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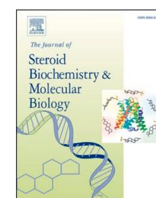

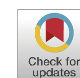

Ecdysterone prevents negative effect of acute immobilization stress on energy metabolism of rat liver mitochondria

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ABSTRACT

Ecdysterone is a naturally occurring steroid hormone, which presents in arthropods and in a number of plants as an insect defence tool. There are many studies showing that application of ecdysterone can alter mitochondrial functions of mammalian cells, however it is not clear whether its effects are direct or mediated by activation of other cellular processes. In our study, we have shown how ecdysterone acts at the mitochondrial level in normal conditions and in certain pathology. We have demonstrated that application of immobilization stress to male rats causes uncoupling of mitochondrial oxidative phosphorylation, the preliminary application of ecdysterone prevents negative effect of immobilization stress on mitochondria. In-vitro experiments with isolated mitochondria have shown that ecdysterone can increase mitochondrial coupling and hyperpolarise mitochondria but without a noticeable effect on ADP/O ratio. Molecular docking experiments revealed that ecdysterone has high binding energy with mitochondrial F_OF_1 ATP synthase, but further biochemical analysis have not revealed either stimulatory or inhibitory effect of ecdysterone on F_OF_1 ATPase activity of the enzyme. Thus, ecdysterone can directly affect mitochondrial bioenergetics, though we assume that its preventive effect on mitochondria during immobilization stress is also coupled with the activation of some other cellular processes.

1. Introduction

Ecdysterone or 20-hydroxyecdysone is a naturally occurring hormone, which regulates the development of arthropods. Many plants in the process of evolution started to synthesize it to protect themselves against insects. For many years, ecdysterone was considered a dietary supplement for athletes, which boosts muscle strength and mass during training process, reduces fatigue and shortens the recovery time between the training sessions, but it was never considered a doping before. Different receptors, such as androgen, estrogen and glucocorticoid receptors, were experimentally checked as possible targets for ecdysterone in mammalian cells [1,2]. However, recently it was shown that the anabolic effect of ecdysterone in muscle cells is associated with its binding to the estrogen receptors β (ERβ) [3]. Many reports have shown its positive effect on sport performance in animals and humans [3–5], which contributed to the idea that ecdysterone should be under the anti-doping control of the World Anti-Doping Agency as a potential performance-enhancing drug [6–8]. Starting from 2020 ecdysterone was included into the WADA Monitoring Programme.[1]

Ecdysterone as a signalling molecule can modulate different processes in insects through activation of genomic and nongenomic signal transduction pathways. Interestingly, it was shown that ecdysterone can exhibit some of its functions through calcium signalling [9–13], which was not yet demonstrated for mammals. Studies with mammalian cells have shown that ecdysterone has great pharmacological potential, bioavailability and pharmacokinetics [14]. Thus, ecdysterone can inhibit breast cancer growth by suppressing glycolytic and mitochondrial bioenergetics and induction of cell autophagy [15] and apoptosis

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[1] https://www.wada-ama.org/en/resources/science-medicine/monitoring-program

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[16] of cancer cells but not of control cells. It showed high neuroprotective potential in cellular [17,18] and mouse models [19] of Parkinson's disease. It protected PC12 cells against MPP^+-induced toxicity via increasing Akt kinase activity and the Akt signalling pathways, including glycogen synthase kinase 3-β inactivation, nuclear translocation of Nrf2 and upregulation of HO-1 expression [17]; and SH-SY5Y cells against 6-hydroxydopamine-induced apoptosis by a mitochondria-dependent pathway - via downregulation of Bax and PUMA, suppressing of 6-hydroxydopamine-induced $\Delta\Psi_m$ loss, inhibiting cytochrome c release, and attenuating caspase-9 activation [18]. Further *in-vivo* studies showed that ecdysterone can protect dopaminergic neurons in the substantia *nigra* and striatum against MPTP-induced toxicity. Administration of ecdysterone reduced motor incoordination, postural imbalance and bradykinesia of mice treated with MPTP [19]. Moreover ecdysterone inhibited β-amyloid induced mitochondrial apoptotic pathway through Akt and JNK signaling, which protected SH-SY5Y cells from death [20].

Administration of ecdysterone had positive effects in experimental diabetes induced by streptozotocin. Application of ecdysterone restored the activity of antioxidant system, which positively affected the memory deficits of rats with diabetes [21]; reduced the level of lipid peroxidation in the pancreas and stimulated pancreatic β-cells to release more insulin, which had beneficial effect on glucose homeostasis in streptozotocin-treated gerbils [22].

Thus, ecdysterone has been shown to have positive effects in pathologies associated with mitochondrial dysfunction and oxidative stress. Moreover, its positive effects are often associated with the modulation of mitochondrial functions, through alteration of different cellular processes. However, it is not known whether ecdysterone can affect mitochondria directly.

Thus, the aim of our study was to check the effects of ecdysterone *in-vivo* and *in-vitro* on respiration and oxidative phosphorylation of isolated mitochondria in health conditions and during immobilization stress - a pathology, which was proven to have negative effect on mitochondrial functions. Immobilization causes severe oxidative stress [23] and mitochondrial dysfunction in different tissues [24–26]. The cellular source of oxidative stress during immobilization in different tissues is not fully studied, however it was shown that mitochondrial targeted antioxidants protected muscle fibres from immobilization-induced atrophy and oxidative stress [27].

2. Materials and methods

2.1. Materials and reagents

All chemicals used in the study are manufactured by Sigma Aldrich, unless otherwise stated. Rhodamine 123 is from Invitrogen. For the study, we used ecdysterone (purity 95–98 %), which was isolated at the Institute of Chemistry of Plant Substances (Tashkent, Uzbekistan) from the roots of *Rhaponticum carthamoides*. [28].

