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EFFECT OF INDUCED RELATIVE HYPOXIA ON RETICULOCYTE COUNT IN ONCOLOGICAL ABDOMINAL SURGERY: A SINGLE-CENTRE, CONTROLLED, RANDOMIZED PILOT STUDY.

Clinical Research

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ABSTRACT

Erythropoietin (EPO) stimulating agents are known as alternatives to transfusion. However, they expose patients to thrombosis and are expensive. A phenomenon, "the normobaric oxygen paradox" (NOP), has been described. A transient hyperoxia followed by a prolonged return to normoxia acts as an effective trigger for EPO production. The mechanism depends on free O2 radicals and on reduced gluthatione (GSH) availabilities. Also, N-acetylcystein (NAC) is known to regenerate the stock of GSH. Our study sought to determine whether a NOP regime administered alongside NAC could produce an increase in reticulocyte count via an increase of EPO production, in patients undergoing oncological abdominal surgery. Prospective, controlled trial randomized 78 patients in 3 parallel groups. The first group (NOP) received 60% oxygen for two hours on days 1,3 and 5 postoperatively via a venti-mask. The second group (NOP+NAC), in addition to the NOP oxygen regime, received 300 mg IV once a day of N-acetyl-cysteine (NAC) on the first day postoperatively and 200 mg orally once a day until the fifth day post-op. The third group received neither NOP nor NAC. On postoperative day 6, reticulocytes were measured and compared to the baseline values. The 95 percent confidence intervals of the mean percentage change from baseline revealed, that the increase in reticulocyte counts was statistically significant for the NOP Group and NOP+NAC Group, whereas it was not significant for the control group. These data suggest that relative hypoxia by means of oxygen gradient is an effective stimulus for reticulocyte production.

KEYWORDS

Normobaric oxygen paradox (NOP); Induced relative hypoxia; N-acetylcystein (NAC); Anaemia: alternative transfusion strategies

Introduction

Anaemia is a frequent complication in cancer patients. More than 50% of patients become anaemic independently of their treatment and approximately 20% of all patients receiving chemotherapy are subject to transfusion of more than one blood unit. (Jelkmann, 1992; Masson, Willam, Maxwell, Pugh, & Ratcliffe, 2001; Mercadante, Gebbia, Marrazzo, & Filosto, 2000)

Over the last decade, the increasing demand for blood transfusions and hence a sufficient supply of donated blood, has presented a challenge that is likely to persist for the foreseeable future. The demand is due essentially to the development of more aggressive treatment in oncology and the spreading of cancer in general. To compound the problem, the number of blood donors is dropping and the risks associated to blood transfusion are still present.

Recombinant human erythropoietin (rhEPO) is considered an option for the treatment of anaemia due to chronic renal failure and that induced by chemotherapy. Erythropoietin (EPO) is a precursor that induces red blood cell production via red bone marrow progenitor cell activation (Gunga et al., 2007; Jelkmann, 1992, 2011). However, rhEPO is expensive and several side effects have been reported upon its pharmacological administration (Bohlius et al., 2009). It is also difficult to obtain in certain countries. These facts have prompted scientists and clinical researchers to try and develop alternatives in this field.

Klausen and Ebert (Ebert & Bunn, 1999; Klausen et al., 1996) have shown that EPO is mostly modulated by renal tissue hypoxia. Based on this observation, Balestra described a new phenomenon, known as the 'normobaric oxygen paradox' (NOP) (Balestra, Germonpre, Poortmans, & Marroni, 2006). The technique used to induce this effect involves the simple administration of a high concentration of oxygen (O2) at normobaric pressure to subjects breathing spontaneously and has been shown to increase significantly the production of endogenous erythropoietin. One possible mechanism for this phenomenon works via a cellular based model adapted to hypoxia (Haddad, 2002). It is dependent on the availability of oxygen free radicals or so-called reactive oxygen species (ROS). The key element of this concept is the complex formed by hypoxia-inducible factor 1 alpha (HIF-1α) and the tumor-suppressing Von Hippel Lindau protein (VHLp)(Ivan et al., 2001; Jaakkola et al., 2001). This complex is constantly bound to ubiquitin ligase.

