„VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY FROM TIMIȘOARA
FACULTY OF MEDICINE
DEPARTMENT XII – OBSTETRICS-GYNECOLOGY

FELICIA FIAT

PhD THESIS

PHYSICAL AND TOXICOLOGICAL EVALUATION OF PHYSIOTHERAPEUTIC AND THERAPEUTIC INTERVENTIONS IN PREGNANCY

Scientific Coordinator

ASSOCIATE PROF. PhD. ELENA SILVIA BERNAD

T i m i ș o a r a
2024

TABLE OF CONTENTS

LIST OF PUBLISHED SCIENTIFIC PAPERS……..**Error! Bookmark not defined.**

LIST OF ABBREVIATIONS AND SYMBOLS……..**Error! Bookmark not defined.**

LIST OF FIGURES……..**Error! Bookmark not defined.**

LIST OF TABLES……**Error! Bookmark not defined.**

DEDICATION.....**Error! Bookmark not defined.**

INTRODUCTION.....**Error! Bookmark not defined.**

GENERAL PART.....**Error! Bookmark not defined.**

1. PHYSIOLOGICAL AND PATHOLOGICAL CHANGES ENCOUNTERED DURING PREGNANCY……**Error! Bookmark not defined.**

1.1. Physiological changes encountered during pregnancy……**Error! Bookmark not defined.**

1.1.1. Musculo-skeletal changes encountered in pregnancy……..**Error! Bookmark not defined.**

1.2. Pathological changes encountered during pregnancy……**Error! Bookmark not defined.**

2. CANCER DURING PREGNANCY……..**Error! Bookmark not defined.**

3. THERAPEUTIC AND PHYSIOTHERAPEUTIC INTERVENTIONS ENCOUNTERED IN PREGNANCY……..**Error! Bookmark not defined.**

3.1. Pain and anticoagulation treatment in the case of pregnant women……...**Error! Bookmark not defined.**

3.2. Anti-cancer therapy during pregnancy……**Error! Bookmark not defined.**

3.3. Kineto-therapeutic interventions in pregnancy……**Error! Bookmark not defined.**

SPECIAL PART .....**Error! Bookmark not defined.**

4. RETROSPECTIVE STUDY OF THE CONSUMPTION OF HEPARIN AND ITS DERIVATIVES IN THE CASE OF PREGNANT WOMEN.....**Error! Bookmark not defined.**

4.1. Introduction……**Error! Bookmark not defined.**

4.2. Materials and methods……**Error! Bookmark not defined.**

4.3. Results and discussions……..**Error! Bookmark not defined.**

4.4. Conclusions……**Error! Bookmark not defined.**

5. IN VITRO TESTING OF HEPARINS ON HCT 116 COLORECTAL CARCINOMA CELLS.....**Error! Bookmark not defined.**

5.1. Introduction……**Error! Bookmark not defined.**

5.2. Materials and methods……..**Error! Bookmark not defined.**

5.3. Results and discusions……..**Error! Bookmark not defined.**

5.4. Conclusions……**Error! Bookmark not defined.**

6. IN VITRO CHARACTERIZATION OF THE CYTOTOXIC PROFILE OF LIDOCAINE ON A-375 CUTANEOUS MELANOMA CELLS AND HACAT KERATINOCYTES **Error! Bookmark not defined.**

6.1. Introduction……..**Error! Bookmark not defined.**

6.2. Materials and methods…….....**Error! Bookmark not defined.**

6.3. Results and discusions……**Error! Bookmark not defined.**

6.4. Conclusions……...**Error! Bookmark not defined.**

CONCLUSIONS

…….Error! Bookmark not defined.

REFERENCES

…….Error! Bookmark not defined.

PUBLISHED ARTICLES *IN EXTENSO*

…….Error! Bookmark not defined.

Introduction

Pregnancy is a special period in the women's life, which involves continuous physiological, psychological, and sometimes pathological changes. These changes often lead to the emergence of pain, musculo-skeletal pain being the most common. To relieve aches in pregnant women, medication such as anesthetics, analgesics, and anti-inflammatory drugs can be used depending on the stage of pregnancy. However, it is crucial to carefully evaluate the risk-benefit ratio. Therefore, it is recommended that pregnant women use safe medication to treat musculoskeletal pain and consider physiotherapy as an alternative if possible.