2.2. Acute immobilization stress model

All the experiments were carried out on white male mongrel rats, kept in normal conditions with standard diet. For all the experiments we used fully grown but not old male rats (150−200 g, 6–7 month old), to exclude unnecessary hormonal and age effects. For the stress model, animals were immobilized in a prone position on a board for 16 h. The limbs were fixed by the adhesive tape and heads were inserted into wire loops which were attached to the board as was described in [29]. Ecdysterone was administered orally to experimental group of animals for 10 days before the start of the experiment at a daily dose of 5 mg/kg. All the procedures were performed by a highly qualified technician according to the rules of the Institute of the Chemistry of Plant Substances ethical committee, Tashkent, Uzbekistan. Animals were sacrificed within 1 h after the end of immobilization stress.

All the animals were divided into three groups: **Control** (3 rats) – normal animals, without immobilization stress and ecdysterone; **Stress** (4 rats) – animals that have been subjected to immobilization stress; **Stress + ecdysterone** (4 rats) – 5 mg/kg of ecdysterone orally for 10 days before the immobilization stress.

2.3. Isolation of rat liver mitochondria

Mitochondria were isolated from the liver by differential centrifugation as described in [30] with some modifications. The animals were euthanized by cervical dislocation, the liver from one rat was excised, thoroughly minced with scissors and homogenized using Teflon homogenizer and resuspended in 50 mL of isolation buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris−HCl, pH 7.4). All the procedures were performed on ice. Tissue fragments, unbroken cells and nuclei was centrifuged for 9 min at 1000×g, at +1 °C. The resulting supernatant was centrifuged for 18 min at 6000×g. All supernatant after second centrifugation was carefully removed; resulting pellet (mitochondria) was re-suspended in 500 μL of isolation buffer without EDTA and put on ice. Protein content was measured by the Biuret test with BSA as the standard. All the experiments with mitochondria were performed during first 4 h after isolation. Animals were sacrificed according to the protocol approved by the Ethical committee of the Centre for Advanced Technologies, Tashkent, Uzbekistan, in compliance with the legislation of the Republic of Uzbekistan.

2.4. Oxygen consumption

Oxygen consumption was measured by the polarographic method with the help of Mitocell S200 respirometry system (Strathkelvin Instruments, North Lanarkshire, Scotland). Respiration rate was assessed according to Chance and Williams [31] as was described in [30] by different respiration states: $\mathbf{V_2}$ – "resting respiration", mitochondrial respiration in the presence of glutamate and malate but without ADP; $\mathbf{V_3}$ – mitochondrial respiration rate during oxidative phosphorylation (after addition of 200 μmoles of ADP); $\mathbf{V_4}$ –respiration rate after all added ADP were converted into ATP; $\mathbf{V_{CCCP}}$ – maximal respiration in presence of 2 μM of CCCP (Carbonyl cyanide 3-chlorophenylhydrazone, Sigma-Aldrich). Functional status of mitochondria was assessed by Respiratory Coefficient (RC) – V_3/V_4 respiration rates ratio; and ADP/O – ratio of 200 μmoles of added ADP to consumed O_2 (μg) during V_3 state. All the experiments were performed over time, at 25 °C and constant stirring in incubation medium: 120 mM KCl, 5 mM glutamate, 5 mM malate, 10 mM Tris−HCl, 1 mM KH_2PO_4, 1 mM EGTA, pH 7.1.

For *in-vitro* experiments, all the results were calculated as a percentage. Respiration rate in V_2 (mean value) of control mitochondria was taken as 100 % in every experimental day, all the other parameters were calculated against it. The results of different experimental days were compared in percent.

2.5. Measurement of mitochondrial membrane potential

For measurements of $\Delta\Psi_m$, isolated mitochondria were loaded with 20 μM of Rhodamine 123 (Invitrogen) on ice in the dark for 30 min. Measurements were carried out on Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, U.S.A.). For the registration of the $\Delta\Psi_m$ signal, the suspension of mitochondria, loaded with Rhodamine 123, was excited at 505 nm and the emission signal was collected at 534 nm. All experiments were performed at 25 °C, in 3 mL quartz cuvettes, with constant stirring in incubation media: 120 mM KCl, 5 mM glutamate, 5 mM malate, 10 mM Tris−HCl, 1 mM KH_2PO_4, 1 mM EGTA, pH 7.1. Mitochondria were added to the cuvette at a concentration of 0.5 mg/mL in terms of protein.

2.6. Molecular docking

For docking experiments we used ecdysterone molecule downloaded from PubChem (20-Hydroxyecdysone, PubChem CID - 5,459,840). The energy minimization was performed with the help of ChemOffice tools and converted into. pdb format with the help of OpenBabel software [32]. Target (receptor) molecules were taken from Protein Data Bank: F_OF_1-ATP synthase (6ZQM, 2XND and 1BMF), Mitochondrial uncoupling protein 2 (2LCK). Energy minimization and geometry optimization of all proteins were performed with the help of UCSF Chimera tool – Dock Prep [33]. During protein preparation, all ligands and solvents were removed from the receptors, missing hydrogens and charges were added. All prepared molecules were saved in. pdb format. Docking experiments were performed with help of AutoDock Vina [34]. Results of docking were analysed and figures were prepared in UCSF Chimera 1.15 [33] and UCSF ChimeraX [35]. Docking experiments were performed in two rounds. During first round of docking we covered a big part of protein to identify possible binding site – F_O region, F_1 region of F_OF_1 ATP synthase, and whole UCP protein. In the second round, we focused our docking experiments on the binding sites, which we identified during the first round.

