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The effect of darbepoetin alfa on the glomerulus of new-born mice with intrauterine growth restriction

Утицај дарбепоетина алфа на гломеруле новорођених мишева са интраутерином рестрикцијом раста

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SUMMARY

Introduction/Objective Intrauterine growth restriction is a condition in which a fetus is not able to achieve its genetic potential for growth. It has been linked with diseases of adulthood, such as hypertension, insulin-independent diabetes mellitus and dyslipidemia.

The objective of the study was to investigate whether the application of darbepoetin alfa during pregnancy and in first week of life affects the number or size of the kidney glomerulus of mice with intrauterine growth restriction.

Methods We used animal model of intrauterine growth restriction. Darbepoetin alfa was administered to the pups on the 1st and 7th day of life (dose 1, 4 and 10 μg/kg). Two of seven groups represented the offspring of the mothers who received darbepoetin alfa during pregnancy. Four weeks after the birth, kidney samples were taken, and morphological and stereological analysis of the glomeruli was performed.

Results Administration of darbepoetin alfa to newborn mice with intrauterine growth restriction led to faster weight gain in the first 7 days of life. Mice born with intrauterine growth restriction had reduced glomerular surface and reduced cortical thickness. The application of darbepoetin alfa immediately after the birth and on the 7th day of life (4 and 10μg/kg) in mice with intrauterine growth restriction led to glomerular hypertrophy and increased thickness of the renal cortex. The application of darbepoetin alfa had no effect on the number of glomeruli.

Conclusion The administration of darbepoetin alfa to mice with intrauterine growth restriction significantly increases the surface area of the kidney glomeruli and cortical thickness.

Key words: intrauterine growth restriction; darbepoetin alfa; kidney; glomerulus

САЖЕТАК

Увод/Циљ Интраутерина рестрикција раста је ситуација у којој фетус није у стању да оствари свој генетски потенцијал за раст. Повезан је са болестима одраслог доба, као што су хипертензија, инсулин-независни дијабетес мелитус и дислипидемија.

Циљ Испитати да ли примена дарбепоетина алфа током трудноће и у првој недељи живота утиче на број или величину гломерула бубрега мишева са интраутерином рестрикцијом раста.

Методе Користили смо животињски модел интраутерине рестрикције раста. Дарбепоетин алфа је апликован штенцима мишева првог и седмог дана живота у различитим дозама
(дозе 1, 4 и 10 *μg/kg*). Две од седам група су обухватале младунце женки које су примале дарбепоетин алфа и током трудноће. Четири недеље након порођаја узети су узорци бубрега и урађена морфолошка и стереолошка анализа гломерула.

Резултати Примена дарбепоетина алфа новорођеним мишевима са интраутерином рестрикцијом раста довела је до бржег повећања телесне тежине у првих седам дана живота. Мишеви рођени са интраутерином рестрикцијом раста имали су смањену површину гломерула и смањену дебљину кортекса. Примена дарбепоетина алфа након рођења и седмог дана живота (4 и 10 *μg/kg*) код мишева са интраутерином рестрикцијом раста довела је до хипертрофије гломерула и повећања дебљине кортекса бубрега. Примена дарбепоетина алфа није утицала на број гломерула.

Закључак Примена дарбепоетина алфа код новорођених мишева са интраутерином рестрикцијом раста значајно повећава површину гломерула и дебљину кортекса бубрега.

Кључне речи: интраутерина рестрикција раста; дарбепоетин алфа; бубрег; гломерул

INTRODUCTION

Intrauterine growth restriction (IUGR) is a condition characterized by failure of a fetus to achieve its genetic potential for growth [1]. IUGR is defined as a fetus, and then also as a newborn, whose birth weight is below the 10th percentile for its gestational age [1]. IUGR occurs when delivery of gases and nutrients to the fetus is not sufficient for adequate intrauterine development [1]. It is known that IUGR is a significant risk factor for type 2 diabetes, obesity, hypertension, dyslipidemia, and insulin resistance (metabolic syndrome) later in life,

what ultimately leads to the premature development of cardiovascular disease [1, 2, 3]. The Barker's hypothesis "The developmental origins of adult disease" states that adverse effects in early development, especially during intrauterine life, can lead to permanent physiological and metabolic changes, resulting in an increased risk of morbidity in adulthood [4]. Brenner et al. hypothesized that the fundamental kidney abnormality leading to elevated blood pressure is reduced filtration surface area [5]. This could be due to a decrease in the number of kidney nephrons and/or a decrease in renal filtration surface per nephron, which is characteristic for kidneys of persons born with IUGR. When the renal functional reserve due to the reduced number of nephrons is greatly decreased, glomeruli will reach the limit of physiological compensatory hypertrophy and pathological mechanisms will be initiated, which can lead to hypertension. Prolonged hyperfiltration of hypertrophic glomeruli can ultimately lead to glomerular sclerosis and possibly loss of glomeruli [6].