In addition, during pregnancy, several changes occur in a woman's body that affect the coagulation system. While pregnancy is a state of increased tendency for blood clotting to prevent excessive bleeding during childbirth, it can also predispose some women to certain types of coagulopathies. Heparins (HEP) are considered medication in this case, that is able to interfere with the body's natural blood clotting processes.

Cancer during pregnancy is another subject of interest, although it does not have such a high frequency, the effects of these conditions can be much more aggressive, both for the mother and for the fetus. Medication plays a crucial role in this case, but during pregnancy, the use of medications, particularly chemotherapy and other systemic treatments, requires careful consideration due to potential risks to the developing fetus. Thus, the goal is to balance the mother's health needs with the well-being of the developing baby, ensuring the safest possible outcome for both. Treatment decisions involve a multidisciplinary approach including oncologists, obstetricians, and other specialists. Factors such as cancer type, stage, gestational age, and the mother's health are considered. Treatment may include surgery, chemotherapy, radiation therapy (avoided during certain trimesters), or a combination, with adjustments made to minimize harm to the fetus. The main concern during cancer treatment in pregnancy is the potential impact on fetal development. Chemotherapy, for example, can affect the rapidly dividing fetal cells and might lead to developmental issues or pregnancy complications.

Emerging from the above, the aim of the present thesis is to analyze the safety status of use and to investigate the toxicological profile of different drugs widely accepted among pregnant women, from a therapeutic point of view in the niche of cancer diseases. To achieve this goal, the following objectives were pursued:

- the analysis of heparins' safety profile during/after consumption in case of pregnant women hospitalized at the "Pius Brînzeu" Emergency Clinical Hospital from Timisoara, Obstetrics and Gynecology Clinic I, between 01.01.2022 and 31.12.2022

- testing heparins from the point of view of their ability to interfere with HCT 116 colorectal carcinoma cell line activity;

-testing lidocaine on HaCaT human immortalized keratinocytes cell line and A-375 melanoma cell line in order to identify possible modifications induced on cellular morphology, nuclear morphology, or cell viability.

To meet these requirements, the thesis was divided into 2 parts, each of them in 3 chapters. The general part in the first phase addressed the physiological and pathological changes during pregnancy, then was focused on the topic of tumor diseases in the case of pregnant women and later on the therapeutic possibilities available to this niche of subjects.

In the special part firstly were analyzed the rate of consumption and the safety profile of heparins in case of pregnant women hospitalized during 2022 year at the ”Pius Brînzeu” Emergency Clinical Hospital from Timisoara, Obstetrics and Gynecology Clinic I. The fifth chapter was focused on testing heparins on human colorectal carcinoma cell line, HCT-116. The last chapter addresses to the capacity of lidocaine, one of the anesthetics considered safe to be used during pregnancy, on melanoma cancer (A-375 cell line).

Results

The first study aimed to follow causes and effect of HEP and its derivates administration in case of pregnant women. 92 hospital release notes were analyzed. 51.1% of subjects (n=47) were aged between 21 and 30 years, 34.78% (n=32) were aged between 31 and 40, 10.86 % (n=10) under 20, and 9.38 (n=3) over 41 years (Figure 1). The urban/rural distribution was approximately equal (Figure 2), with an insignificantly increased percentage in the case of patients from rural areas.