2.7. Measurement of ATPase activity in sub-mitochondrial particles

ATP hydrolysing activity of F_OF_1 ATPase was measured in sub-mitochondrial particles prepared by the freezing–thawing method as was described in [36]. Detection of ATP hydrolysis was performed by the pH metric method as described in [36,37], in the following medium: KCl 0.1 M, EDTA 50 μM, $MgCl_2$ 2 mM, Tris−HCl 5 mM (pH 8.0) with the help of Oakton PC 2700 pH meter connected to the laptop via serial port and CoolTerm software. The method is based on the fact that ATP hydrolysis at pH values close to neutral leads to the appearance of H + ions in the sample due to differences in the pKa of dissociating groups of substrate (ATP) and reaction products (ADP and Pi). All recordings were performed at room temperature and constant stirring.

2.8. Statistical analysis

Statistical analysis and curve fitting were performed using Origin 8.6 software (Microcal Software Inc., Northampton, MA). To determine the statistical significance of the results we performed One-Way ANOVA followed by the Tukey post-hoc test. Results were considered significant if the p-value was less than 0.05. Results were expressed as mean ± s.e. m.

3. Results

3.1. Immobilization stress causes dis-balance of mitochondrial oxidative phosphorylation

Immobilization stress caused severe disturbances in the mitochondrial oxidative phosphorylation. Mitochondria of stressed rats had increased substrate respiration in V_2 and V_4 compared to control (Fig. 1 A, C). On the contrary, respiration rate in V_3 was decreased significantly compared to control (Fig. 1 A, C). In some experiments, the transition from V_3 to V_4 wasn't observed at all. Because of the changes in V_3 and V_4 Respiratory Coefficient (RC) of mitochondria of stressed rats decreased significantly against control (Fig. 1 C). Moreover, the RC of stressed mitochondria decreased to 2.5 ± 0.27, which is below the normal RC values (RC of healthy mitochondria is ranged from 3 to 15). The Respiratory Coefficient is an indicator of the coupling of oxidation and phosphorylation processes in mitochondria. RC below 3 indicates that mitochondria are uncoupled and that the energy, which is generated during oxidation is not used for the phosphorylation processes.

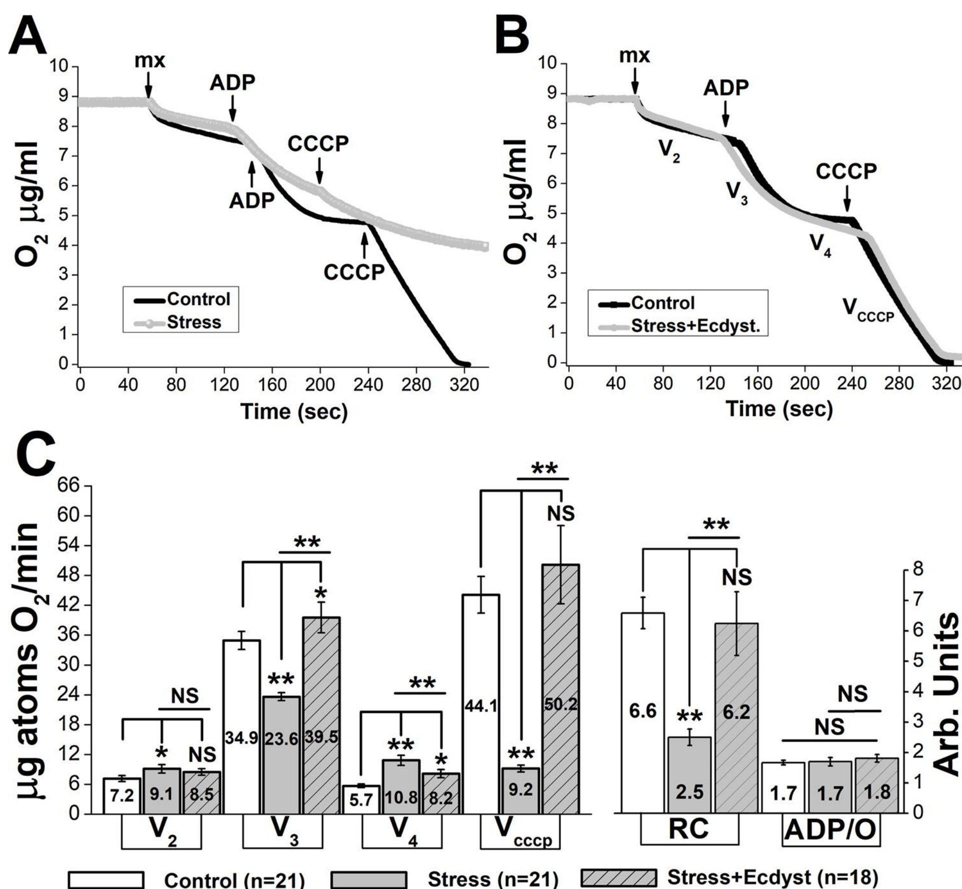

Fig. 1. Ecdysterone prevents negative effect of immobilization stress on mitochondrial energy metabolism. A. Immobilization stress causes severe disturbances in mitochondrial respiration by increasing the respiration rate in V_2 and V_4, decreasing the respiration rate in V_3 and almost eliminating the effect of CCCP, compared to control; **B.** Preliminary administration of ecdysterone eliminates the negative effect of immobilization stress and brings functional parameters of mitochondria almost to the control level; **C.** Changes in mitochondrial respiration caused by immobilization stress leads to the significant decrease of RC compared to control, preliminary administration of ecdysterone restores the RC to control level. Numbers in the center of the bars indicate the mean values of the respiration rate in the corresponding mitochondrial state; *p ≤ 0.05; ** p ≤ 0.01.

Application of the uncoupler (2 μM of CCCP) causes maximal respiration rate, which was clearly seen in the control experiments (Fig. 1 A and C). However, application of the CCCP in experiments with stressed mitochondria did not change the respiration rate much and V_{CCCP} was almost the same as V_4 (Fig. 1 A, C). Interestingly, the ADP/O of stressed mitochondria did not change compared to the control (Fig. 1 C).