Erythropoietin (EPO) is a 165-amino acid peptide. Studies indicate the benefit of EPO therapy in case of brain injury, retinal disease, gastrointestinal and myocardial ischemia. A positive effect of EPO on the kidneys has been demonstrated in various studies. It had been shown that EPO antagonizes endothelial cell apoptosis, increases the sensitivity of endothelial cells and the activity of endothelial nitric oxide synthase (eNOS), stimulates mitogenesis and angiogenesis of endothelial cells, increases renal blood flow, and has an anti-inflammatory effect [7, 8].

The aim of this study is to investigate whether the application of darbepoetin alfa (DA) during pregnancy and in first week of life, affects the number or size of the kidney glomerulus of mice with intrauterine growth restriction.

METHODS

In the experiment, female mice and male and female pups of the NMRI (Naval Medical Research Institute) breed were used. The animals have been grown in standard laboratory conditions with food and water ad libitum. The experimental IUGR model was used. Female mice were mated for 24 hours with fully mature males. The day of conception was determined by serial observation of vaginal swabs by light microscopy. When the presence of spermatozoids was confirmed, it was recorded as a zero day of gestation. Gestation in NMRI mice breeds lasts for 19–21 days. From 15th to 21st day of gestation, dexamethasone (diluted in 0.9% NaCl) was administered to pregnant females. Dexamethasone was applied subcutaneously, at a dose of

100 μg/kg per day (0.2 ml). After spontaneous delivery, the pups were weighted and left on natural nutrition. Only the newborn mice who were born from pregnancies with 6–10 fetuses (newborns) were included in the experiment. Criteria for exclusion from the study were as follows: mice born in pregnancies with less than 6 fetuses and mice born from pregnancies with more than 10 fetuses. According to the random selection method, the pups were classified into one of the following groups:

Group 1 (Control group 1) – 10 mice with IUGR that received 0.1 ml 0.9% NaCl intraperitoneally;

Group 2 – Eight mice with IUGR that received DA intraperitoneally (10 μg/kg) on the 1st and 7th day of life;

Group 3 – Eight mice with IUGR, that received DA intraperitoneally (4 μg/kg) on the 1st and 7th day of life;

Group 4 – Eight mice with IUGR, that received DA intraperitoneally (1 μg/kg) on the 1st and 7th day of life;

Group 5 – Eight mice with IUGR whose mothers received DA subcutaneously (10 μg/kg) on 15th postconceptual day;

Group 6 – Eight mice with IUGR whose mothers received DA subcutaneously (10 μg/kg) on 15th post conceptual day, and who received DA intraperitoneally (10 μg/kg) on the 1st day of life;

Group 7 (Control group 2) – 10 mice who were born and raised without any prior intervention during the fetal period and after the birth.

Four weeks after the birth, the experimental mice were sacrificed by decapitation. The kidneys were removed from the sacrificed animals and tissue samples were fixed in the solution of 10% buffered formalin, dehydrated and molded into paraffin. After rehydration, the samples were cut at 5 μm thick slices and stained with the hematoxylin-eosin. Histological examination (morphological and stereological analyses of glomeruli, intermedial and juxtamedullary zone of the renal cortex) was performed using light microscope at 400x and 100x magnification. Morphometric and stereological analyses included measurement of the surface of the glomerulus and the thickness of the renal cortex, as well as determining the numerical density of the glomerular

profiles using commercial image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). The area of 821–1109 glomerular profiles per group was measured. Every 5th section was a slide mounted. Only well-preserved structures that do not cut the "forbidden" lines of the test system were considered.

