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Cytological Alterations of Aortic Adventitial Fibroblasts *in vitro* Under the Action of Methionine in Sand Rats, *Psammomys obesus*

Alteraciones Citológicas de Fibroblastos Adventicios Aórticos *in vitro* Bajo la Acción de la Metionina en Ratas de Arena, *Psammomys obesus*

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SUMMARY: Methionine is an essential amino acid involved in critical metabolic process, and regulation of methionine flux through metabolism is important to supply this amino acid for cell needs. Elevation in plasma methionine commonly occurs due to mutations in methionine-metabolizing enzymes, such as methionine adenosyltransferase. This elevation could cause a hyperhomocysteinemia (HHcy), an independent risk factor of cardiovascular diseases, including atherosclerosis. Several lines of evidences suggest that HHcy due to methionine supplementation can affect the matrix production by involving cell types, such as vascular smooth muscle cells and fibroblasts. To this end, aortic adventitial fibroblasts primary cultures were exposed to methionine. Methionine at a concentration of 20 mM produced definite effects on the in vitro grown fibroblasts after 72 hours. The most evident were retraction of cell processes, vacuolation and fragmentation of the cytoplasm, production of irregular contour of the nucleus and nucleolus, decrease of cytoplasmic basophilia and the presence of pycnosis.

KEY WORDS: Aortic adventitial fibroblasts; In vitro; Methionine; Morphology.

INTRODUCTION

Cardiovascular disease remains the largest cause of death in the world (Lüscher, 2016). Atherosclerotic plaque stability is now recognized as a major player in patient morbidity and mortality; however, there currently is no accurate model to study atherosclerotic plaque stabilization (Zulli & Hare, 2009). An unstable plaque (thin fibrous cap, high macrophage low smooth muscle cell content) can rupture and cause thrombosis and also platelet thrombosis, and this event usually occurs on the shoulders of the plaques (Wang *et al*., 2022).

Methionine is an essential amino acid, important for the synthesis of cysteine and S-adenosyl methionine (SAM) which is a methyl group donor for methylation reactions. SAM donates its methyl group to a methyl acceptor, thereby forming a methylated product and S-adenosylhomocysteine (SAH). SAH is then converted into homocysteine (Zaric *et al*., 2019). Hyperhomocysteinemia (HHcy) is regarded as an important cardiovascular risk factor (Guieu *et al*., 2022). One of the hypotheses explaining the association between HHcy and atherosclerosis is that HHcy may be a marker for altered methylation of cellular substrates that utilize SAM as a methyl donor (Dubey *et al*., 2022). Therefore, it has been suggested that the association between HHcy and cardiovascular disease may be explained by high methionine diet (Chaturvedi *et al*., 2016; Guieu *et al*. 2022). Indeed, decades of investigation in human patients have established a close correlation between Hhcy and cardiovascular diseases and subsequent complications such as heart attacks and strokes have been described previously (Baszczuk & Kopczyn´ski, 2014). Moreover, a diet rich in methionine has been shown to induce cardiovascular system damage through oxidative stress, inflammation, and extracellular matrix remodeling (Chaturvedi *et al*., 2016).

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Extensive experimental data have indicated that hyperhomocysteinemia may result from homocysteine or methionine supplementation (Rahimi *et al*., 2021). Furthermore, it has been shown that a diet rich in methionine in animal models induces on the one hand a hyperhomocysteinemic state (Hidiroglou *et al*., 2004; Rahimi *et al*., 2021) and on the other hand, damage to the cardiovascular system through oxidative stress, inflammation and remodeling of the extracellular matrix (Chaturvedi *et al*., 2016; Rahimi *et al*., 2021).

In a previous study, we showed that a daily intraperitoneal injection of 70 mg/kg of methionine for 6 months can cause in sand rats (*Psammomys obesus*), a hyperhomocysteinemia accompanied by vascular alterations, especially marked by a reorientation and proliferation of smooth muscle cells (SMCs), an accumulation of connective tissue at both media and adventitia layers, associated with the formation of aneurysms (Zerrouk *et al*., 2022).

Additionally, This result was confirmed *in vitro* after SMCs cultures was treated with methionine, showing the switch from a quiescent contractile phenotype to a synthetic proliferative phenotype, essentially characterized by an increase in the cells size. In addition, the nuclear size and the number of nucleoli increased suggesting an increase in the transcription activity of the SMCs treated with methionine which is confirmed by the increase in the extracellular compartment (ECC) total protein levels (Zerrouk *et al*., 2022), which could explain the increase of connective tissue, especially collagens.