Thus, in this series of experiments, it was demonstrated that immobilization stress causes severe disbalance in mitochondria by uncoupling the processes of oxidation and phosphorylation.

3.2. *In-vivo application of ecdysterone prevents negative effect of immobilization stress on mitochondrial oxidative phosphorylation*

To check the possible preventive effect of ecdysterone on immobilization stress we fed animals with 5 mg/kg of ecdysterone on the daily basis during 10 days before the immobilization stress. Application of ecdysterone did not change ADP independent respiration (V_2), though it increased significantly ADP dependent respiration compared to stressed mitochondria (Fig. 1 B, C). Respiration rate in V_4 was slightly decreased compared to stressed mitochondria, but it still was higher than in control experiments (Fig. 1 B and C). Positive effect of ecdysterone on respiration rates in V_3 and V_4 restored RC to control level but didn't affect ADP/O significantly (Fig. 1 B, C). In the presence of ecdysterone mitochondria restored its ability to respond to the uncoupler, the respiration rate in V_{CCCP} returned to the control level (Fig. 1 B, C).

To check whether the positive effect of ecdysterone on oxidative phosphorylation during immobilization stress is caused due to the direct effect of the substance on mitochondria we performed in-vitro experiments.

3.3. *Effect of ecdysterone on mitochondrial oxidative phosphorylation in-vitro*

For the experiments, we used two concentrations of ecdysterone (10 and 100 μM). In the first series of experiments, we applied ecdysterone immediately with mitochondria to check quick effects. In the second series of experiments, we incubated isolated mitochondria with 10 and 100 μM of ecdysterone for 1 h before the start of experiments.

Addition of 10 μM of ecdysterone led to an increase in mitochondrial respiration in the V_2, V_3, V_4 functional states, but not in V_{CCCP}, compared to control (Fig. 2 A). The increase in respiration rate was almost proportional in V_3 and V_4, so there were no significant changes in RC and ADP/O compared to the control (Fig. 2 A). Application of 100 μM of ecdysterone did not significantly affect the respiration rate, mitochondrial coupling (RC), and the efficiency of mitochondrial oxidative phosphorylation (ADP/O) compared to the control (Fig. 2 A).

In the next series of experiments, we incubated mitochondria with ecdysterone for 60 min, and then monitored mitochondrial respiration. Pre-incubation of mitochondria with 10 μM of ecdysterone increased the respiration rate in V_3 state by 7.2 % compared to the V_3 of mitochondria in control experiments, and also reduced V_4 by 18.5 % compared to the control V_4 (Fig. 3.2 B). Despite the fact that after pre-incubation with 10 μM of ecdysterone, the changes in respiration rates (V_3 and V_4) were not statistically significant compared to control, their effects summarized and this led to a significant increase in RC, from 7 (control) to 10.1 (Fig. 2 B). The respiration rate in other functional states, as well as ADP/O parameter, did not change significantly compared to control (Fig. 2 B).

Pre-incubation of mitochondria with 100 μM of ecdysterone reduced the respiration rate in all functional states of mitochondria, but as far as this reduction was proportional, this did not lead to significant changes in RC and ADP/O parameters (Fig. 2 B).

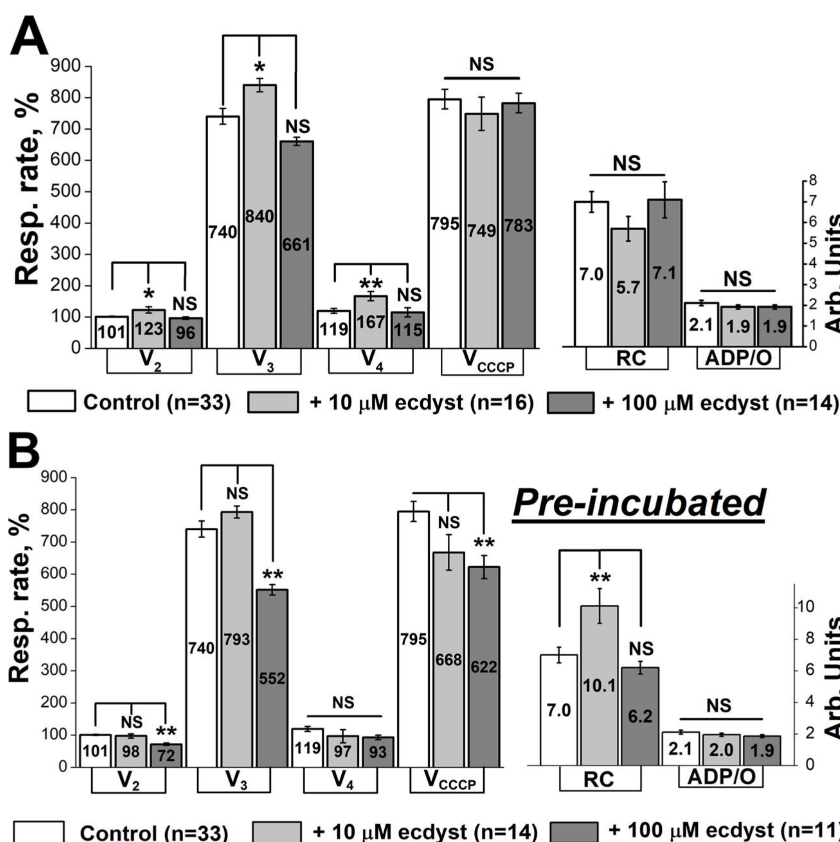

Fig. 2. Effect of ecdysterone applied *in-vitro* on mitochondrial respiration. A. Application of 10 and 100 μM of ecdysterone during the experiment doesn't change significantly functional parameters of mitochondria; **B.** Pre-incubation of mitochondria with 10 of ecdysterone for 1 h led to the increase of respiration rate in V_3 and decrease in V_4, which led to the significant increase of RC; pre-incubation with 100 μM of ecdysterone proportionally reduced respiration rate in all functional states, however that didn't cause significant changes in RC and ADP/O. Numbers in the center of the bars indicate the mean values of the respiration rate in the corresponding mitochondrial state in %, mean value in V_2 of control group was taken as 100 %, all calculations were made against this parameter; $*p \leq 0.05$; $** p \leq 0.01$.