The numerical density (*Nv*) was determined by observing 15 microscopic fields per animal [9]. It was calculated using Weibel and Gomez formula [10]:

$$Nv = \frac{\kappa}{\beta}\left[\frac{Na^3}{Vv}\right]^{1/2}$$

$$Vv = \frac{Pp}{Pt}$$

where: Na – number of glomerular profiles in a test-area; Vv – volume density of glomerules; κ – the size distribution coefficient assuming 10% coefficient of variation (1.01); β – the shape coefficient for sphere (1.38); Pp – number of points that hit the glomerules; Pt – total number of points.

The thickness of the cortex was measured as the length of the line positioned at a right angle between the parallel tangent lines on the surface of the kidney and the boundary of the cortex and the core of the kidney on 41-83 fields.

Statistical data processing was performed using StatSoft, Inc. software packages (2007) STATISTICA (data analysis software system), version 8.0, and Glantz, Stanton A. Primer of Biostatistics, 5th Edition, McGraw-Hill, 2002.

The experiment was approved by the Ethics Committee on Animal Care and Use of the University of Novi Sad (Ethics Committee approval number I-2013-05) and were performed and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

RESULTS

It was found that birth weight of newborn mice of the Group 7 (mice that gave birth spontaneously without previous intervention on the mother) was statistically significantly higher compared to the Group 1 (IUGR) (1.675 g *vs.* 1.253 g; $p < 0.05$), Group 2 (IUGR) (1.675 g *vs.* 1.2

g; $p < 0.05$), Group 3 (IUGR) (1.675 g *vs.* 1.089 g; $p < 0.05$), Group 4 (IUGR) (1.675 g *vs.* 1.156 g ; $p < 0.05$) and Group 5 (IUGR and DA on the 15th day of pregnancy) (1.675 g *vs.* 1.35 g; $p < 0.05$). Statistical significance was not established by comparing the birth weight of newborn mice between Group 6 (IUGR and DA on the 15th day of pregnancy) and Group 7 (mice that gave birth spontaneously without previous intervention on the mother) (1.75 g *vs.* 1.675 g; $p>0.05$).

The birth weight of newborn mice in Group 6 (IUGR and DA on the 15th day of pregnancy) is statistically significantly higher compared to Group 1 (IUGR) (1.75 g *vs.* 1.253 g; $p < 0.05$), Group 2 (IUGR) (1.75 g *vs.* 1.2 g; $p < 0.05$), Group 3 (IUGR) (1.75 g *vs.* 1.35 g; $p < 0.05$), Group 4 (IUGR) (1.75 g *vs.* 1.156 g; $p < 0.05$) and Group 5 (IUGR and DA on the 15th day of life) (1.75 g *vs.* 1.35 g; $p < 0.05$).

The average birth weight of newborn mice with IUGR (Group 1) was 1.253 g, what is 25.2% less in comparison with spontaneously delivered pups without IUGR (Group 7) whose average birth weight was 1.675 g.

The average weights of mice on the 7th day of life are shown in Table 1.

The average body weight of the pups in Group 7 (mice that gave birth spontaneously without prior intervention on the mother and without intervention on them after birth) on the 7th day of life was statistically significantly higher compared to the pups in other groups ($p < 0.05$), except the pups in Group 2.

Mice pups in Group 2 (IUGR and DA 10 μg/kg) had a statistically significantly higher body weight on the 7th day of life compared to the pups in Group 1 (IUGR) (3.625 g *vs.* 2.32 g; $p < 0.05$), Group 3 (IUGR and DA 4 μg/kg) (3.625 g *vs.* 3.18 g; $p < 0.05$) and Group 4 (IUGR and DA 1 μg/kg) (3.625 g *vs.* 2.733 g; $p < 0.05$).

The average body weight of mice pups on the 7th day of life in the Group 1 (IUGR) was statistically significantly lower compared to all other groups, $p < 0.05$.

Average values of glomerulus area size of mice are shown in Table 2.

By comparing the surface area of the kidney glomeruli, it was determined that there is no statistically significant difference between Group 2 (IUGR and DA 10 μg/kg) and Group 7 (mice

that gave birth spontaneously without previous intervention on the mother), (2471 μm^2 *vs.* 2425 μm^2; $p > 0.05$).

Mice pups of in Group 5 (IUGR and DA on the 15th day of pregnancy) had a statistically significantly higher kidney glomerular surface compared to mice in Group 3 (IUGR and DA 4 μg/kg) (2319 μm^2 *vs.* 2201 μm^2; $p < 0.05$) and Group 4 (IUGR and DA 1 μg/kg) (2319 μm^2 *vs.* 1954 μm^2; $p < 0.05$).

