulated endothelial cell growth and increased vascular permeability (Cao et al., 2001). It was found that leptin is involved in wound healing due to its pro-angiogenic activity, for example in the oral cavity in humans (Aydin et al., 2005).

Although leptin was found in many vertebrate species, the existence of avian leptin had been unclear for a long time (Sharp et al., 2008, Londraville et al. 2017). The genes coding short and long forms of leptin receptors are present also in birds (Dunn et al., 2000) and mammalian leptin is functional in avian organism (Adachi et al. 2008; Yosefi et al., 2010). Dose dependent proangiogenic effect of murine recombinant leptin applied *in ovo* and *ex ovo* was confirmed using Japanese quail CAM experimental model (Vyboh et al., 2010). Another study on the influence of leptin on angiogenesis and the involvement of the STAT-3 pathway stated the opposite – an inhibitory effect of leptin (Su et al., 2012).

Heparins play a role in vascular endothelial cell function and they are able to modulate the activities of angiogenic growth factors by facilitating their interaction with receptors and promoting their activation (Norrby, 2006). Direct effect of heparins on angiogenesis is not fully elucidated and may be antagonistic. In most cases the effect of low-molecular-weight heparin (LMWH) on angiogenesis is inhibitory. Presumably, heparins are able to interact with various angiogenic factors depending on the sulfate modifications in their glycosaminoglycan chains and molecular weight (Rema et al., 2012).

Using human umbilical vein endothelial cells and the chicken CAM model, the anti-angiogenic properties of LMWH tinzaparin were investigated, and were dose and relative molecular weight dependent (Mousa and Mohamed, 2004). The opposite, a proangiogenic effect of LMWH dalteparin sodium, was later demonstrated by Norrby and Nordenhem (2010). Dalteparin stimulated the VEGF-mediated angiogenesis in vivo, however, in combination with epirubicin (chemotherapeuticum) it produced an inhibitory effect on angiogenic processes.

In the study of Dogan et al. (2011), the antiangiogenic effect of four commercially available LMWH (tinzaparin sodium, nadroparin calcium, enoxaparin sodium and bemiparin sodium) were studied on the chicken CAM model. All studied heparins showed a dose dependent antiangiogenic effect. On the other hand, high molecular weight heparin may exhibit proangiogenic response. Rema et al. (2012) revealed that the diffusion of 100 μM heparin (15 kDa) into the stroma region of chick CAM model had a direct impact on angiogenesis and could favour blood vessel sprouting.

Due to inconclusive results from other studies, further experiments of targeted and selective inhibition of angiogenesis are needed. Our aim was to analyze the effect of leptin and high and low molecular heparins on angiogenesis using Japanese quail CAM model. We used topical application, fractal dimension coefficient analysis (Df), the changes in CAM thickness and expression analysis of genes involved in this complicated process.

2. Materials and methods

2.1. Quail CAM in vivo model

Fertilized Japanese quail (*Coturnix japonica*) eggs (n = 200) were incubated in a forced draught incubator without egg rotation at 37 °C and humidity 50–60%. To prepare *ex ovo* culture, on embryonic day (ED) 3 the surface of the egg was disinfected with 70% ethanol, carefully opened under sterile conditions and the total volume of the egg was transferred into a six-well tissue culture plate (TPP, Switzerland). The embryos were kept in a humidified incubator (Binder, USA) at 37 °C and 90% humidity until ED7. Out of 170 transferred embryos, 135 survived and were used for treatment.

2.2. Experimental treatment

At ED7 the CAMs were treated with 5 μg murine recombinant leptin (PeproTech, UK), 75 IU of heparin sodium (Zentiva, Czech Republic) and 47 IU of nadroparin calcium (GlaxoSmithKline, UK). The drugs were dissolved in 500 μl/embryo of pre-warmed sterile phosphate buffered saline solution (PBS) and uniformly pipetted onto the surface of the individual membranes. The control group was treated with PBS only. The group size ranged from 30 to 37 embryos.

2.3. Fractal analysis

24 h after the treatment, the membranes (n = 78; 16–22/group) were fixed with a pre-warmed 4% paraformaldehyde and 2% glutaraldehyde dissolved in PBS. After 48 h, the fixation solution was removed, CAM was carefully separated from the embryo, washed in PBS, mounted on a slide and dried. Slides were photographed with digital camera (Canon EOS 5D with Canon MP-E 65 mm f/2.8 1-5x Macro lens, Canon, Japan) using Slimlite 5000 K Lightbox (Kaiser, Germany) as a source of homogenous white light.