During the hyperoxic state, the production of agents protecting against ROS will be met by the augmentation of gluthatione (GSH) via the stimulation of gluthathione synthethase enzyme activity. The GSH produced scavenges the ROS. When returning to the normoxic state, the extra GSH produced will neutralize the ROS, diminish their availability and finally lead to the dissociation of the complex, permitting the HIF-1 α to be dimerised with a unity β. This complex formed is responsible for the cascade of the EPO gene expression, subsequently leading to EPO hormone production. (De Bels et al., 2012; Masson et al., 2001)

Since GSH is involved in this cascade, some workers have suggested that an adjuvant might be made available to augment its availability. This would permit the neutralization of extra ROS and thus probably increase EPO production. Known for its redox state, N-acetyl-L-cysteine (NAC) could have the capacity to regenerate GSH. This approach was shown to be effective in the Zembron-Lacny trial (Zembron-Lacny, Slowinska-Lisowska, Szygula, Witkowski, & Szyszka, 2010), but the study did not include oxygen sessions. However, Momeni et al have evaluated the effect of a single dose of NAC on the EPO production in a hyperoxic model. This study demonstrated a significant EPO increase, but it was limited to 48 hours and used healthy volunteers as subjects rather than patients (Momeni, De Kock, Devuyst, & Liistro, 2011). The same results were also shown in one hypoxic model using NAC (Hildebrandt, Alexander, Bartsch, & Droge, 2002)though few clinical trials have been able to reproduce this effect in studies using relative hypoxia.

The use of a pure O2 breathing gas to induce the NOP effect and treat anaemia has been supported in recent publications (Balestra, Germonpre, Lafere, & Van der Linden, 2010; Burk, 2007; De Bels, Corazza, Germonpre, & Balestra, 2011). However, other studies have shown contradictory results, though a major difference is they used healthy volunteers (Keramidas et al., 2011; Keramidas, Norman,

Gustafsson, Eiken, & Mekjavic, 2012). Given these confounding results, it is likely that a positive NOP effect is specifically dose and time dependent. Clinical trials in this field are rare and we are yet to fully understand exactly how NOP effect functions (Lafere, Schubert, De Bels, Germonpre, & Balestra, 2013; Rocco, Ditri, De Bels, Corazza, & Balestra, 2014). One very recent study, made in clinical setting on acutely ill patients, confirms previous positive results. The authors observed an increase in EPO and reticulocytes after one session of 2-hour period of normobaric hyperoxia (FiO2 1.0) (Donati et al., 2017)

Our pilot study sought to determine whether a NOP regime administered alongside NAC could produce an increase in reticulocyte counts via an increase of EPO production, in patients undergoing oncological abdominal surgery.

Materials and Methods

This prospective single-centre, controlled, randomized pilot study was compliant with the declaration of Helsinki and approved by the local ethics committee of the Institut Jules Bordet, Belgium (approval number CE 1805). All participants signed a written informed consent. Inclusion criteria consisted of patients who were at least 18 years old and eligible for abdominal oncological surgery. Exclusion criteria included: severe renal insufficiency (GFR < 60 ml/minute and creatinine >2 mg/dl), bleeding necessitating iterative transfusion per or postoperatively, severe respiratory syndrome requiring constant oxygen administration and intolerance to an oxygen mask. All data were collected at the Institut Jules Bordet, Belgium.

A total of 78 patients scheduled for abdominal surgery were enrolled to participate in the study at the Institute between February and November 2011. Randomization took place at the end of the surgery. Patients were randomized in a 1:1:1 ratio to any of three parallel groups. The first group (NOP) received 60% oxygen for two hours on days 1,3 and 5 postoperatively via a venti-mask. The second group (NOP+NAC), in addition to the NOP oxygen regime, received 300 mg IV once a day of N-acetyl-cysteine (NAC) on the first day postoperatively and 200 mg orally once a day until the fifth day post-op. The third group (control) received neither NOP nor NAC. An exception to this rule permitted all patients to receive oxygen during the first 24 hours postoperatively according to their needs and it could also be administered during surgery. The treatment was open to all except laboratory staff.