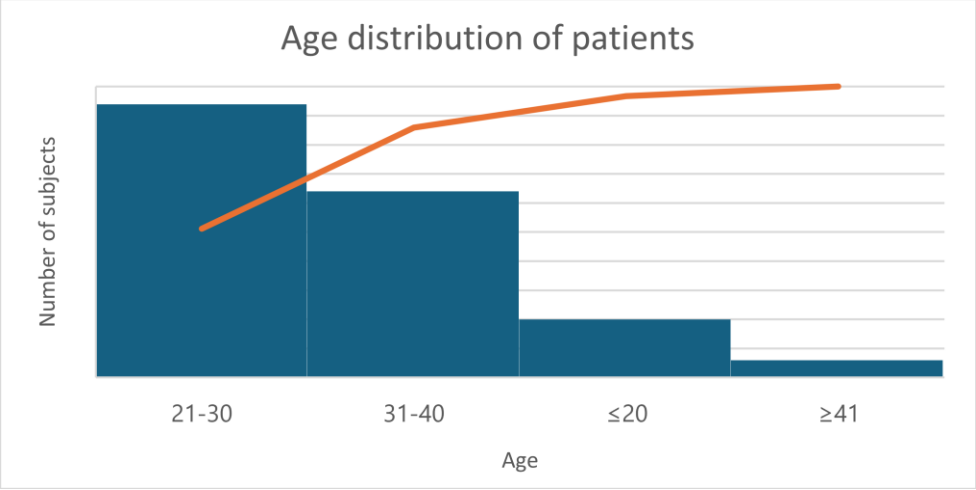


Figure 1. Age distribution of the subjects.

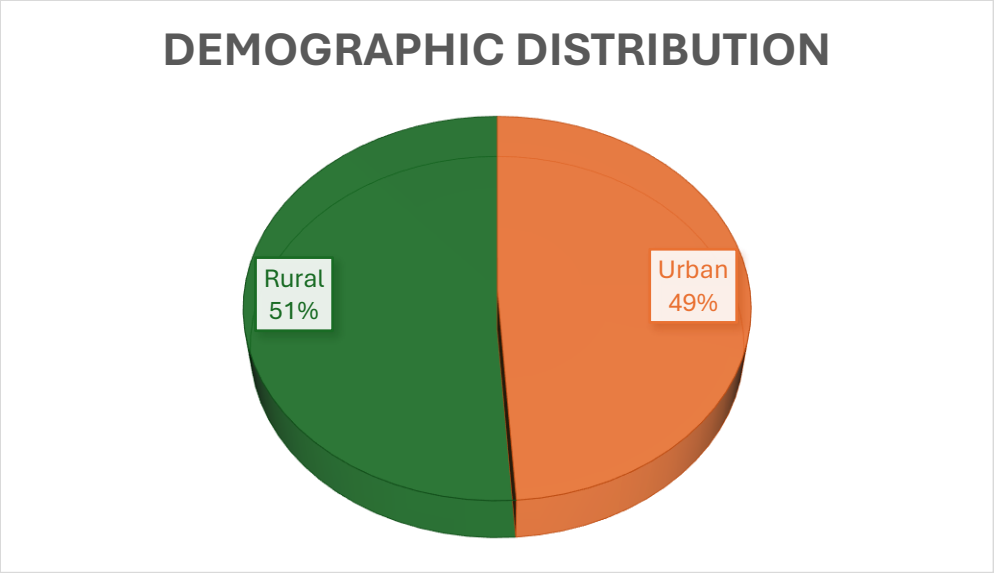


Figure 2. Rural/urban area distribution of subjects.

Clinical data

All the analyzed subjects were pregnant, 15 with a pregnancy of less than 36 weeks (16.30%), 2 ectopic pregnancies and 2 spontaneously aborted, and the rest of the patients with pregnancies between 37 and 41 weeks. 44.57% received anticoagulant treatment with low-molecular weight heparin (LMWHep) (Table 1). The reasons for approaching this therapy are specified in Table 2, the most frequent being the care given to the mother for the uterine scar due to a previous surgery (41.5%). 34. 78% (n=32) gave birth naturally and 46.74% by caesarean intervention. 8 cases of death of newborns were registered without causal relationship between the administration of LMWHep and the recorded data. However, most newborns presented a very good Apgar index (70.65%).

Table 1. Characteristics of subjects according to their discharge note

	Diagnostic		LMWHep treatment	The type of birth		Apgar index		Aborted/ dead
	Pregnancy < 36 weeks	Pregnancy > 36 weeks		N	C	0-7	8-10	
Patients (n)	15	73	41	32	43	17	65.00	8
Patients (% of total)	16.30	79.35	44.57	34.78	46.74	18.48	70.65	16.30

N-natural birth, C-caesarian intervention

Table 2. Summarized diagnostic and laboratory results according to the discharge ticket