Digital images were processed using ImageJ 1.51 software (Abramoff et al. 2004). For the fractal analysis a square region (512x512 pixels) from the area with distal arterial branches was selected. The images were binarized, skeletonized and the fractal dimension coefficient (Df) was calculated following the procedures described by Parsons-Wingerter et al. (2000).

2.4. Histological analysis

Six CAMs per group were fixed (4% paraformaldehyde), dehydrated in ethanol and soaked in paraffin (melting point 56 – 58 °C, Merck). After 24 h the samples were overlaid with paraplast Plus for tissue embedding (56 – 57 °C, Sigma) in casting molds and solidified in a refrigerator (for at least 2 h). Serial sections (4 μm) were cut on a microtome (Hyrax M40, Zeiss), stained with hematoxylin-eosin and photographed (Leica DM5500, 100x magnification). CAM thickness was measured at 6 different locations in 6 serial cross sections of the sample using ImageJ 1.51 software (Abramoff et al. 2004).

2.5. RNA isolation

Approximately 50 mg of liquid nitrogen-frozen CAM tissue (n = 33; 8–9/group) was homogenized using TRI-Reagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's protocol (Chomczynski and Sacchi, 1987). The tissues were briefly homogenized using UltraTurrax T18 (IKA-Labortechnik, Germany). Subsequently 200 μl of chloroform was added to each sample, mixed and phases were separated by centrifugation. Chloroform phase was discarded and the extracted RNA (aqueous phase) was precipitated with freeze-dried isopropanol, washed by 75% ethanol and dissolved in 50 μl of RNAse free water. RNA samples were treated with DNAse (Thermo Fisher Scientific) to prevent contamination with genomic DNA. The concentration and purity of RNA was measured spectrophotometri-

cally using a Multiscan Go (Thermo Fisher Scientific). Each sample was tested for RNA degradation on an agarose-formaldehyde gel.

2.6. Reverse transcription and qPCR analysis

cDNA was synthesized from 2 μg of total RNA in 40 μl volume. The reaction mixture contained 2 μg RNA, oligo $(dT)_{18}$ primer (100 μM, Thermo Fisher Scientific), random hexamer primer (100 μM, Thermo Fisher Scientific), RT buffer 5x (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ with 0.1 M DTT, Thermo Fisher Scientific), RiboLockRNase Inhibitor (40U/ μl, Thermo Fisher Scientific), DNTP Mix (10 mM, Thermo Fisher Scientific), RevertAid Reverse Transcriptase (200U/μl, Thermo Fisher Scientific). cDNA was diluted 10 times, and a 5 μl aliquot was used for qPCR analysis using FastStart DNA Master SYBR Green I (Roche). The reaction was done in 20 μl according to the manufacturer directions with an annealing temperature 55 °C. qPCR primers are listed in Table 1. Quek1, VEGFA, β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts were analyzed on the LightCycler® Nano (Roche). The final data were normalized to β-actin and GAPDH mRNA levels. Control group mRNA levels were set to 1.

2.7. Statistical analysis

The data were statistically evaluated using the one-way ANOVA followed by Fisher LSD post hoc test (fractal dimension) and the Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparison test (CAM thickness and relative expression levels of *VEGF-A* and *Quek1*). We report the results as the mean ± SEM. The analysis was performed using SigmaPlot 13 (Systat Software, San Jose, CA, USA).

3. Results

The local application of tested substances did not affect the survival of the embryos, whose mortality did not exceed 22% between ED5 and ED8.

The indicator of changes in the vasculature was fractal dimension (Df) coefficient. Df after leptin administration (Fig. 1) was significantly increased ($p < 0.001$) compared to the control. There was a trend towards an increase of Df values after the application of heparin sodium compared to the control ($p = 0.08$). The administration of nadroparin had the opposite effect than heparin ($p < 0.01$). We also observed a significant difference compared to the leptin group ($p < 0.001$).