In this study, we examined the effects of methionine supplementation in culture on aortic adventitial fibroblasts, responsible for the synthesis of adventitial connective material.

MATERIAL AND METHOD

Culture of aortic adventitial fibroblasts. The aortic adventitial fibroblasts of sand rats, *Psammomys obesus*, were cultured by the explant technique (Bouguerra *et al*., 2001). The control aorta was removed and immediately plunged in a Petri dish containing Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal calf serum (FCS) supplemented with 1 % antibiotics (streptomycin 50 mg/mL, penicillin 50 IU/mL, Sigma), 1.2 % glutamine (Sigma) and 5 % HEPES to maintain pH of 7.4. The aortic lumen was then emptied of blood. The aorta was incubated for 20 min at 37 °C in 0.1 % collagenase I (Sigma) in a serum-free medium to remove the endothelium and facilitate the separation of media and adventitia and cut into 1 mm explants. 8–10 explants were placed in culture 25 cm^3 flasks and incubated in the presence of DMEM containing 20 % FCS, 1.2 % glutamine, and 1 % antibiotics and placed in the incubator at 37°C under a humidified atmosphere with 95 % air and 5 % CO2. The cultivation of explants is the primary culture.

On the second culture passage and at the 14^{th} passage and at a confluence, the cells were resuspended after trypsinization. They have seeded in 6-well plates at a rate of 0.8×10^6 cells/mL/well in DMEM supplemented with 10 % FCS, 1.2 % glutamine, and 1 % antibiotics and incubated in the presence of 20 mM (3 mg/mL) DL-methionine for 72 h (Benavides *et al*., 2007). At confluence, the medium was removed; the control fibroblasts and fibroblasts cultured with methionine were reincubated in 1.5 mL DMEM without FCS for 24 h. The mediums of the extracellular and intracellular compartments were used for the determination of total protein (Bradford, 1976).

Quantification of aortic adventitial fibroblasts proliferation rate. The aortic adventitial fibroblasts were seeded in 6-well plates at a rate of 0.8×10^6 cells/mL/well in DMEM supplemented with 10 % FCS, 1.2 % glutamine, 1 % antibiotics. Six fibroblasts plated well were treated with 20 mM of DL-methionine and incubated for 72 h (Benavides *et al*., 2007). Another six fibroblasts plated well were incubated without methionine and used as control. The cell proliferation rate was then performed on 100 µL cell suspension in the presence of trypan blue by counting in Malassez chamber.

Morphometry of cultured aortic adventitial fibroblasts. To analyze the effect of methionine on aortic adventitial fibroblasts (14^{th} passage), the cells were plated in 6-well plates at 0.8×10^6 cells/mL/well in the presence of DL-methionine (20 mM) for 72 h. After this period the milieu was removed, and the cells were washed once in phosphate-buffered saline (PBS) and fixed in Bouin's fixative for 30 min. After rinsing with PBS and ethanol (96 %), the cells were stained for 10 min with a solution of Giemsa (1 % in methanol) and May Grunwald (0.7g/L) (V/V, 1:1) stains, diluted to 1/3 in distilled water. The excess of stain was removed by washing with PBS.

In order to assess the phenotypic state of aortic adventitial fibroblasts subjected to methionine, we carried out 20 measurements concerning certain cellular and nuclear parameters, namely, cellular major axis, major and small nuclear axes, number of nucleoli in each nucleus. Each parameter was measured in different fields of view and in several wells.

Ethical approval. The present study was approved by the institutional Animal Care Committee of the National

Administration of the Algerian Higher Education and Scientific Research (DGRSDT; https://www.dgrsdt.dz) and Use Committee of the University of Bab Ezzouar, and was carried out in accordance with the Algerian legislation (Law number 12-235/2012) relating to animal protection, the recommendations of the Algerian Association of Experimental Animal Sciences (AASEA 45/DGLPAG/DVA/SDA/14), and the EU Directive 2010/63/EU for animal experiments.

Statistical analysis. Quantitative results were analyzed by GraphPad Prism 8.0 software (GraphPad Inc., San Diego, CA, USA). The values were expressed as a mean and mean's standard error (SEM). The Student's t-paired test was used for aortic adventitial fibroblasts culture study. When the values of P were lower than 0.05, the difference was considered statistically significant.