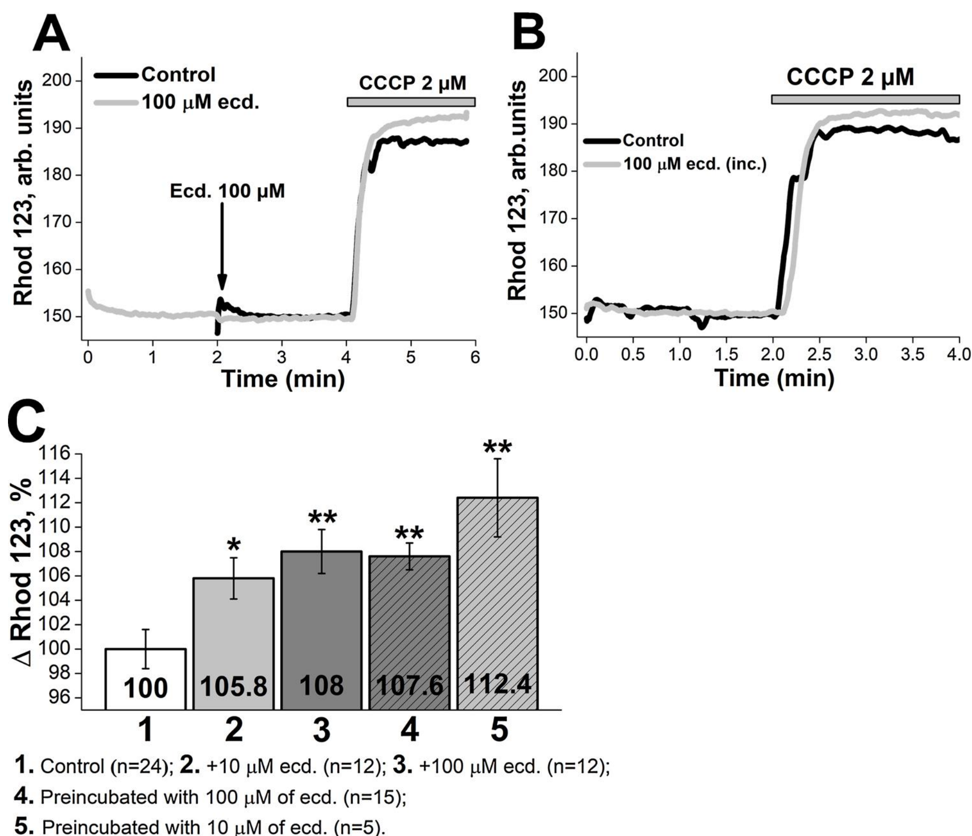

Fig. 3. In-vitro application of ecdysterone increases mitochondrial membrane potential. In-vitro effect of ecdysterone on mitochondrial membrane potential applied during experiment **(A)** and pre-incubated for one hour **(B)**, representative traces; **C.** Application of ecdysterone increases mitochondrial membrane potential, statistical analysis of the results. Numbers in the center of the bars indicate the mean values of the ΔRhod 123 in the corresponding group; mean value in control group was taken as 100 %, all calculations were made against it; $^*p \le 0.05$; ** $p \le 0.01$.

3.4. Ecdysterone increases mitochondrial membrane potential in-vitro

During experiments with the immobilization stress model, it was shown that ecdysterone restores V_{CCCP} to control values. V_{CCCP} of stressed mitochondria was at the V_4 level (Fig. 1 B and C). One of the possible reasons for such a sharp decrease in V_{CCCP} may be a disruption of the mitochondrial membrane integrity, which will affect the permeability of protons. To study whether ecdysterone affects the permeability of the mitochondrial membrane to protons, we measured the mitochondrial membrane potential, using fluorescent dye Rhodamine 123 (Rhod 123). Using this method, it is possible to monitor the overall bionergetic status of mitochondria, as well as changes in the mitochondrial membrane permeability for protons. An increase of Rhod 123 intensity indicates a decrease in membrane potential, which was clearly seen in control experiments after addition of the uncoupler CCCP (Fig. 3 A, B). For these series of experiments, we also used two concentrations of ecdysterone (10 and 100 μM) and two types of its application, similarly to previous experiments.

Application of 10 and 100 μM of ecdysterone caused mitochondrial coupling, which was observed after the addition of CCCP (Fig. 3 A, C). In the presence of ecdysterone the effect of CCCP was higher by 5.8 and 8 %, for 10 and 100 μM respectively, which indicates the hyperpolarization of mitochondria by ecdysterone (Fig. 3 A, C). Pre-incubation of mitochondria with ecdysterone for 1 h, also increased the effect of the CCCP by 12.4 and 7.6 % compared to control (Fig. 3 B, C). For these series of experiments, the effect of ecdysterone was more significant as the curve had more noticeable negative slope during the first two minutes of the experiment (Fig. 3 B, C). Thus, ecdysterone hyperpolarized mitochondria, but the effect was not depended on the pre-incubation time.

3.5. Structure-based docking studies

In our previous experiments, ecdysterone increased mitochondrial membrane potential and mitochondrial coupling *in-vitro*. This could be due to mild inhibition of F_OF_1 ATP synthase or due to a decrease in proton leakage across the inner mitochondrial membrane.