After 24 h administration we observed a clear effect on the structure and thickness of the quail CAM. In the leptin and heparin groups we observed accumulation of fibroblasts in the mesenchyme (Fig. 2). Both groups showed a significantly greater thickness compared to the control and nadroparin group ($p < 0.001$) (Fig. 3), with the average thickness of 66.4 μm (control), 98.3 μm (leptin), 107.5 μm (heparin) and 72.6 μm (nadroparin). We saw an increased number of blood vessels in the leptin group. The administration of nadroparin calcium had no significant effect on the thickness and morphology of the CAM after 24 h of exposure (Fig. 3) when compared to the control. The effect, however, was opposite to that of heparin ($p < 0.001$).

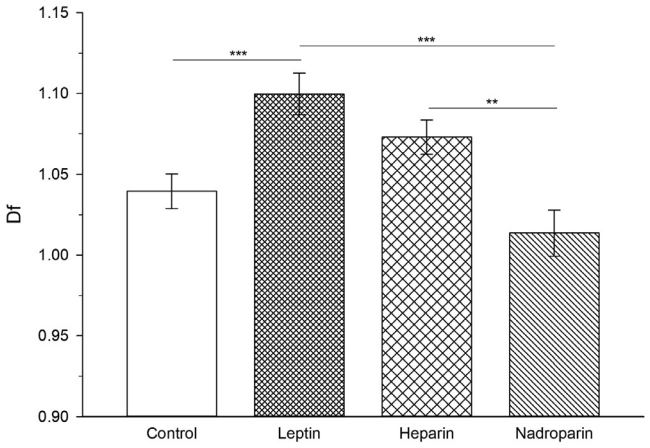

Fig. 1. Changes in fractal dimension (Df) after administration of leptin, heparin sodium and nadroparin calcium at ED7 on quail CAM. Mean values ± SEM, n = 16 – 22, $^{***}p < 0.001$; $^{**}p < 0.01$.

There were no significant changes in the expression of *VEGF-A* 24 h after treatment (Fig. 4). In *Quek1* expression (Fig. 5), we did not discover any statistically significant changes among the control and experimental groups, but we observed a similar pattern of results corresponding to our previous results (Df and CAM thickness), with the highest values for leptin and heparin. The administration of nadroparin had an opposite effect to that of leptin administration and caused a decrease of *Quek1* levels ($p < 0.05$).

Table 1
The primer sequences and amplified characteristics.

Gene	Primers (5′-3′)	Gene bank accession number	T_m (°C)
Quek1 - for	CATCAATGCGAATCATACAGTTAAG	X83288	60.9
Quek1 - rev	CATTCACAAGCAGGGTGAATG		59.4
VEGFA - for	CGGAAGCCCAATGAAGTTATC	P67965	59.4
VEGFA - rev	GCACATCTCATCAGAGGCACAC		64.0
β-actin - for	CACCACTGGTATTGTGATGGAC	AF199488	62.1
β-actin - rev	GTGGTGAAGCTGTAGCCTCTCT		64.0
GAPDH - for	GAACGCCATCACTATCTTCCAG	Z19086	62.1
GAPDH - rev	GGGCTGAGATGATAACACGC		60.5

4. Discussion

Our experiments confirmed a significant positive effect of leptin on angiogenesis in contrast to the variable effects of heparin molecules. The topical application of tested substances did not have a significant negative effect on embryo survival and coincided with general *ex ovo* survival, which corresponds to 80% after ED7 (Auerbach et al., 1974).

The calculation of the fractal dimension after VEGF administration was used by Kurz et al. (1994) who showed the suitability of this method for assessing vascular growth. In our experiment we analyzed the topical *ex ovo* application of recombinant murine leptin, heparin sodium and nadroparin calcium on the quail embryo CAM surface. The highest fractal dimension coefficient was observed in the leptin group, indicating a stimulatory effect for new vessel formation under given conditions.