RESULTS

Effects of methionine on cultured aortic adventitial fibroblasts. After 72 h of incubation with DL-methionine (20 mM), the aortic adventitial fibroblasts primary cultures exhibited morphological and functional alterations marked by cells' hypertrophy, cytosolic vacuolization due to oncosis, chromatin's hypercondensation, and fragmented nuclei indicating apoptosis alterations after MGG staining (Figs. 1A, B and C).

However, the aortic adventitial fibroblasts became significantly larger after methionine treatment, by 311.4 % ($P < 0.01$) (Fig. 1 B and C; Table I). The cell nuclei also significantly increased in size, by 189.17 % ($P < 0.05$) of the nuclear major axis and 97.38 % ($P < 0.05$) of the nuclear small axis (Table I). In addition to the bigger size, the nuclei of the aortic adventitial fibroblasts showed an increased number of nucleoli compared to corresponding controls (8.58 ± 0.45 vs. 3.17 ± 0.12, $P < 0.01$) (Table I).

In addition, the quantification of the proliferation rate of adventitial fibroblasts showed a significant decrease in the proliferation of the cells after methionine treatment compared to control cells ($P < 0.0001$; Fig. 1D) while the assessment of the total protein in the extracellular (23.74 ± 1.56 vs. 20.13 ± 2.71 µg/mL, $P < 0.01$) and intracellular (22.76 ± 1.48 vs. 20.39 ± 1.5 µg/mL, $P < 0.05$) compartments were significantly increased after methionine treatment suggesting an increase in protein secretion by adventitial fibroblasts (Fig. 1E).