		PATIENTS (N)	PATIENTS (% OF TOTAL)
DISCHARGE DIAGNOSIS	Care given to the mother for the uterine scar due to a previous surgery	17	41.46
	Medical induction of labor failure	6	14.63
	Birth by caesarean section	2	4.88
	Other diagnosis*	15	36.59
HEMOGLOBIN VALUE	Under 12 g/dL	3	7.32
	Over 16 g/dL	32	78.05
HEMATOCRIT VALUE	Under 35 %	13	31.71

COAGULATION/FIBRINOGEN VALUE	Over 46%	0	0.00
	Under 200 mg/dL	0	0.00
	Over 400 mg/dL	41	100.00

Other diagnostics include: tubal pregnancy (n=1), care given to the mother for the insufficient growth of the fetus (n=1), premature detachment of the placenta (n=1), severe or delayed hemorrhage following abortion and ectopic and molar pregnancy (n=1), premature rupture of the membranes, with the onset of labor in 24 h (n=1), care given to the mother for a disproportion due to an abnormally large fetus (n=1), care given to the mother for a pelvic presentation (n=1), maternal care for fetal injuries resulting from a viral disease (n=1), labor and birth complicated by unspecified fetal distress (n=1), care given to the mother for the intrauterine death of the fetus (n=1), gestational hypertension without specific proteinuria (n=1), physiological lause (n=1), hereditary hemorrhagic telangiectasia (n=1), maternal care for signs of fetal hypoxia (n=1), and partial retention of the placenta and membranes, without hemorrhage (n=1.)*

The next study evaluated the capacity of HEP and fraxiparine, as representative of LMWHep (FRAX) to interfere with the cellular viability of human colorectal carcinoma cells (HCT 116 cell line). Thereby cells were treated with 4 concentrations from each substance (10, 25, 50, and 100 UI), for 72h, then the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5- bromide diphenyltetrazolium) assay was applied. Obtained results show a slight stimulated effect up to the concentration of 50 UI (up to 120%) in case of HEP, and a gentle inhibitory effect at 100 UI (90%). When about FRAX, similar results were obtained, however with an attempt at more observable linearity. Thus, at all tested concentrations, the viability was approximately like that observed in the case of untreated control cells (around 100%) (Figure 3).

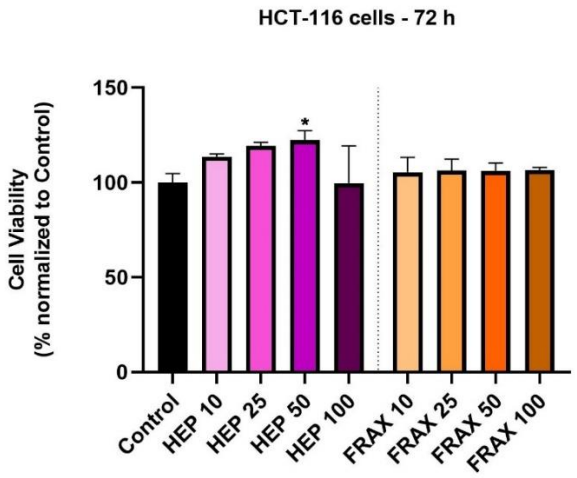
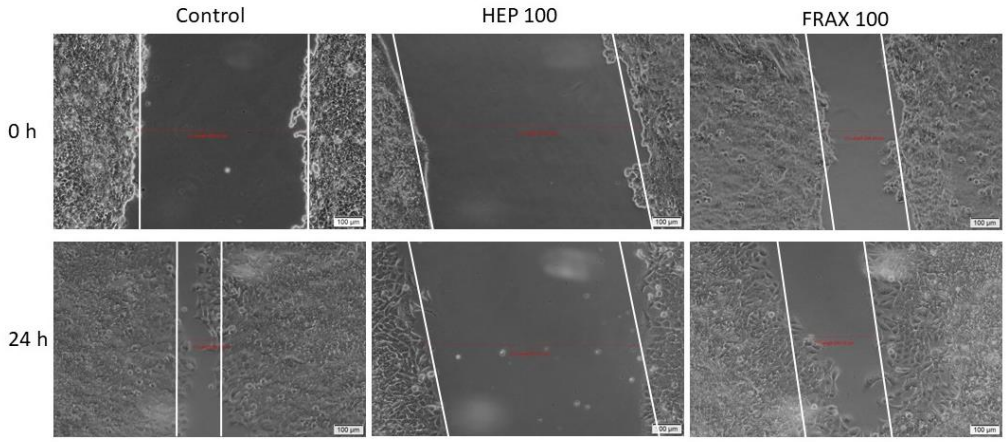