Considering the above mentioned we decided to perform docking experiments to understand whether ecdysterone can bind to mitochondrial F_OF_1 ATP synthase and UCP protein. F_OF_1 ATP-synthase is a complex enzyme, which consist from several functional parts. We performed docking experiments separately with F_O and F_1 regions, peripheral stalk and OSCP. Interestingly, in the F_O region ecdysterone docked inside the c ring, closer to matrix side with Vina score -8.4 and formed bonds with four serines and one threonine of different c subunits (Fig. 4 A, B and Table 1). For docking with F_1 region we chose three different structures available in PDB - 6ZQM [38], 2XND [39] and 1BMF [40]. 6ZQM is one of the latest structure of an enzyme obtained by electron cryo-microscopy, but it has two mutations in α subunits. In 2XND focus was made on the structure of catalytic sites and it is mutations free. 1BMF is a structure of F_1 ATPase and it also has mutations in α subunits. All three structures have native ligands and/or inhibitors in active sites, which were removed for docking experiments. Our calculations showed that ecdysterone could potentially bind to the α subunit of F_1 in the place of inhibitor/ligand binding, which was shown with all three structures (Fig. 4 A, C, D, E and Table 1). The highest binding energy was with α_{DP} subunit, however ecdysterone did not bind to the catalytic site of F_1 but to the opposite side of the α_{DP} subunit (Fig. 4 F, red molecule). For better understanding we merged our docking results with original protein structures and saw that ecdysterone binds exactly to the place where ligands and/or inhibitors locate (Fig. 4 C, D, E), however ecdysterone didn't interact with Mg^{2+}. To understand that our calculations are adequate we performed docking experiments with AMP-PNP (Adenylyl-imidodiphosphate) - a non-hydrolysable analogue of ATP,