In the past, the angiogenic effect of leptin was examined in various models with mutually conflicting results. In birds, leptin receptors responding to mammalian leptin were detected and mammalian leptin is still frequently used in avian model experiments (Henderson et al., 2018; Ferver et al., 2020). Leptin administration significantly promotes healing of the oral cavity in rabbits, accelerates the migration of epithelial cells and histological analysis confirmed more dense distribution of the vessels in the leptin group (Umeki et al., 2014). It was shown that *in ovo* administered mouse recombinant leptin significantly reduced the density and number of blood vessels in the CAM of the chicken embryos but only in females (Su et al., 2012). Leptin (0.5 μg) was injected into the albumen before incubation, and the results were analyzed dur-

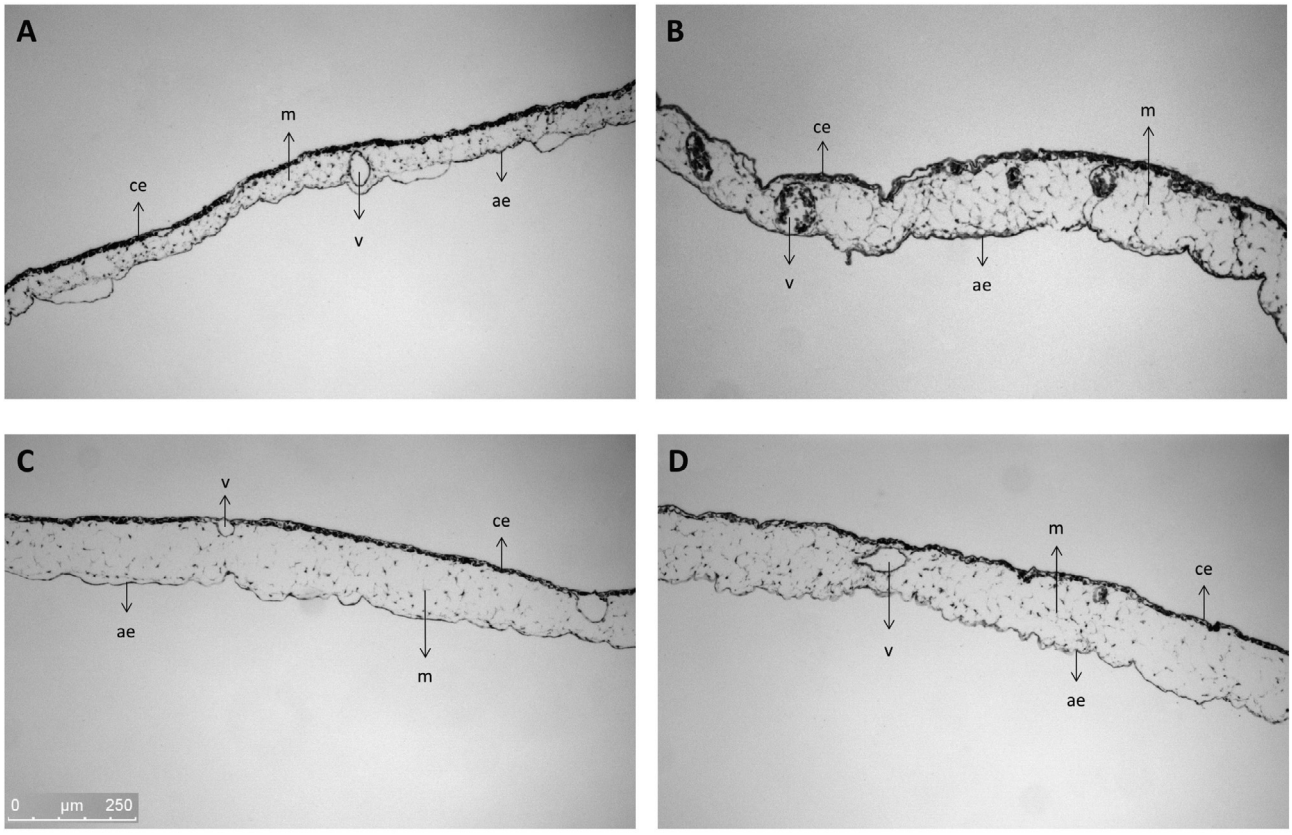

Fig. 2. Effect of leptin, nadroparin calcium and heparin sodium administered at ED7 on CAM morphology. **(A)** CAM treated with 500 µl PBS shows normal morphology with thin chorionic (ce) and allantoic (ae) epithelial layer. **(B)** CAM treated with 5 µg of murine recombinant leptin in 500 µl PBS shows increased thickness, more blood vessels (v) and accumulation of fibroblasts. **(C)** Group treated with 75 IU heparin sodium in 500 µl PBS shows fibroblast accumulation in mesenchyme (m). **(D)** Treatment with 47.5 IU nadroparin calcium slightly increased thickness of CAM. Representative tissues are 4 µm in thickness stained with hematoxylin-eosin and images were taken at 100x magnification. Bar is 250 µm.