No sample size calculation was performed for this pilot study as the objective was to evaluate the magnitude of the treatment effect. The sample size was similar or superior to other studies performed previously.

All patients received an oral benzodiazepine before surgery. All patients underwent general anaesthesia which could also involve an epidural technique. Standard anaesthesia monitors (electrocardiogram, pulse oximetry, end-tidal carbon dioxide concentration, and oscillometric arterial blood pressure) were used. The anaesthetist was free to choose his or her technique. During the surgery, all patients were ventilated with FiO2 between 40 and 50%. All patients were extubated at the end of the surgery and were admitted to the intensive care unit (ICU) for at least 24 hours. During this period, oxygen was given to all patients if their saturation fell below 98 percent. The oxygen protocol started 24 hours after admission to the ICU. The transfusion threshold was set to 8 g/dl together with clinical signs.

Clinical information collected on each patient included age, gender, tobacco use, ASA score (physical status classification system approved by the American society of anaesthesia) and body mass index. Surgery specific data included type of intervention, start and end time of surgery. Laboratory data including reticulocyte count, haemoglobin, haematocrit, urea, creatinine, ferritin, transferrin saturation and iron were collected at baseline (D0) and on the sixth day (D6). The occurrences of postoperative complications were also recorded.

Study outcomes

The primary endpoint was reached once the reticulocyte count was evaluated (percentage change) from baseline to D6, within each group. The secondary end points included haematocrit and haemoglobin levels measured at baseline and D6.

Statistical analysis

Mean percentage changes from baseline for reticulocyte counts, haemoglobin and haematocrit levels are presented along with their 95% 2-sided confidence intervals (Wald method). The differences between groups in reticulocyte counts were assessed by fitting an ANCOVA model to the percentage changes from baseline, including terms for the treatment group and baseline level of reticulocytes. Furthermore, an ANCOVA analysis of the percentage change from baseline in haematocrit and in haemoglobin, using terms for group, baseline value and duration of surgery, was used to demonstrate the impact of duration of surgery on these parameters.

Results

A total of nine subjects were excluded from the analysis due to their need for blood transfusion within the study period. Missed data and premature patient withdrawal were responsible for a variation in the number of observation between the groups. Premature patient withdrawal was due to personal and not for medical reason. The baseline and perioperative characteristics of the subjects are detailed in Table 1.

Table 1 Baseline and perioperative characteristics

Group		NOP	NOP+NAC	Control
Age	N	20	22	21
	Mean (SE)	63 (1.9)	66 (1.6)	63 (1.4)
	Median	63	68	63
	Min; Max	46; 78	49; 85	53; 79
BMI (Kg.m2(-1))	N	20	22	21
	Mean (SE)	27.8 (1.19)	24.8 (0.68)	28.1 (1.09)
	Median	28.9	24.4	27.7
	Min; Max	13.75; 38.06	18.70; 31.14	18.51; 37.90
Sex	Male	17 (85%)	19 (86%)	17 (81%)
	Female	5 (15%)	3 (14%)	4 (19%)
Tobacco use	Yes	5 (25%)	3 (14%)	2 (10%)
	No	15 (75%)	19 (86%)	19 (90%)
Diabetes	Yes	0	3 (14%)	5 (24%)
	No	20 (100%)	19 (86%)	16 (76%)
Hyperlipidaemia	Yes	9 (47%)	7 (32%)	9 (43%)
	No	10 (53%)	15 (68%)	12 (57%)
ASA	1	1 (5%)	0	0
	2	18 (90%)	18 (86%)	18 (86%)
	3	1 (5%)	3 (14%)	3 (14%)
Aspirin intake	0	16 (80%)	22 (100%)	21 (100%)
	1	4 (20%)	0	0
History of hypertension	0	10 (50%)	12 (55%)	11 (52%)
	1	10 (50%)	10 (45%)	10 (48%)
Duration of surgery (min)	N	19	22	21
	Mean (SE)	317 (17.3)	316 (14.5)	331 (15.7)
	Median	312	302	322
	Min; Max	216; 443	204; 475	237; 506

Values are mean, standard error, number of patients or median (minimum-maximum)

Baseline laboratory variables were generally balanced across the three treatment groups (Table 2) with the exception being iron, which was slightly lower on average in the group NOP+NAC.