The glomerular surface of the mice in Group 6 (IUGR and DA on the 15th day of pregnancy and after birth 10 μg/kg) was statistically significantly higher compared to mice in Group 3 (IUGR and DA 4 μg/kg) (2289 μm^2 *vs.* 2201 μm^2; $p < 0.05$) and Group 4 (IUGR and DA 1 μg/kg) (2289 μm^2 *vs.* 1954 μm^2; $p < 0.05$).

It was found that the glomerular surface of mice in Group 3 (IUGR and DA 4 μg/kg) is statistically significantly higher than the glomerular surface of mice in Group 4 (IUGR and DA 1 μg/kg), (2201 μm^2 *vs.* 1954 μm^2; $p < 0.05$).

It was found that there is no statistically significant difference in the numerical density of glomeruli between all experimental groups, $p > 0.05$ (Table 3).

The obtained results indicate that the thickness of the kidney cortex was statistically significantly higher in mice from the Group 2 (IUGR and DA 10 μg/kg) compared to all other groups, $p < 0.05$ (Table 4).

DISCUSSION

Unfavorable environmental conditions during prenatal or early postnatal period may increase the susceptibility to chronic diseases in later life. In 1988, Brenner et al. pointed out that a small number of nephrons, acquired in utero, could be a common factor in the population prone to hypertension and kidney disease [5]. A kidney with fewer nephrons, and therefore a low filtration surface area, has a reduced ability to excrete sodium, which leads to hypervolemia, which contributes to the development of hypertension. Animal experiments and epidemiological data support the "nephron number" hypothesis [11]. It is known that IUGR in humans can lead to a reduction in the number of nephrons. In newborns with IUGR, the kidney is of altered shape (thin, sausage-like) resulting from a smaller number of concentric layers [11, 12]. Brenner et

al. were the first to highlight the potential consequences of a reduced number of nephrons at birth on kidney disease in later life. They also pointed out that nephropenia, as a result of IUGR, could create a disproportion between a body size and excretory capacity leading to vasodilatation, glomerular hypertension, and progressive loss of nephrons due to glomerulosclerosis [13].

Numerous studies have shown a significant reduction in the number of nephrons as a result of IUGR [11]. There are numerous studies, both, in humans and experimental animal models, showing the link between low birth weight (LBW) and long-term increase in blood pressure and the threat of developing kidney disease. The risk of kidney dysfunction later in life increases in infants with IUGR who were born prematurely [14]. A systematic review of eighty studies conducted in children, adolescents and adults between 1996. and 2000. which examined the relationship between blood pressure and birth weight showed a decrease in blood pressure with increased birth weight (a decrease in blood pressure of about 2mmHg for each kilogram of birth weight) [15]. A relative increase in blood pressure of 2 mmHg is associated with a 6% higher risk of coronary artery disease and a 15% higher risk of stroke [16]. There is an inverse relationship between gestational age at birth and blood pressure levels. The study of Cooper et al. has shown a decrease in systolic blood pressure by 0.53 mmHg with each additional week of gestational age at birth [17]. White et al. published a meta-analysis of 31 relevant studies and concluded that people born with low birth weight (LBW) are at 70% higher risk of developing kidney disease [18].

Numerous experiments were conducted where IUGR was induced in an animal model and its effect on the kidneys was examined [19]. In our experiment, IUGR animal model was induced by glucocorticoids. Pups with IUGR had statistically significantly lower birth weight (25.2%) than those in the control group. LBW in combination with a sudden increase in body weight ("catch-up phenomenon") after the birth increases the risk of hypertension and cardiovascular disease in later life. Several studies on animal models investigated effects of "catch-up phenomenon" and demonstrated that increased oxidative stress, shortening of the telomeres and accelerated kidney, heart and aortic aging occurring in those animals is associated with premature death. Also, there are data suggesting that accelerated aging and increased oxidative stress are features of people born with LBW [11]. In our experiment, newborn mice were measured on the 7th day of life. Newborn mice from the control groups had statistically significantly higher body weight compared to all other (IUGR) groups, except when compared to those IUGR mice who were treated with high dose of DA (10 μg/kg). Also, mice from the latter

group had larger glomeruli. Further studies, as well as long term follow-up, are needed to prove the benefits or adverse effects of this rapid increase in body weight.