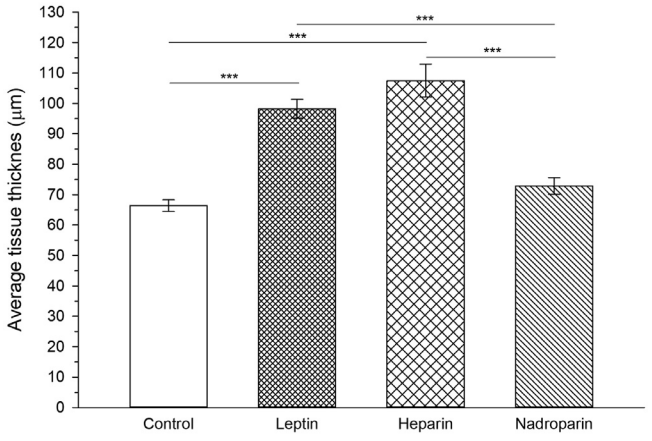

Fig. 3. Effect of leptin, heparin sodium and nadroparin calcium administered at ED7 on the CAM thickness. CAM paraffin sections (4 µm) were stained with hematoxylin-eosin, photographed (Leica DM5500, 100x magnification), and measured the CAM thickness at six different locations from 6 serial cross sections of the sample (ImageJ software). Mean values ± SEM, n = 6, $^{***}p < 0.001$.

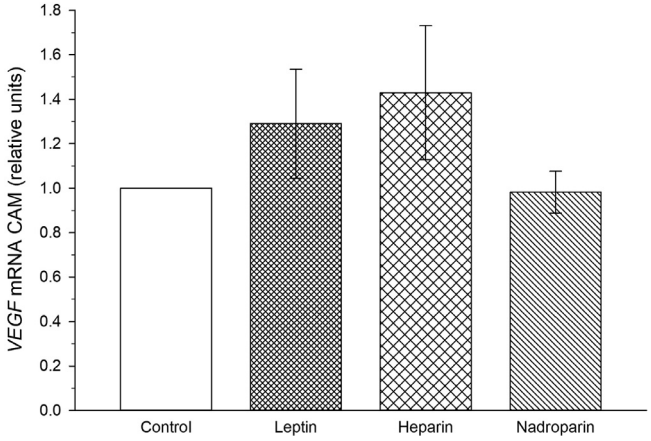

Fig. 4. Relative expression levels of *VEGF-A*. Mean values ± SEM, n = 7–9.

ing ED12. The angiogenic effect of applied substances on the CAM model is usually observed over a relatively short period of time, 24–48 h after application, as it was in our case (24 h). The angiogenic potential of *in ovo* applied human recombinant leptin (HRL) was most pronounced after 72 h and exhibited a dose-dependent effect, with the maximal response after the administration of 3 µg HRL (Manjunathan and Ragunathan, 2015). In our study, 5 µg of murine recombinant leptin had a significant stimulatory effect on vessel growth (expressed by Df). A similar effect, even after application of lower concentrations, was reported by Vyboh et al. (2010) who applied 10, 100 and 1000 ng of mouse recombinant leptin on the quail CAM.

Unfractionated heparin sodium stimulated the growth of blood vessels, whereas the administration of low molecular nadroparine caused its inhibition. This is in accordance with the findings indicating a higher survival rate (lower percentage of metastasis for-

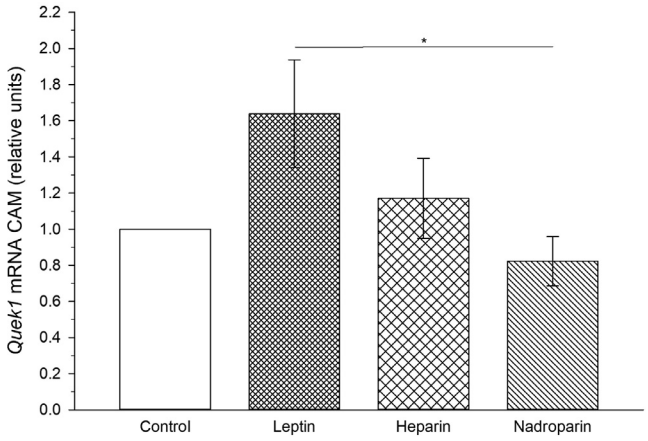

Fig. 5. Relative expression levels of *Quek1*. Mean values ± SEM, n = 8–9. $^{*}p < 0.05$.