Table 2 Baseline laboratory parameters

Variable	N	Mean	Std.	Median	Minimum	Maximum
Urea (mg.dl-1)						

NOP group	20	40.6	2.25	40	24	64
NOP+NAC group	22	37.3	1.86	37.5	16	56
Control group	21	36.2	1.67	34	26	54
Creatinine (mg.dl-1)						
NOP group	20	0.98	0.040	0.96	0.67	1.43
NOP+NAC group	22	0.93	0.053	0.96	0.25	1.6
Control group	21	0.95	0.038	0.92	0.71	1.47
Iron (g.dl-1)						
NOP group	19	121.8	12.89	125	23	218
NOP+NAC group	21	103.4	11.21	101	32	249
Control group	20	118.8	11.73	127.5	33	213
Ferritin (g.dl-1)						
NOP group	19	226.5	28.03	256	9	490
NOP+NAC group	20	213.7	42.60	169	53	901
Control group	20	197.2	43.23	142	17	763
Transferrin (mg.dl-1)						
NOP group	19	233.7	10.55	237	173	327
NOP+NAC group	21	234.6	8.22	244	165	302
Control group	21	249.4	10.15	249	176	371
Transferrin saturation (%)						
NOP group	19	35.8	3.72	32	6	64
NOP+NAC group	21	31.7	3.71	27	8	87
Control group	20	33.2	3.35	35	10	54

Laboratory parameters at day 6 (Table 3) were similar across the three treatment groups.

Table 3 Efficacy results of laboratory parameters on Day 6

	N	Mean	SD Error	Median	Minimum	Maximum
Urea (mg.dl-1)						
NOP group	20	33.5	2.53	29.5	20	63
NOP+NAC group	22	33.5	1.83	32.5	12	52
Control group	20	26.6	1.77	27.5	12	43
Creatinine (mg.dl-1)						
NOP group	20	0.91	0.042	0.915	0.59	1.27
NOP+NAC group	22	0.89	0.057	0.895	0.23	1.64
Control group	20	0.82	0.039	0.85	0.5	1.06
Iron (g.dl-1)						
NOP group	20	41.9	4.71	32	15	92
NOP+NAC group	22	57.3	7.42	52	13	131
Control group	20	43	5.29	38	14	105
Ferritin (g.dl-1)						
NOP group	18	408.2	50.33	461.5	76	843
NOP+NAC group	20	304.1	36.02	284	75	791
Control group	19	380.3	62.82	297	80	1112
Transferrin (mg/dl)						
NOP group	20	206.6	5.94	202.5	168	268
NOP+NAC group	22	201.5	10.39	197	89	295
Control group	20	202.9	7.67	202	151	275
Transferrin saturation (%)						
NOP group	20	14.4	1.58	12	5	31
NOP+NAC group	22	21.5	3.48	18	6	80
Control group	20	14.9	1.75	12.5	6	37

Baseline levels (D0), as well as D6 and percentage change from baseline of reticulocyte counts, haematocrit and haemoglobin, are respectively presented along with efficacy results in Tables 4, 5 and 6. The ninety-five percent confidence intervals of the mean percentage change from baseline revealed by excluding 0, that the increase in reticulocyte counts was statistically significant (at 0.05 level) for the NOP Group and NOP+NAC Group, whereas it was not significant for the control group (Table 4).

Table 4 Efficacy results of reticulocytes.

		NOP	NOP+NAC	Control
Baseline Reticulocytes (103.l-1)	N	18	21	21
	Mean (SE)	65.06 (5.278)	73.04 (4.419)	69.46 (5.169)
Day 6 Reticulocytes (103.l-1)	N	19	22	21
	Mean (SE)	83.24 (7.573)	83.15 (5.806)	78.06 (6.909)
Percent change from baseline	N	17	21	21
	Mean (SE)	39% (10.8)	24% (11.2)	27% (16.9)
	Median	34%	9%	22%
	Min; Max	-33; 123	-20; 216	-92; 275
	95% CI	[16; 62]	[1; 48]	[-8; 63]
	p-value for comparison with 0 (t-test)	0.0026	0.0405	0.1213