Figure 3. In vitro HCT 116 cell evaluation after 72 h of incubation with HEP and FRAX (10, 25, 50, and 100 UI) by performing the MTT assay. The data are presented as viability % normalized to untreated cells (expressed as average values ± SD of three independent experiments). One-way ANOVA analysis and the Dunett's multiple comparisons post-test were conducted to identify the statistical differences between obtained data (* $p < 0.1$).

To assess the impact of HEP and FRAX on the migratory properties of colorectal carcinoma cells, a wound healing assay was applied. HCT 116 cells were treated with the highest concentration (100 UI) of each substance (Figure 4). The results were depended on the tested HEP, the most potent effect being observed in case of HEP 100, with wound healing rates of 2,6%, significantly more potent than in case of control, where the wound healing rates was 85,9%. When about LMWHep, these manifested also a good inhibitory property of the cell migration, with a wound healing rates of 14.52%.

A)



B)

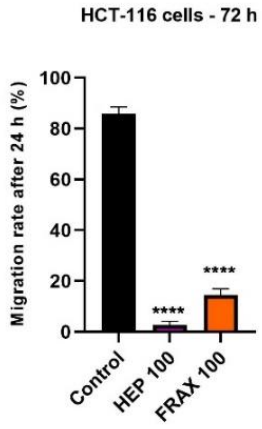


Figure 4. A) Pictures illustrating the migratory capacity of HCT 116 cells after treatment with HEP 100UI and FRAX 100UI for 24h. The scale bars represent 50 µm. B) Graphic depiction of the migratory potential of HCT 116 cells after the treatment with HEP 100UI and FRAX 100UI. The bar graphs are exposed as % of wound closure after 24 hours compared to the initial distance at t0. The results are expressed as averages ± SD of 3 independent experiments. One-way ANOVA analysis and the Dunett's multiple comparisons post-test were applied to follow the statistical differences between obtained results (** $p < 0.0001$).**

The last step of the in vitro study was the analysis of nuclear morphology, to identify if the cellular death occurred by necrosis or apoptosis. For this experiment, two concentrations were tested: 10 and 100 UI of each type of HEP. Hoechst reagent was used to counterstain the cell nuclei, and treated cells were compared with unstimulated (control) cells. In the case of the lowest concentration, no significant changes were observed, the nuclei present a round and regular form, without signs of fragmentation. Instead, in case of the highest concentration, signs of apoptosis were observed. HEP 100 induced membrane blebbing, nuclear condensation and fragmentation (highlighted with the yellow arrows in Figure 5). FRAX 100 also induced chromatin condensation and nuclei fragmentation (Figure 5).