EPO is a hematopoietic growth factor whose production is regulated by hypoxia. It is a pleiotropic cytokine that exhibits various biological effects in many non-hematopoietic tissues. EPO exhibits its effects in different organs such as brain, heart, lung, kidneys and liver. A growing body of evidence indicates that EPO reduces glomerular and tubular injury and dysfunction caused by severe ischemia and reperfusion (I/R), both in experimental animals and in neonates. Mechanisms through which EPO exhibits its protective effect against renal I/R injury are as follows: an increase in renal blood pressure, and an increase in diuresis due to an increase in cortical perfusion and intraglomerular pressure. EPO also protects proximal tubular epithelial cells from cellular damage and death by its anti-apoptotic, antioxidant, anti-inflammatory, and pro-angiogenic effects [7].

An experimental study conducted by Spandau et al. has proven the protective effect of EPO in the sense of its inhibiting the apoptotic death of proximal and distal tubule cells [20]. Administration of EPO at the time of renal I/R injury significantly reduces the damage of cell function and protects against cell death [21].

Darbepoetin alfa (DA) is a long acting hyperglycolized EPO derivative, with a half-life of about 3 times longer than recombinant human erythropoietin (rHuEPO) and with similar effects as rHuEPO. It has been proven that DA has an anti-apopotic effect in both toxic and hypoxic kidney damage [22].

Brenner et al. have hypothesized that the fundamental kidney abnormality that leads to elevated blood pressure is reduced filtration surface. This can be caused by a decrease in the number of kidney nephrons, as well as by a decrease of renal filtration area per nephron [5]. In our experiment, morphological and stereological analysis showed that treatment with DA significantly increases glomerular area, but do not influence the number of glomeruli. Experimental animals that received DA (1 μg/kg or 4 μg/kg at 1st and 7th day of life) had statistically significant increase of the glomerular area compared to IUGR offspring's who were not treated. However, animals that were spontaneously delivered, without any previous intervention, had a greater glomerular area compared to all three IUGR groups (group 1 (IUGR); group 3 (IUGR and DA 4 μg/kg); and group 4 (IUGR and DA 1 μg/kg). But also, those experimental animals with IUGR that received DA in a high dose of 10 μg/kg at 1st and 7th day of life, showed an increase

in glomerular area to the extent that there was no statistically significant difference when compared with the group of healthy offsprings.

The numerical density of glomeruli in experimental animals was also investigated. Although there are differences between the observed groups, they are not statistically significant.

In the world literature, there are no clinical or experimental studies examining the effect of erythropoietin on kidney glomeruli in IUGR. This research was conceived as a pilot study which would be the basis for further investigations on this topic.

CONCLUSION

Based on the results of our experiment, we can assume that higher doses of EPO administered immediately after birth to mice with IUGR have a positive effect on the growth of glomeruli.

Further studies are needed to determine the benefit of this effect later in the lives of IUGR-born children, as well as on comorbidity, with an emphasis on blood pressure.

Conflict of interest: None declared.

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Table 1. Comparison of body weights of pups between all experimental groups on the seventh day of life

Groups	Body weight (g)	p
7 *vs.* 1	3.825 *vs.* 2.32	0.000
2 *vs.* 1	3.625 *vs.* 2.32	0.000
7 *vs.* 4	3.825 *vs.* 2.733	0.000
5 *vs.* 1	3.45 *vs.* 2.32	0.000
6 *vs.* 1	3.438 *vs.* 2.32	0.000
3 *vs.* 1	3.18 *vs.* 2.32	0.000
2 *vs.* 4	3.625 *vs.* 2.733	0.000
7 *vs.* 3	3.825 *vs.* 3.18	0.000
5 *vs.* 4	3.45 *vs.* 2.733	0.000
6 *vs.* 4	3.438 *vs.* 2.733	0.000
4 *vs.* 1	2.733 *vs.* 2.32	0.005
3 *vs.* 4	3.18 *vs.* 2.733	0.005
2 *vs.* 3	3.625 *vs.* 3.18	0.007
7 *vs.* 6	3.825 *vs.* 3.438	0.010
7 *vs.* 5	3.825 *vs.* 3.45	0.013
5 *vs.* 3	3.45 *vs.* 3.18	0.097
6 *vs.* 3	3.438 *vs.* 3.18	0.114
7 *vs.* 2	3.825 *vs.* 3.625	0.177
2 *vs.* 6	3.625 *vs.* 3.438	0.272
2 *vs.* 5	3.625 *vs.* 3.45	0.305
5 *vs.* 6	3.45 *vs.* 3.438	0.941