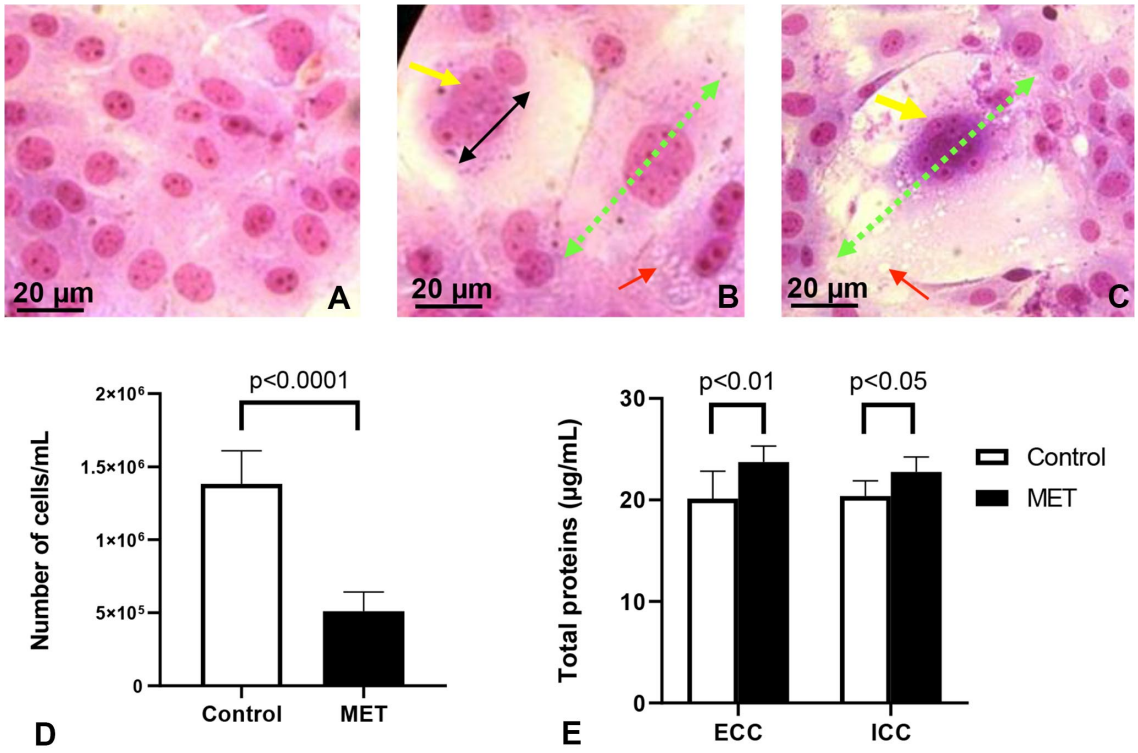

Fig. 1. *Psammomys obesus* aortic adventitial fibroblasts of control group (A) and aortic adventitial fibroblasts exposed at 20 mM of Methionine (B, C) for 72 hours. Green double-headed arrow, increase size of fibroblast; black double-headed arrow, fragmentation and enlargement of nuclei; yellow arrow, increase of nucleoli number with hypercondensation oh chromatin; red arrow, cytoplasmic vacuolization. D. Proliferation aortic adventitial fibroblasts Met-group vs control group; E. Total protein (µg/10^6 cells) in the extracellular (ECC) and intracellular (ICC) compartments of aortic adventitial fibroblasts Met-group vs control group. Data are presented as mean ± SD; *, **, **** statistically significant differences between the control and Methionine group; $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively.

Table I. Morphometric study of the cultured aortic adventitial cells, in the control group and Methionine group.

Morphometric parameters	Control group	Methionine group
Cellular major axis (µm)	32.45 ± 1.39	133.5 ± 24.6**
Nuclear major axis (µm)	21.7 ± 1.46	62.75 ± 12.48*
Nuclear small axis (µm)	13.4 ± 2.04	26.45 ± 3.98*
Number of nucleoli	3.17 ± 0.12	8.58 ± 0.45**

Data are presented as mean ± SD. *, **, statistically significant differences at the same time between the control and Methionine treated cells at 20mM for 72 h, $p < 0.05$ and $p < 0.01$, respectively. (n = 5; 6 sections per animal).

DISCUSSION

Red meat, a component of the routine diet, contains high methionine level which is absorbed very efficiently and enters the plasma until removed and metabolized by the different tissues (Chaturvedi *et al*., 2016). Since the cardiovascular disease increases in the population with high meat intake, the question is raised whether methionine directly or through its metabolites is implicated in the pathophysiology of cardiovascular diseases. For instance, given that methionine is a donor of methyl groups, it has been shown that excess of methionine in diet content alters the DNA methylation levels thus altering the gene expression (Park *et al*., 2008). More importantly, the accumulation of Hcy, a methionine-derived metabolite, causing Hhcy has been demonstrated to be a cardiovascular disease risk (Chaturvedi *et al*., 2016; Guieu *et al*. 2022).

Numerous experimental data that showed a methionine-enriched diet to induce a Hhcy in different animal models (gerbil, mouse and rat), thus causing atherosclerotic-type alterations (Hidiroglou *et al*., 2004; Zulli & Hare, 2009; Mendes *et al*., 2014). Previously, we reported that a chronic methionine administration induced HHcy associated with cardiovascular disease phenotype in the sand rat, *Psammomys obesus*. HHcy induced a significant increase in the extracellular matrix components particularly collagens which accumulated in the interstitial and perivascular spaces in heart and aorta indicating a developing fibrosis. Further analysis of the aorta showed that HHcy also induced vascular alterations including SMCs reorientation and proliferation associated with aneurysm formation (Zerrouk *et al*., 2022).

The main finding of the present *in vitro* study showed that a 20 mM (3 mg/mL) of the methionine supplementation after 72 hours induced cytological alterations in adventitial fibroblasts grown *in vitro* and also promoted a highly decrease in cell's proliferation rate ($P < 0.0001$). According to Yoneda & Krasnoschecoff (1958), the action of methionine and its homologue ethionine (48 hr, 0.016 M) on fibroblasts of heart explants grown *in vitro*, pronounced mitosis-inhibiting effect in addition to producing cytological modifications. Furthermore, Matsuura *et al*. (2015), showed that methionine free culture in the process of cardiac cell sheet fabrication is useful for elimination of remaining undifferentiated human induced pluripotent stem cells (iPS) while maintaining the viabilities of cardiomyocytes and noncardiomyocytes in cardiac cell sheets.

Benavides *et al*. (2007) reported that high concentrations of methionine (1 to 5 mg/mL) inhibit cellular growth and gene expression in the human breast tumor-derived MCF-7 cells. These inhibitory effects on cellular growth are, in part, due to inhibition of cellular proliferation via a p53-dependent pathway.