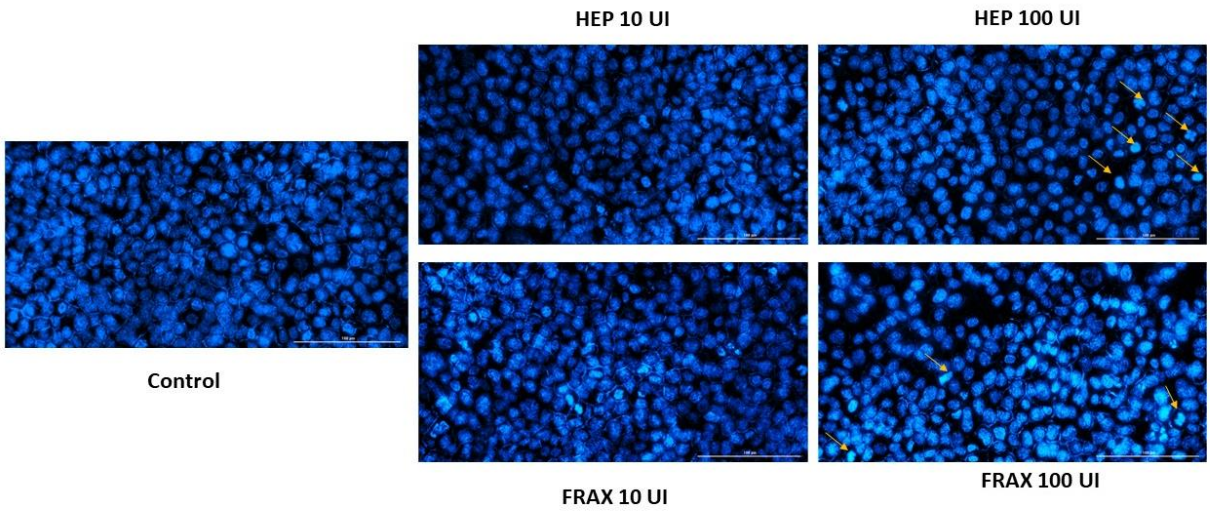


Figure 5. Pictures of the HCT 116 cellular nuclei counterstained with Hoechst 33342 reagent after 72 h of treatment with HEP and FRAX 10 and 100UI. The yellow arrows assign nuclei with apoptotic signs. The scale bars represent 100 µm.

The last study followed the cytotoxic profile of lidocaine (LID) on A-375 cutaneous melanoma cells. As LID is usually used as a local anaesthetic, to test the substance on melanoma cells, it was preliminarily tested on human immortalized keratinocytes. Following the MTT method, it was observed that at all concentrations (0.025%, 0.05%, 0.125% and 0.2%), except the highest one (0.25%), the viability was maintained over 90% (Figure 6). At 0.25%, the percentage of viable cells was significantly reduced to 13.5%, highlighting the cytotoxic potential of LID at this concentration.

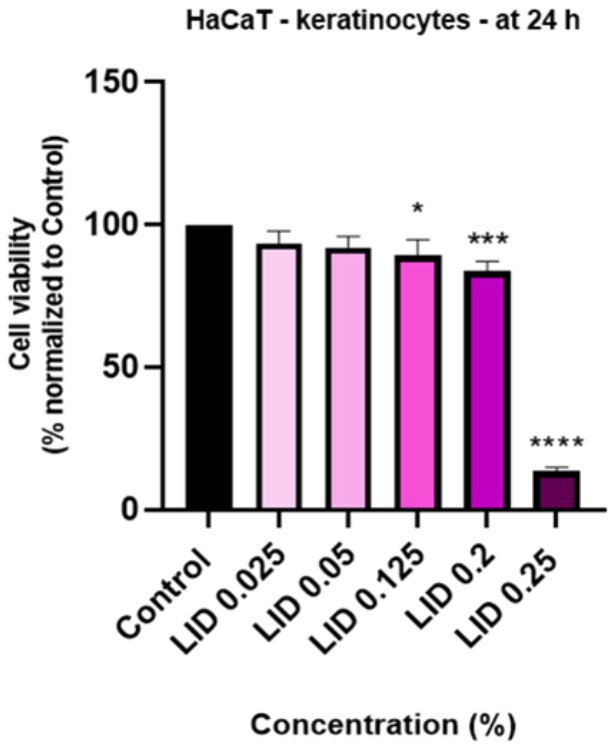


Figure 6. In vitro assessment of the effect LID (0.025, 0.05, 0.125, 0.2 and 0.25 %) exerts on the viability of HaCaT cells after 24 h of treatment by applying the MTT assay The data are presented as viability percentages (%) normalized to control (untreated cells) and expressed as means ± SD of three independent experiments performed in triplicate. The statistical differences between the control and the LID- treated groups were identified by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (* $p < 0.1$, *** $p < 0.001$; **** $p < 0.0001$).

When analyzing the viability of A-375 melanoma cells following the 24 h treatment (Figure 7), it was observed that at 0.025%, 0.05% and 0.125% LID exerted no cytotoxic effect, the viability maintaining over 95%. However, a significant drop in viability was obtained at higher concentrations (0.2% and 0.25%), when the percentage of viable cells was reduced to 24.33% and 20.90%, respectively.