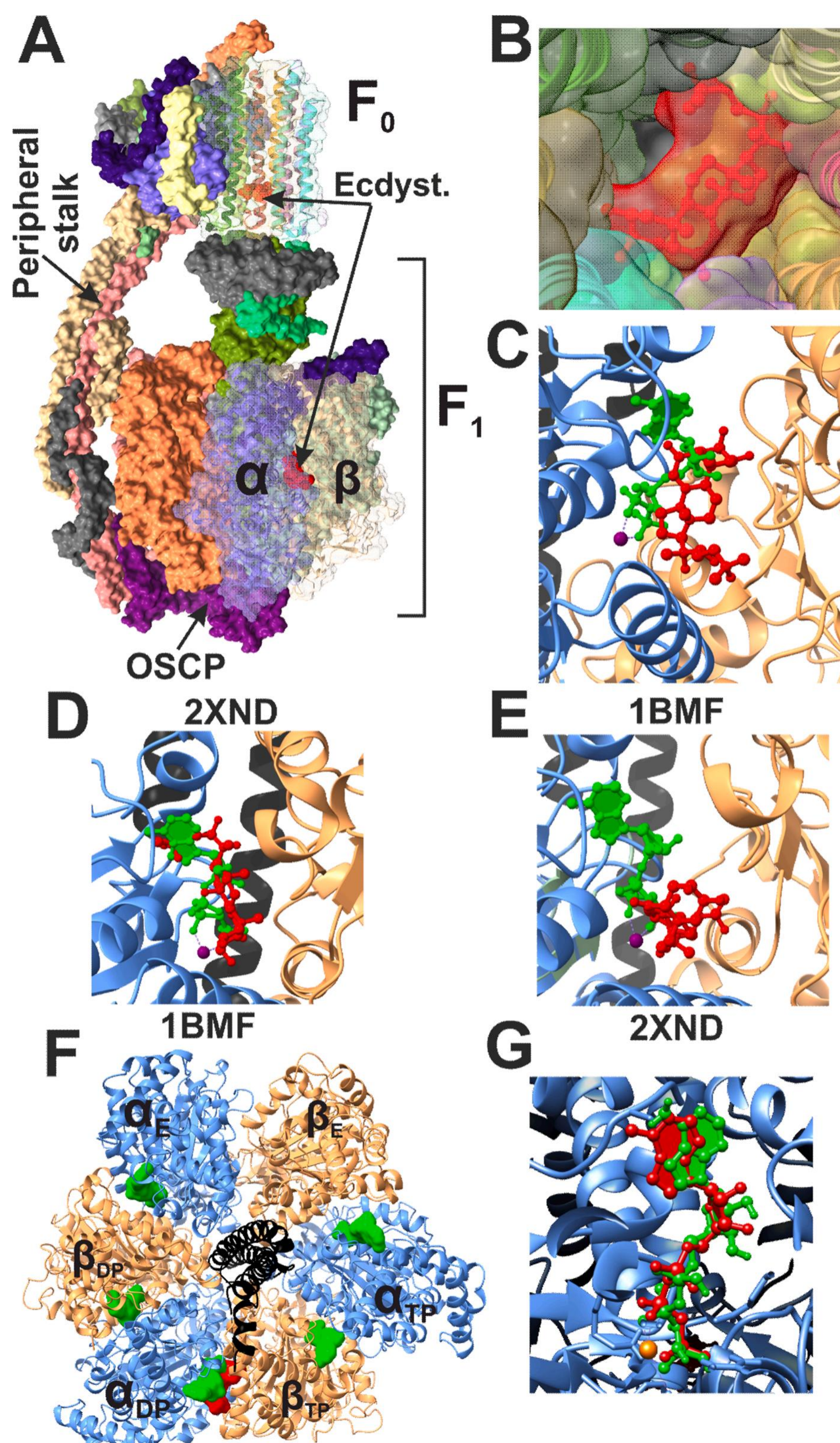

Fig. 4. Ecdysterone can potentially interact with mitochondrial F_0F_1 ATP-synthase – a view from molecular docking results. **A.** Ecdysterone can potentially bind to the F_O and F_1 region of mitochondrial ATP synthase - general view; **B.** In F_O region ecdysterone docked inside the c ring, closer to matrix side and formed bonds with four serines and one threonine of different c subunits - spatial position of the ecdysterone in the F_O region; **C, D, E.** In F_1 region ecdysterone binds to the α-subunits of 6ZQM (**A**), 2XND (**D**) and 1BMF (*E*), exactly to the place where native ligands and/or inhibitors locate – ecdysterone is red, ATP and AMP-PNP are green, Mg^{2+} is purple; **F.** The highest energy of binding of ecdysterone was with α_{DP} subunit, however ecdysterone binds not to the catalytic site of F_1 but to the opposite side of α_{DP} subunit - ecdysterone is red, other ligands are green; **G.** Docking of AMP-PNP with the F_1 region of 2XND proved that our calculations are adequate – docked AMP-PNP almost perfectly superimposed the AMP-PNP, which was present in the crystal structure of 2XND. Native AMP-PNP is green, docked one is red, Mg^{2+} is orange.

which was present in the active sites of 2XND and 1BMF. Merging of our docking results with the original structures showed almost perfect position match of docked AMP-PNP with the original one (Fig. 4 G). Docking of ecdysterone with UCP protein showed low binding energy (Table 1).

3.6. F_OF_1 ATPase activity

Docking experiments showed that ecdysterone can potentially bind to F_OF_1 ATP synthase, in both F_O and F_1 regions, which can lead to the modulation of the enzyme activity. Considering that, we decided to check how ecdysterone affect the ATPase activity of mitochondrial F_OF_1 ATP synthase. For these series of experiments, we pre-incubated the sub-mitochondrial particles with 100 μM ecdysterone for 2 and 60 min

Table 1
Docking results of ecdysterone with mitochondrial proteins.

Target		$\Delta G_{binding}$ (kcal/mol)	Amino acids
F_OF_1-ATP synthase, state 2 (6ZQM)	F_O	−8.4	SER P:31, SER L:31, SER K:31, THR K:27, SER R:31
	F_1	−8.7	ARG D:356, GLY A:174, SER D:355, THR D:354, ARG D:372
	Peripheral stalk	−7.6	—
	OSCP	−6.4	—
F_OF_1-ATP synthase (2XND)	F_1	−9.7	ARG C:219, ARG F:356, SER C:177, TYR F:368, ARG F:372, ARG C:362
F_1-ATPase (1BMF)	F_1	−9.0	GLN C:172, GLN C:432, TYR C:433, ARG C:219, MG C:601
Mitochondrial uncoupling protein 2 (2LCK)		−7.2	THR A:229, PHE A:226, PHE A:218, PHE A:222, LEU A:294, TYR A:291

before the start of the experiment, at room temperature. Our experiments showed that ecdysterone doesn't affect the ATPase activity of F_OF_1 (Fig. 5 A and B) in both experimental modes. Application of 2 μg/mL oligomycin - selective inhibitor of F_OF_1 ATPase, blocked the ATPase activity in the presence of 1 mM ATP (Fig. 5 A and B). Thus, despite the fact that *in-silico* experiments showed high binding energy of ecdysterone to F_OF_1 ATP synthase, biochemical analysis did not reveal either inhibition or activation of ATPase activity in the presence of ecdysterone.

4. Discussion

In this study, we have shown that immobilization stress causes disruption of mitochondrial oxidative phosphorylation. Administration of ecdysterone for 10 days prior to immobilization stress prevents stress dependent mitochondrial dysfunction. It is well-known that acute and chronic immobilization stress causes generation of ROS and down-regulation of antioxidant system in many tissues including brain, liver, skeletal muscles etc. [26,27,41–43]. Moreover, these changes significantly inhibit the activity of I and II mitochondrial respiratory chain complexes in NO dependent manner [26]. However, it should be noted that in previous studies the activity of the mitochondrial complexes and "mitochondrial" respiration were performed with submitochondrial particles rather than functional, coupled mitochondria [26] or mitochondrial respiration was assumed with help of MTT Assay [25], which cannot give the full picture of mitochondrial oxidative phosphorylation. Interestingly, it was shown that immobilization stress increases UCP1 expression in brown adipose tissue [44]. The function of UCP1 is mainly related to thermogenesis, however, other isoforms of UCP proteins are widely expressed in different tissues and play various roles including stimulation of mitochondria production, regulation of ROS production in health and disease [45] etc. The level of UCP depends on such hormones as thyroid hormone, norepinephrine, epinephrine, and leptin [46]. Interestingly, the level of all of these hormones increases after immobilization stress [47,48]. Thus, immobilization stress increases the level of ROS and different hormones, which in turn can elevate the level of UCP proteins. Since UCP proteins are expressed in different tissues, the uncoupling effect of immobilization stress on rat liver mitochondria might be associated with increased UCP expression in rat liver cells. Elevated level of UCP and concomitant mitochondrial uncoupling can be a protection tool against oxidative stress caused by immobilization [45]. In our experiments we showed that mitochondria isolated from stressed rats had increased respiration rate in "resting" V_2 state, reduced V_3 and almost had no effect after addition of uncoupler (Fig. 1). Administration of ecdysterone restored mitochondrial coupling (Fig. 1). Mitochondrial uncoupling during immobilization stress might be due to increased leakage of mitochondrial membrane for protons, which will undoubtedly cause $\Delta\Psi_m$ loss. In previous studies it was shown that ecdysterone can suppress 6-hydroxydopamine-induced $\Delta\Psi_m$ loss [18]. However, the possible influence of ecdysterone on the level of hormones, UCP proteins or direct effects on mitochondria should not be excluded.