Table 2. Comparison of glomerulus area sizes between the groups

Groups	Glomerulus area size (μm^2)	p
2 *vs.* 4	2471 *vs.* 1954	0.000
7 *vs.* 4	2425 *vs.* 1954	0.000
6 *vs.* 4	2289 *vs.* 1954	0.000
5 *vs.* 4	2319 *vs.* 1954	0.000
1 *vs.* 4	2247 *vs.* 1954	0.000
2 *vs.* 3	2471 *vs.* 2201	0.000
3 *vs.* 4	2201 *vs.* 1954	0.000
2 *vs.* 1	2471 *vs.* 2247	0.000
7 *vs.* 3	2425 *vs.* 2201	0.000
2 *vs.* 6	2471 *vs.* 2289	0.000
7 *vs.* 1	2425 *vs.* 2247	0.000
7 *vs.* 6	2425 *vs.* 2289	0.000
2 *vs.* 5	2471 *vs.* 2319	0.001
5 *vs.* 3	2319 *vs.* 2201	0.013
6 *vs.* 3	2289 *vs.* 2201	0.015
7 *vs.* 5	2425 *vs.* 2319	0.025
5 *vs.* 1	2319 *vs.* 2247	0.128
2 *vs.* 7	2471 *vs.* 2425	0.224
1 *vs.* 3	2247 *vs.* 2201	0.225
6 *vs.* 1	2289 *vs.* 2247	0.244
5 *vs.* 6	2319 *vs.* 2289	0.504

Table 3. Comparison of the numerical density of glomeruli between all experimental groups

Groups	Numerical density of glomeruli (mm^{-2})	p
4 *vs.* 3	2.705 *vs.* 2.381	0.061
1 *vs.* 3	2.712 *vs.* 2.381	0.064
4 *vs.* 2	2.705 *vs.* 2.449	0.166
1 *vs.* 2	2.712 *vs.* 2.449	0.167
6 *vs.* 3	2.594 *vs.* 2.381	0.237
5 *vs.* 3	2.6 *vs.* 2.381	0.267
1 *vs.* 7	2.712 *vs.* 2.534	0.306
4 *vs.* 7	2.705 *vs.* 2.534	0.308
7 *vs.* 3	2.534 *vs.* 2.381	0.388
6 *vs.* 2	2.594 *vs.* 2.449	0.448
5 *vs.* 2	2.6 *vs.* 2.449	0.467
1 *vs.* 6	2.712 *vs.* 2.594	0.504
4 *vs.* 6	2.705 *vs.* 2.594	0.515
1 *vs.* 5	2.712 *vs.* 2.6	0.565
4 *vs.* 5	2.705 *vs.* 2.6	0.576
7 *vs.* 2	2.534 *vs.* 2.449	0.652
2 *vs.* 3	2.449 *vs.* 2.381	0.725
5 *vs.* 7	2.6 *vs.* 2.534	0.732
6 *vs.* 7	2.594 *vs.* 2.534	0.732
1 *vs.* 4	2.712 *vs.* 2.705	0.967
5 *vs.* 6	2.6 *vs.* 2.594	0.975

Table 4. Comparison of the thickness of the renal cortex between all experimental groups

Group	1 862.42 µm	2 1086.3 µm	3 988.21 µm	4 931.54 µm	5 922.62 µm	6 924.49 µm	7 958.73 µm
1		0.000000	0.000417	0.748833	1.000000	0.596013	0.027405
2	0.000000		0.039828	0.000237	0.000143	0.000004	0.001626
3	0.000417	0.039828		1.000000	0.997114	0.398601	1.000000
4	0.748833	0.000237	1.000000		1.000000	1.000000	1.000000
5	1.000000	0.000143	0.997114	1.000000		1.000000	1.000000
6	0.596013	0.000004	0.398601	1.000000	1.000000		1.000000
7	0.027405	0.001626	1.000000	1.000000	1.000000	1.000000	