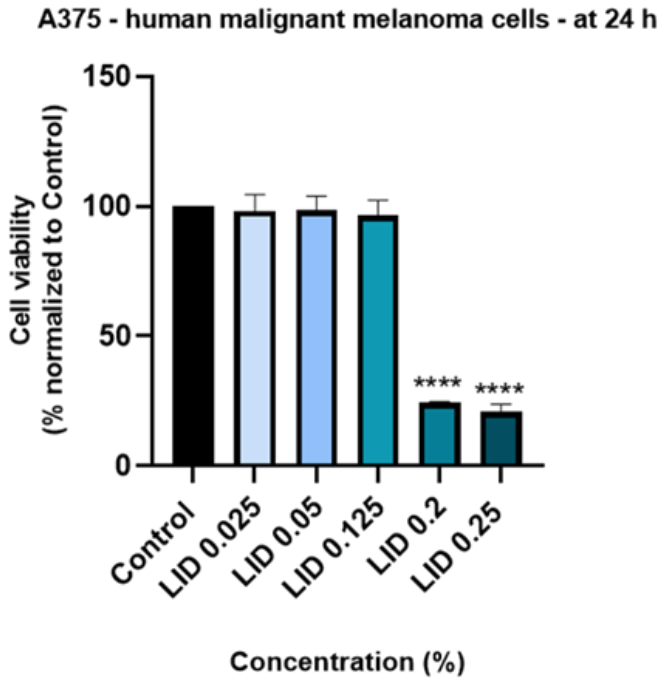


Figure 7. In vitro assessment of the effect LID (0.025, 0.05, 0.125, 0.2 and 0.25 %) exerts on the viability of A-375 cells after 24 h of treatment by applying the MTT assay The data are presented as viability percentages (%) normalized to control (untreated cells) and expressed as means ± SD of three independent experiments performed in triplicate. The statistical differences between the control and the LID- treated groups were identified by applying the one-way ANOVA analysis followed by the Dunett's multiple comparisons post-test (** p < 0.0001).**

The last step in the present study was to evaluate the potential cell death mechanisms underlying the cytotoxic effect of LID by examining the morphology of HaCaT and A375 cells' nuclei which were stained using the Hoechst 33342 dye. As shown in Figure 8, the 24 h treatment of HaCaT cells with LID at concentrations up to 0.2% induced no changes in the nuclear aspect, their shape remaining similar to control - regular and round, without signs of fragmentation or condensation.

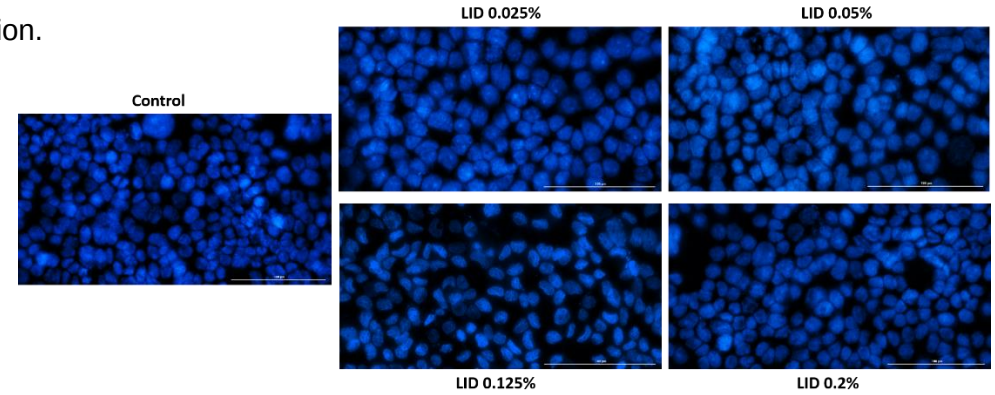


Figure 8. Images of the cellular nuclei stained using Hoechst 33342 reagent in HaCaT cells following the 24 h treatment with LID 0.025, 0.05, 0.125 and 0.2%. The scale bars represent 100 µm.

However, following the 24 h treatment with LID on melanoma cells (A-375), several changes in the aspect of the cellular nuclei were observed only at the highest evaluated concentration - 0.2% (Figure 9). LID induced nuclear fragmentation, chromatin condensation and membrane blebbing, modifications indicated by the white arrows.