mation) of oncological patients receiving chemotherapy together with low-molecular heparin (von Tempelhoff et al., 2000; Norrby, 2006). Nonetheless, the antiangiogenic effect of low-molecular heparins may vary depending on the size of the fragment, the process of its production, or the experimental model used (Norrby, 2006). Heparin derived from natural sources is frequently used to prevent thrombosis, however, other effects of unfractionated heparin have been described. Shen et al. (2011) found that heparin (no molecular weight has been reported) attenuates miR-10b expression and induces HoxD10 expression in vivo while inhibiting angiogenesis and impairing the growth of MDA-MB-231 tumor xenografts. Rema et al. (2012) compared three different doses of unfractionated heparin (50, 100, 150 μM) applied on the surface of chicken CAM by gelatin sponge. The maximal stimulatory effect was observed after 72 h using 100 μM concentration. Mousa and Mohamed (2004) described a dose dependent antiangiogenic effect of low-molecular heparin tinzaparin.

Histological analysis was another method we used to determine the influence of tested substances on CAM vasculature. In the leptin group we found a significant increase in membrane thickness, higher number of blood vessels, as well as accumulation of fibroblasts in the mesenchymal region. Increased numbers of fibroblasts, along with the presence of other cells such as macrophages and mast cells, are associated with an angiogenic tissue response (Ribatti et al., 2001). Their accumulation results in the irregular appearance of the membrane observed in the leptin and high molecular heparin groups. In the study of Manjunathan and Ragunathan (2015), leptin administration induced comparable morphological changes in the chicken CAM. Similarly, administration of 100 μM heparin (for 72 h) significantly increased the average tissue thickness of chicken CAM and the number of fibroblasts compared to the control and other used concentrations of heparin (Rema et al., 2012). The presence of fibroblasts in the stroma might favor endothelial cell migration, which is considered the most important step in angiogenesis. Histological analysis of undersulfated LMWH glycol-split heparins (ST2184) confirmed the inhibitory effect of angiogenic response triggered by VEGF on chicken CAM (Pisano et al., 2004). Contrastingly, heparin did not affect the angiogenic activity of VEGF. In our study LMWH nadroparin did not significantly affect the CAM structure and thickness.

To complement the study of morphological changes in CAM, we investigated the expression of two angiogenic genes (*VEGF-A* and *Quek1*) after the administration of leptin and two different types of heparin using qRT-PCR. We assumed that the expression of a key angiogenic factor – *VEGF-A*, will be affected by the administered agents. Contrarily, we did not find any significant changes. Su et al. (2012) measured *VEGF* mRNA expression 12 days after *in ovo* administration of leptin. Surprisingly, leptin reduced the expression of this gene, but only in female embryos. In their study on chicken CAM, Manjunathan and Ragunathan, (2015) explored the effect of human recombinant leptin treatment on the expression of two VEGF isoforms (VEGF121, VEGF165). The expression of both isoforms was significantly increased 72 h after administration of 3 μg HRL. We used tissues that were exposed to the substances for 24 h time period, which might not have been long enough for the changes in the expression to take effect. The expression of the VEGFR2 quail homologue, referred to as Quek1, was affected, however, these changes were not statistically significant. The only significant difference found was between the leptin and nadroparin groups. These differences, however, corresponded to results of fractal dimension calculation.

A long-term monitoring of gene expression would probably provide more information. Another possibility to consider is the fact that studied substances might have primarily influenced the expression of other factors involved in angiogenesis, which could be verified by extending the pool of analyzed genes.

5. Conclusion

Our experimental results documented a modulatory effect of leptin, high and low molecular heparin on the angiogenesis in the Japanese quail chorioallantoic membrane model. The leptin group showed a significant increase in Df compared to the control group. Furthermore, we also observed a stimulatory effect of heparin sodium and an inhibitory effect of nadroparin calcium. Further investigation of VEGF-A and Quek1 expression are needed, in order to examine the long-term effect of leptin and heparin administration. The modulatory effect of these tested substances might be used in the area of cancer therapy and wound healing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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