However, we were also able to induce cellular morphological changes consistent with necrosis by incubating fibroblasts with methionine for 72 hours. These changes were essentially marked by cells' hypertrophy, chromatin's hypercondensation, and fragmented nuclei indicating apoptosis alterations. The homologue of methionine, ethionine, at a concentration of 0.016 M produced definite effects on the *in vitro* grown Iibroblasts after 48 hours. The most evident were retraction of cell processes, vacuolation and fragmentation of the cytoplasm, rounding and shortening of the mitochondria, production of irregular contour of the nucleus and nucleolus, decrease of cytoplasmic basophilia and pycnosis (Yoneda & Krasnoschecoff, 1958).

The cytoplasmic vacuolization, nuclear fragmentations and chromatin condensation that we observed are signs of oncosis which precedes apoptosis. According to Tsai *et al*. (1994), homocysteine, amino acid derived from methionine, could induce apoptosis of endothelial cells. Similarly, Sharma *et al*. (2006), reported that homocysteine could cause apoptosis by involving the mechanism of caspase action.The reactive oxygen species (ROS) are responsible for hypercondensation of chromatin. ROS in particular superoxide ion (2O) allows interaction with the NO- and the genesis of peroxynitrite (ONOO-) a cause for damage to DNA (Sharma *et al*., 2006; Jiang *et al*., 2023). These are detected by the p53 tumor suppressor, to be overexpressed (Jiang *et al*., 2023). The p53 is a molecular switch that detects DNA breaks and commits cells in the process of repair or apoptosis (Jiang *et al*., 2023).

Interestingly, our current study showed that the number of nucleoli was sifgificatly increased in the incubating fibroblasts with a 20 mM of methionine (8.58 ± 0.45 vs. 3.17 ± 0.12, $P < 0.01$). This increase is indicative of increased synthesis of cellular protein. While the assessment of the total protein in the extracellular and intracellular compartments were significantly increased ($P < 0.01$ and $P < 0.05$, respectively). Yao & Sun (2014), demonstrated that

incubation of rat aortic adventitial fibroblasts with L-Hcy significantly increased collagen type 1 and AT1R (Angiotensin II Type 1 Receptor) expression, suggesting that adventitial fibroblasts may play an important role in the accumulation of the extracellular matrix and vascular adventitial remodeling.

After injury, adventitial fibroblasts are activated and differentiated to myofibroblasts, which secrete extracellular matrix proteins, such as collagen (Sluijter *et al*., 2004). HHcy exacerbates vascular constrictive remodeling by accelerating neointima formation and collagen accumulation in the adventitia (Kumbasar *et al*., 2001). In our previous study, we demonstrated that prolonged methionine administration in *Psammomys obesus* induced hyperhomocysteinemia associated with an atherosclerosis phenotype including the typical alterations in the aortal adventitia causing an aneurysm; where we noticed a matrix very rich in collagen probably synthesized by fibroblasts (Zerrouk *et al*., 2022). According to Liu *et al*. (2012), HHcy induces abdominal aortic aneurysm formation in mice via activation of the adventitial fibroblast NADPH oxidase 4.

CONLUSION

In summary, we found that the action of methionine on aoric adventitial fibroblasts grown *in vitro* was studied. It was observed that methionine inhibits mitosis in addition to producing cytological modifications. The mechanism of action of his amino acid is discussed.

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RESUMEN: La metionina es un aminoácido esencial que participa en procesos metabólicos críticos, y la regulación del flujo de metionina, a través del metabolismo, es importante para suministrar este aminoácido para las necesidades celulares. La elevación de la metionina plasmática ocurre comúnmente debido a mutaciones en las enzimas que metabolizan la metionina, como la metionina adenosiltransferasa. Esta elevación podría causar una hiperhomocisteinemia (HHcy), un factor de riesgo independiente de enfermedades cardiovasculares, incluida la aterosclerosis. Varias líneas de evidencia sugieren que la HHcy por suplementación con metionina puede afectar la producción de matriz al involucrar tipos celulares, como células musculares lisas vasculares y fibroblastos. Para este fin, cultivos primarios de fibroblastos adventicios aórticos fueron expuestos a metionina. La metionina a una concentración de 20 mM produjo, después de 72 horas, efectos definidos sobre los fibroblastos cultivados *in vitro*. Los más evidentes fueron retracción de los procesos celulares, vacuolación y fragmentación del citoplasma, producción de contorno irregular del núcleo y nucléolo, disminución de la basofilia citoplasmática y presencia de picnosis.

PALABRAS CLAVE: Fibroblastos adventicios aórticos; *in vitro*; Metionina; Morfología.

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