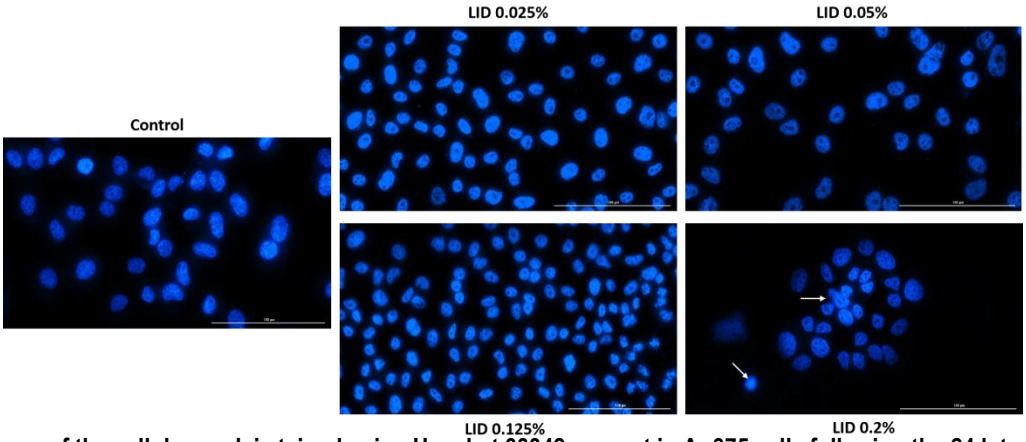


Figure 9. Images of the cellular nuclei stained using Hoechst 33342 reagent in A- 375 cells following the 24 h treatment with LID 0.025, 0.05, 0.125 and 0.2%. The arrows indicate nuclei expressing apoptotic features. The scale bars represent 100 µm.

Conclusions

In the first study, HEP and LMWHep have been analyzed from the point of view of safety in administration for pregnant women. These medications are frequently used for this category of patients, because of their physiological downregulations of the hemostatic system into a hypercoagulable state, posing elevated potential to develop coagulation problems, that must be managed. In the retrospective study, 92 discharge tickets of pregnant patients admitted to the "Pius Brînzeu" hospital in Timișoara were investigated. The results of the analysis highlighted the fact that almost half of the patients were treated with anticoagulant medication, and the most frequent cause of administration was the care given to the mother for the uterine scar due to previous surgery, without side effects, and with a good Apgar index observed in newborns.

In the second study, heparin and its derivatives were tested on HCT-116 cell line. A colorectal carcinoma cell line was chosen due to the fact that this form of malignancy is of interest especially in case of pregnant women because of the symptoms that can be easily confused with the physiological symptoms encountered during pregnancy, the complexity of diagnosis and identification of safe treatment solutions for both mother and fetus. Thus, it was observed that HEP and LMWHep manifested significant anti-migratory effect and good pro-apoptotic potential, suggesting the possible potential use in the case of colorectal cancer, especially in the segment of pregnant patients, for whom the current medication is limited.

The goal of the last in vitro study was to characterize the cytotoxic profile of lidocaine on skin healthy and cancer cells, as a basis for further investigations regarding its potential efficiency for cutaneous melanoma management in the case of patients with strict restrictions to classical treatment, such as pregnant women. The primary findings of the study demonstrate the safety of the anaesthetic on skin keratinocytes (HaCaT) at concentrations up to 0.2% when applied for 24 hours. The cell viability values were consistently over 90%, and there were no observable morphological changes or apoptotic-like nuclear features. However, at the concentration of 0.2%, LID exhibited increased cytotoxicity against A-375 cells. This was evident through a significant reduction in cell viability, alterations in cell shape and confluence, and the presence of apoptosis-specific nuclear characteristics. These findings provide a foundation for further research to investigate the specific mechanism of action responsible for the anti-melanoma effect of LID. Additionally, it opens up possibilities for repurposing LID in clinical practice for skin cancer therapy.

In conclusion, the obtained results highlight the fact that the drugs considered safe during pregnancy and used for various current ailments, can show important effects on other health problems, less common but with an imposing impact, such as cancer diseases, for which the treatment schemes in the case of pregnant women are not quite bright. Supplementary investigations are necessary to strengthen the findings and to identify the exact mechanisms of action.