Direct application of ecdysterone to isolated mitochondria slightly modified oxidative phosphorylation. Moreover, *in-vitro* application of ecdysterone increased mitochondrial membrane potential by 6–12 %, depending on the concentration and experimental conditions. The increase in the mitochondrial membrane potential might occur due to the coupling of oxidative phosphorylation, inhibition of the activity of the 5th complex of the respiratory chain (which uses the membrane potential for ATP synthesis), or a decrease in proton leakage across the membrane. In all our *in-vitro* experiments, the ADP/O parameter in the presence of ecdysterone decreased by 5–9 % compared to the control (Fig. 2 A, B, the effect was not statistically significant), which may indicate a slight inhibition of the 5th complex of the respiratory chain. Physiological proton leakage across the mitochondrial membrane can be modulated by decreasing of the level of lipid peroxidation. Kuzmenko et. al in 1997 showed that ecdysterone is able to inhibit the processes of lipid peroxidation *in-vitro* via unknown mechanism [49]. Molecular docking results showed high binding energy of ecdysterone in F_O and F_1 regions of the fifth respiratory complex, but subsequent biochemical analysis did not reveal any effects. The binding of the substance to the protein largely depends on its environment – solvents, pH, temperature etc. The absence of any changes in ATP-ase activity in the presence of ecdysterone might be due to limitations of the method. At the same time F_OF_1 ATP synthase is an enzyme that produces majority of ATP in our cells, which can work in two directions - ATP synthesis and ATP hydrolysis. In our experiments, we checked only ATP hydrolyzing activity of F_OF_1, so there is a possibility that the enzyme in our experiments was in a conformation not favourable for ecdysterone binding. Nowadays F_OF_1 ATP synthase is a leading candidate as the mitochondrial

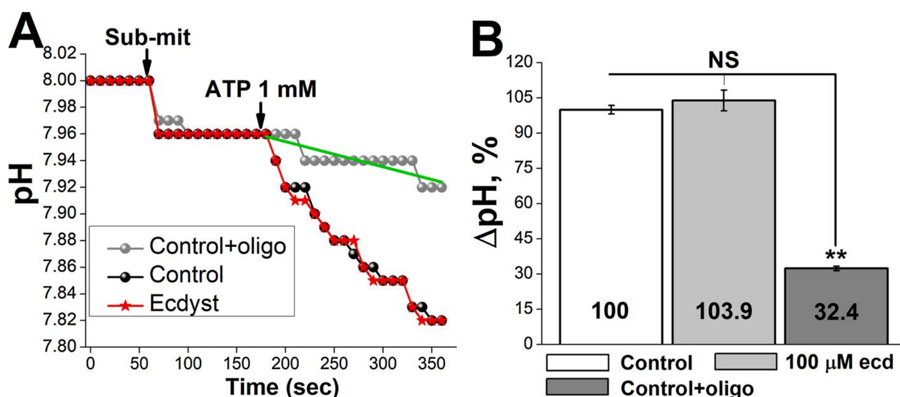

Fig. 5. Hydrolyzing activity of mitochondrial F_OF_1 ATP-ase in the presence of ecdysterone. Pre-incubation of sub-mitochondrial particles with 100 μM of ecdysterone doesn't affect the hydrolyzing activity of mitochondrial F_OF_1 ATP-ase, application of 2 μg/mL oligomycin significantly inhibited ATP-ase activity – **(A)** representative traces; **(B)** Statistical analysis of the results, n = 16 for control, n = 20 for experiments with ecdysterone; n = 5 for experiments with oligomycin. Numbers in the center of the bars indicate the mean values of the ΔpH in the corresponding group; mean value in control group was taken as 100 %, all calculations were made against it; ** p ≤ 0.01.

permeability transition pore (PTP) [50], considering that interaction of ecdysterone with F_OF_1 can lead to the modulation of PTP functioning.

In 2015 Parr et.al. showed that ecdysterone exhibits some of its properties in mammalian cells through interaction with ERβ [3]. It is well known that activation of estrogenic receptors has a positive effect on mitochondrial functions. The activation of estrogenic receptors by 17β-estradiol can protect human neuroblastoma cells against ATP depletion, the decrease in the mitochondrial membrane potential, and the generation of reactive oxygen species [51]. Nilsen et.al showed that 17β-estradiol treatment protects primary hippocampal neurons against glutamate excitotoxicity by stimulating Bcl-2 expression and promoting mitochondrial Ca^{2+} load tolerance [52]. Burstein et.al in 2018 showed that estrogen receptor beta can modulate PTP throw direct interaction with PTP regulator cyclophilin D, which causes differences in calcium retention capacity of mitochondria in males and females [53].

Thus, our results demonstrate that ecdysterone can directly affect mitochondrial energy metabolism, which will undoubtedly contribute to the general understanding of the mechanisms of action of ecdysterone in mammals. However, the mitochondrial targets for ecdysterone are still to be discovered.

Author contributions

A.Y.B., Y.V.L. and Ch.O.S. contributed to the study design; A.Y.B., Y. V.L., Ch.O.S., Z.A.Kh. and V.N.S. provided experimental tools; Z.A.Kh. and V.N.S. were responsible for *in-vivo* models; A.Y.B., Ch.O.S., F.A.Kh., K.S.N. and M.T.R. performed *in-vitro* experiments; A.Y.B., and A.R.M. performed *in silico* experiments; all co-authors were responsible for data analysis; A.Y.B., F.A.Kh. and A.R.M. prepared figures; A.Y.B. was responsible for drafting and revising the manuscript.

Ethical approval

All animal experiments were conducted according to protocols approved by the ethical committees of Institute of the Chemistry of Plant Substances and Centre for Advanced Technologies, Tashkent, Uzbekistan, in compliance with the legislation of the Republic of Uzbekistan.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare that there are no competing interests associated with the manuscript.

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