Values are mean, standard error, number of patients or median (minimum-maximum)

Table 5 Efficacy results of haematocrits

		NOP	NOP+NAC	Control
Baseline Haematocrit (%)	N	20	22	21
	Mean (SE)	41.99 (1.223)	42.08 (1.030)	43.08 (0.806)
Day 6 Haematocrit (%)	N	20	22	21
	Mean (SE)	[illegible]	36.63 (1.543)	36.74 (1.286)
Percent change from baseline	N	20	22	21

	Mean (SE)	-14% (2.5)	-13% (3.0)	-15% (2.5)
	Median	-13%	-9%	-16%
	Min; Max	-34; 8	-44; 11	-44; 3
	95% CI	[-19; -9]	[-19; -7]	[-20; -9]

Values are mean, standard error, number of patients or median (minimum-maximum)

The mean percentage decreases from baseline in haematocrit and in haemoglobin were similar across all three groups (Table 6).

Table 6 Efficacy results of haemoglobin

		NOP	NOP+NAC	Control
Baseline haemoglobin	N	20	22	21
	Mean (SE)	14.23 (0.449)	14.25 (0.367)	14.55 (0.271)
Day 6 haemoglobin	N	20	22	21
	Mean (SE)	12.03 (0.430)	12.28 (0.517)	12.42 (0.436)
Percent change from baseline	N	20	22	21
	Mean (SE)	-15% (2.7)	-14% (2.8)	-15% (2.4)
	Median	-17%	-10%	-13%
	Min; Max	-37; 11	-40; 7	-43; 1
	95% CI	[-20; -9]	[-20; -8]	[-20; -10]

Values are mean, standard error, number of patients or median (minimum-maximum) None of the pairwise treatment differences were found to be statistically significant (Table 7).

Table 7 Percentage change from baseline in reticulocytes for between group comparisons

Group	p-value
NOP versus NOP+NAC	0.9175
NOP versus Control	0.9876
NOP+NAC versus Control	0.8977

The percentage of change from baseline in haemoglobin and haematocrit were significantly correlated (negatively) with the duration of surgery (Table 8).

Table 8 Effect of surgery duration

	p-value for effect of surgery duration
On haematocrit percentage change from baseline	0.0083
On haemoglobin percentage change from baseline	0.0056

Discussion

Pre- and postoperative anaemia is common in abdominal oncological surgery. Most patients are subject to allogeneic transfusion when excessive blood loss occurs and/or when their vital signs are threatened. To date, the literature shows that transfusion is associated with a higher mortality rate, a longer hospital stay and increased morbidity (Vincent et al., 2008). In the present study, transfused patients per and postoperatively were excluded from statistical analysis as this could alter reticulocyte production (Halvorsen, Haga, & Bechensteen, 1993). They were mostly transfused during surgery and the first day postoperatively.

The possibility that NOP can stimulate the production of reticulocytes and red blood cells is still under debate. Balestra (Balestra & Germonpre, 2011) suggested that 40% O2 might be the threshold concentration of oxygen to trigger the NOP. The administration of 100% oxygen has yielded contradicting results. In our study, we chose an intermediate concentration of inspired O2 (60%) in order to try to determine an effective gradient of oxygen.

An increase in absolute reticulocyte counts was observed in all groups. This effect could probably be attributed to peroperative oxygen administration (FiO2 40-50%), then followed by a decrease in the concentration of O2 breathed at the end of surgery. In other words, an unavoidable, relative hypoxia was administered to all subjects across the groups, as dictated by surgical protocol. However, within the groups, the mean percentage change from baseline to day 6 (D6) in reticulocyte counts was only statistically significant in the NOP and NOP+NAC groups (Table 4). This leads us to believe that repeated relative hypoxia brought about some changes. The oxygen gradient induced is most probably behind this observed trend since in these two groups and during the postoperative period the 40% oxygen variation (Fi O2 60% to FiO2 21%) was much bigger than that in the control group (10%). However, we did not notice a significant difference between groups, as we postulated before that NAC could positively influence the NOP+NAC group. Another hypothesis is that the NOP+NAC group could be influenced by the slight elevated baseline reticulocyte counts, although no statistically significant difference was observed.

This result is in agreement with that of Zembron trial (Zembron-Lacny et al., 2010). They noted that a daily intake (1200 mg) of NAC augmented EPO, haemoglobin and haematocrit significantly, as well as diminishing reticulocytes counts. They suggested that NAC, by way of reducing H2O2, could both induce EPO gene expression and inhibit gene transcription. Another clinical trial evaluated the effect of NAC and hyperoxia on EPO production in healthy subjects. A single NAC dose (600 mg) per day was administered to subjects breathing 100% oxygen for 60 minutes. Once again, an EPO elevation was noted without a significantly augmented haemoglobin level since the follow up period was limited to fourty-eight hours (Momeni et al., 2011). The hypothesised effect of NAC on EPO production was derived from the NOP intracellular mechanism (Haddad, 2002).

The control group did not show any significant reticulocyte elevation one week after surgery. A similar result was also observed by Van Iperen et al (van Iperen, Biesma, van de Wiel, & Marx, 1998) and Lafere et al (Lafere et al., 2013) where a small, non-significant increase in reticulocyte count one week after surgery was observed. Another factor that could impact the increase in reticulocyte count is the inflammatory reaction induced by surgery. This inflammatory reaction is thought to increase hepcidin synthesis, an iron regulatory peptide (Verga Falzacappa et al., 2007). This will make iron less available to promote an increase in reticulocyte count and consequently impact the action of EPO (Pak, Lopez, Gabayan, Ganz, & Rivera, 2006). In the present study, these two mechanisms could influence our results in a different way since we do not have a control on the inflammatory process.

In our study, the percentage of change from baseline in haemoglobin and haematocrit were significantly correlated (negatively) with the duration of surgery. In fact, the more the duration was the more the patients were exposed to oxygen. We can assume from what was previously published, that giving too much oxygen will blunt EPO synthesis for a while and thus compromise reticulocyte production (De Bels et al., 2011; De Bels et al., 2012; Revelli et al., 2013).

In the present study, renal function was not altered and was well balanced across the three treatment groups. Thus, it is not likely to have influenced reticulocyte production. However, we noticed that the level of iron was slightly lower on average in the NOP+NAC group. This could influence erythropoiesis negatively (Cavill, 2002).

The haemoglobin results did not show any significant difference with respect to the baseline values. These results were in parallel with those previously observed in healthy volunteers, where no rise in haemoglobin was noted despite a significantly elevated level of EPO (Balestra et al., 2006). However, in two case studies, the NOP effect has purportedly caused a significant rise in haemoglobin, though the results may be caused by two specific characteristics of those trials. The first is that the patients were anaemic at baseline due to chemotherapy treatment and cancer itself. The second is that the oxygen sessions were delivered over several weeks, while the variation in haemoglobin was noted after only two weeks (Burk, 2007). In our study, the fact that subjects were not anaemic, they were treated over a short period and that the haemoglobin measurement period was not enough prolonged, all probably contributed to the

absence of a significant variation in haemoglobin levels.

A limitation of this study is that no sample size calculation was realised as thought it was a pilot study and thus no sufficient power to make a definitive conclusion. However, our data can certainly be used to project how large a study would be necessary to make that definitive conclusion.

From the observations made above, we can suggest that relative hypoxia could bring about a change in reticulocyte stimulation by means of the oxygen gradient we provoked. The NAC did not significantly influence erythropoiesis. Different factors could influence or modulate this mechanism. However, a longer follow up period and a prolonged relative hypoxia protocol is probably necessary to clarify any effect on haemoglobin levels. Given the slight change in iron levels, another potential route of investigation following this pilot study might be to consider the combination of NAC-Iron-Oxygen (NACIO) administration and/or a comparison with a standard anaemia treatment, for instance erythropoietin stimulating agents.

Conclusions

These data suggest that relative hypoxia by the means of induced oxygen gradient is an effective stimulus for reticulocyte production.

However, it is difficult to tell if the NOP is clinically useful to treat at least partially peri-operative anaemia. So, other studies and protocols are necessary to investigate its impact on haemoglobin production as well as the magnitude of the oxygen gradient.

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